Glycinergic inhibition to the medial nucleus of the trapezoid body shows prominent facilitation and can sustain high levels of ongoing activity

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Mayer F, Albrecht O, Dondzillo A, Klug A. Glycinergic inhibition to the medial nucleus of the trapezoid body shows prominent facilitation and can sustain high levels of ongoing activity. J Neurophysiol 112: 2901–2915, 2014. First published September 3, 2014; doi:10.1152/jn.00864.2013.—Neurons in the medial nucleus of the trapezoid body (MNTB) are well known for their prominent excitatory inputs, mediated by the calyx of Held. Less attention has been paid to the prominent inhibitory inputs that MNTB neurons also receive. Because of their auditory nature, both excitatory and inhibitory synapses are highly active in vivo. These high levels of activity are known to reduce excitatory synaptic currents considerably, such that in vivo synaptic currents produced by the calyx are smaller than typically measured in standard brain slice experiments. The goal of this study was to investigate the properties of the inhibitory inputs in the Mongolian gerbil (Meriones unguiculatus) under activity levels that correspond to those in the intact brain to facilitate a direct comparison between the two inputs. Our results suggest that inhibitory inputs to MNTB are largely mediated by a fast and phasic glycinergic component, and to a lesser degree by a GABAergic component. The glycinergic component can sustain prolonged high levels of activity. Even when challenged with stimulus patterns consisting of thousands of stimuli over tens of minutes, glycinergic inputs to MNTB maintain large conductances and fast decays and even facilitate substantially when the stimulation frequency is increased. The inhibition is mediated by a relatively small number of independent input fibers. The data presented here suggest that inhibitory inputs to MNTB sustain high levels of activity and need to be considered for a full understanding of mechanisms underlying processing of auditory information in MNTB.

auditory brain stem; inhibition; medial nucleus of the trapezoid body; calyx of Held; short-term plasticity

SYNAPTIC TRANSMISSION in the medial nucleus of the trapezoid body (MNTB) has been studied extensively over the last years. Principal neurons in the MNTB receive neural excitation via a giant synapse, the calyx of Held (Borst et al. 1995; Borst and Soria van Hoeve 2012; Forsythe 1994; Held 1892, 1893; Kuwabara et al. 1991; Tolbert et al. 1982). Synaptic currents produced by the calyx are large and fast, and both the calyx and the postsynaptic neuron have a number of adaptations suited to transmit action potentials with very high temporal fidelity (Guinan and Li 1990; Kochubey et al. 2009; Taschenberger and von Gersdorff 2000). The sign inversion is needed because neural inhibition is required in the sound localization process in which the MNTB participates (Brand et al. 2002; Caird and Klinke 1983).

This view is inconsistent with the observation that transmission at the calyx is not entirely fail-safe, and in some cases incoming action potentials do not result in spiking of MNTB principal neurons—at least in some species and under some circumstances (Grande and Wang 2011; Hermann et al. 2007, 2009; Klug 2011; Kopp-Scheinpflug et al. 2003, 2008; Lorteije et al. 2009). Moreover, MNTB neurons themselves receive synaptic inhibition (Albrecht et al. 2014; Awatramani et al. 2004; Green and Sanes 2005; Kuwabara et al. 1991; Smith et al. 1998; Thompson and Schofield 2000), which modifies firing patterns of MNTB neurons (Green and Sanes 2005; Kopp-Scheinpflug et al. 2008; Tolnai et al. 2008).

Although intrinsic projections within MNTB have been suggested as one possible source of inhibitory inputs (Kuwabara and Zook 1991), a recent study has shown that the ipsilateral ventral nucleus of the trapezoid body (VNTB) is their main extrinsic source (Albrecht et al. 2014). VNTB neurons receive excitatory input from stellate cells in the contralateral cochlear nucleus (Thompson 1998; Warr 1969) with tonic/chopping firing patterns (Oertel 2011) and/or excitatory input from globular bushy cells in the contralateral cochlear nucleus (Kuwabara et al. 1991; Spriou et al. 1990) with primary-like or primary-like with notch firing patterns (Smith et al. 1991). Thus both the excitatory and inhibitory inputs to MNTB neurons originate directly or indirectly from the contralateral cochlear nucleus. The significance of this circuit is that both the excitatory and inhibitory inputs are of auditory brain stem nature and respond to sound stimulation with ongoing firing patterns and relatively high activity levels. Moreover, both types of inputs are spontaneously active during periods of acoustic silence (although the exact rates of spontaneous activity in VNTB neurons have not been reported to date). Thus synapses in these pathways never experience prolonged periods of inactivity.

Many studies of MNTB synaptic transmission use standard brain slice conditions that lack the typical levels of chronic and high-frequency activity that are present in the auditory brain stem of the intact brain. Physiological properties of excitatory inputs to MNTB neurons change substantially when this chronic activity is absent from slice preparations (Hermann et al. 2007, 2009). The main question we addressed in this study was how the properties of inhibitory postsynaptic current (IPSC) amplitudes would change when investigated under conditions of chronic activity and high firing rates compared with their properties in silent brain slice...
preparations (Awatramani et al. 2004). The results suggest that inhibitory inputs to MNTB retain substantial synaptic amplitudes even after being challenged with long stimulus trains consisting of thousands of pulses. Inhibitory inputs also show substantial synaptic facilitation. Our data suggest that, under physiologically relevant conditions, the strength of inhibitory inputs to MNTB is significant, and therefore the MNTB may not function as a simple relay station but rather transform spike train information.

METHODS

All animal procedures were approved by the University of Colorado School of Medicine Animal Care and Use Committee and strictly followed all applicable regulations.

In Vitro Recordings

Slice preparation. Slices of brain stem were prepared from Mongolian gerbils (Meriones unguiculatus) of either sex ranging in age from P12 to P18 (all stages after hearing onset). Animals were anesthetized by isoflurane inhalation (IsoFlo, Abbott Laboratories) and decapitated. The brain stem was dissected out and cut into slices from P12 to P18 (all stages after hearing onset). Animals weregolian gerbils (Meriones unguiculatus) of either sex ranging in age.

The internal solution to eliminate postsynaptic sodium currents. Glutamate and glutamine were blocked by 200 nM NMDA receptor antagonist (Tocris Bioscience). In a subset of experiments, glycine currents were blocked by 500 nM strychnine (Sigma).

The majority of recordings performed for this study focused on properties of isolated glycinerergic inputs to MNTB principal neurons, and thus glutamatergic and GABAergic blockers were standardly added to the ACSF, as well as QX-314 to the intracellular solution.

Electrical stimulation of inhibitory inputs. IPSCs were evoked by electrical stimulation in the vicinity of the MNTB principal neuron via an ESC-filled glass pipette with a tip resistance of ~2–3 MΩ. The location and intensity of the stimulus were optimized to obtain the largest IPSCs (Fig. 1A). Unless stated otherwise, stimulus amplitudes were chosen to activate the largest possible number of input fibers (maximum stimulation). Stimuli were 100-μs-long square pulses of 1–90 V delivered with an STG 2004 computer-controlled four-channel stimulator (Multi Channel Systems, Reutlingen, Germany) and a stimulation isolation unit (Iso-flex, AMPI, Jerusalem, Israel). The stimulator permitted completely independent uploading and operation of the four channels, allowing the seamless integration and thus true embedding of simulated auditory signals (i.e., high-frequency bursts) in the simulated spontaneous activity. Spontaneous activity was simulated by using 5-, 10-, 20-, 40-, and 60-Hz near-Poission-distributed stimulus trains. Previous in vivo recordings from the MNTB of Mongolian gerbils (Hermann et al. 2007) determined that spontaneous activity is near-Poission distributed (near-Poission because interstimulus intervals of <2 ms are underrepresented). The frequencies of spontaneous activity ranged from <1 Hz to >100 Hz with a mean of ~25 Hz (Hermann et al. 2007). In light of these observations, the five stimulus trains were created to simulate typical spontaneous activity that a MNTB neuron might experience in the intact brain. Whenever possible, several different background frequencies were tested on the same neuron. A recording session was started using one of the five background stimulus frequencies, and a data set was recorded with that frequency. After several minutes of ongoing stimulation with the background activity, high-frequency trains were seamlessly embedded into the background activity and suprasytic responses to the high-frequency trains were measured. Whenever the neuron/recording was still healthy after the completion of that data set, a different background stimulus rate was programmed and the neuron allowed to adapt to this new background rate, after which a second data set at the new frequency was recorded, and so on.

Sound-evoked activity was simulated by short high-frequency trains consisting of 50 stimuli at 50, 100, and 300 Hz. These trains imitate activity caused by typical sound stimuli (tone bursts) used in in vivo experiments.

For experiments with single-pulse stimulation, the time between pulses was at least 2 s. Experiments to determine the number of current steps covered the voltage range from the first occurrence of an IPSC up to the voltage at which a further increase would not result in larger IPSCs. In few cases, the maximum voltage output of 90 V was reached before a plateau in IPSC amplitude size was seen. These data were excluded from the analysis since maximum amplitudes might not have been reached in these cases.

Glycine puff experiments. For these experiments a third pipette was used and connected to a Picospritzer III (Parker Hannifin, Cleveland, OH). It was filled with ACSF containing 2 mM glycine (glycine HCl, Sigma-Aldrich). The distance to the cell held in whole cell patch-clamp mode as well as the duration and the pressure of the puff (~300 ms, 10 psi) were optimized to evoke the maximum IPSC response. We used the same type of pipettes as for patch-clamp recordings (~2.6- to 2.8-MΩ tip resistance).

Analysis

IPSC amplitudes and kinetics were analyzed in IGOR Pro 6.21 (WaveMetrics), Clampfit 10.3.0.2 (Molecular Devices, Sunnyvale, CA), and Fitmaster (version 2.32, HEKA). Data from all ages were...
GLYCINERGIC INHIBITION TO MNTB SUSTAINS HIGH LEVELS OF ACTIVITY

Fig. 1. Electrical stimulation of inhibitory synapses near medial nucleus of the trapezoid body (MNTB) neurons elicited synaptic currents of several nanamperes. A: the position of the stimulus electrode was optimized such that electrical stimuli recruited inhibitory postsynaptic currents (IPSCs) with maximum amplitudes, and in most experiments stimulus amplitudes were chosen to activate the largest possible number of inhibitory input fibers (maximum stimulation). The image shows a section of a brain slice collected from a posthearing animal (P15) containing a part of MNTB. One MNTB neuron is patch clamped (dotted oval), and a second glass electrode is placed at various locations around the recording site to test for inhibitory inputs ("X" symbols). B and C: the age range tested in this study was P12–18. No significant effects of age on IPSC amplitudes were found [1-way ANOVA with single-day age groups, \( P = 0.93 \); 1-way ANOVA with 3 age-combined groups (P12–13, P14–15, P16–18), \( P = 0.63, n = 52 \); Fig. 1B].

Statistical. Statistical analysis was done with SigmaStat 3.5 (Systat Software, Point Richmond, CA), MS Excel 2010 (Microsoft, Redmond, WA), and SAS 9.2 (SAS Institute, Cary, NC). Statistical significance was tested with a Student’s \( t \)-test, unless otherwise noted. Unless otherwise noted, means and SE are reported.

\( k \)-Means cluster analysis. To determine the number of current steps, the \( k \)-means cluster algorithm in MATLAB 7.9 (MathWorks) was used. This analysis tests different numbers of clusters (in our case, numbers of synaptic inputs) and plots the resulting error as a function of the number of clusters. An increase in the number of clusters will always result in a smaller error, but error reduction becomes very incremental for very large numbers of clusters. The likeliest number of clusters for a given data set is when error reduction becomes very small. In a graphical solution, this corresponds to the point where the plot flattens significantly.

Mathematical Determination of Effects of Conditioning

Because of the variable interstimulus intervals of the Poisson trains and, related to that, a significant amount of variability in the resulting IPSC amplitudes, it was not possible to simply measure IPSC amplitudes at or near the end of the 2-min trains to determine steady-state values. We therefore determined these values mathematically with the following equation:

\[
\begin{align*}
\ln(Y_{it}) &= \ln(e_{it}) + \ln(\delta_{it}) + \ln(C_{ik}) + \ln(a_{k}) \cdot \exp[\ln(b_{k}) \cdot t] \\
&= \ln(e_{it}) - \ln(\delta_{it}) - \ln(C_{ik}) - \ln(a_{k}) - \ln(b_{k}) - t
\end{align*}
\]

where the amplitude \( k \) is the frequency subscript, \( i \) is the cell subscript, \( t = \) time, \( Y \) is the absolute value of the amplitude, and \( e \) is the random error, i.e., noise

\[
\ln(e_{it}) \sim N(0, \sigma_{e}^{2})
\]

\( \delta \) is the random cell effect; observations on a cell are more homogeneous than observations from different cells. On the log scale the random cell effect shifts function for a cell up or down compared with the population mean

\[
\ln(\delta_{it}) \sim N(0, \sigma_{\delta}^{2})
\]

(\( a, b, \) and \( C \) are fixed parameters, possibly dependent on frequency.

On the log scale \( E[\ln(Y(t = 0))] = \ln(C) + \ln(a) \), and as \( t \to \infty \), \( E[\ln(Y(t))] \to \ln(C) \), assuming \( \ln(b) < 0 \).

\( \ln(C) \) is the asymptotic expected log amplitude at large times. \( \ln(a) \) is the difference in expected log amplitudes between time zero and its asymptotic value at large times. \( \ln(b) \), assuming its negative, determines the rate at which the expected log amplitude decays toward its asymptotic value at large times. The more negative \( \ln(b) \) is, the faster the expected log amplitude decays.

Additive differences on the log scale translate to ratios on the original scale. Equations were fit with SAS 9.2 (SAS Institute). There were no appreciable differences among \( \ln(C) \) by frequency. Therefore the equation uses a single parameter for \( \ln(C) \) across all frequencies.

This particular equation is equivalent to an exponential decay with exponentially decreasing rate changes and was found to be the best fit for the data set. For example, it rendered a much better fit for the data set than a standard exponential decay function with a constant asymptotic parameter or a double exponential decay function (i.e., the sum of 2 exponentials). Our very large data sets, with randomly changing interstimulus intervals that covered a time span that started with synapses being completely at rest and ended with a state in which synapses had just been exposed to several thousand stimuli, proved to...
be challenging to fit. Note that the only purpose of this empirically
determined equation was to calculate IPSC steady-state values and the
approximate time it takes to reach these steady-state values, not to
create a physiologically relevant vesicle release model.

Antibodies

The guinea pig polyclonal antibody against synthetic COOH-
terminus antigen from rat glycine transporter 2 (GlyT2) was obtained
from Millipore (catalog no. AB1773, lot no. NG 1809675). The specifici-
ty of this antibody for GlyT2 protein has been determined previously
(Dufour et al. 2010), and an antibody against this particular glycine transporter was chosen because of its specificity for neuronal
glycine transporter (Poyatos et al. 1997). The mouse monoclonal antibody mAb7a against rat NH2-terminus gephyrin antigen was
obtained from Synaptic Systems (catalog no. 147 011). The specificity
of this antibody has been determined previously by Schneider et al.
(2006). The anti-GlyT2 antibody was used at a 1:2,000 dilution, and the
anti-gephyrin antibody was used at a 1:5,000 dilution. The following
secondary antibodies were used (purchased from Invitrogen-Mo-
elcular Probes): goat anti-guinea pig conjugated with Alexa 568
(catalog no. A11075) and goat anti-mouse conjugated with Alexa 488
(catalog no. A11029). All secondary antibodies were used at 1:1,000
dilutions. Cell somas were counterstained with NeuroTrace Fluores-
cent Niissl 640 nm (Molecular Probes, catalog no. N-21483) at a
concentration of 1:100 for 25 min.

Immunohistochemical Procedures

Gerbils were overdosed with intraperitoneal injections of pentobar-
bital sodium (70 mg/kg body wt, Sigma), followed by a transcardial
perfusion with ice-cold phosphate-buffered saline (PBS, in mM: 137
NaCl, 2.7 KCl, 1.76 KH2PO4, 10.0 H3PO4, pH 7.2), followed by a transcardial
perfusion with ice-cold phosphate-buffered saline (PBS, in mM: 137
(52x394)NaCl, 2.7 KCl, 1.76 KH2PO4, 10.0 H3PO4, pH 7.2), followed by a transcardial
perfusion with ice-cold phosphate-buffered saline (PBS, in mM: 137

RESULTS

Two Components of Inhibitory Currents to MNTB

Several anatomical sources for inhibition to MNTB neurons have been suggested (Albrecht et al. 2014; Kuwabara and Zook
1991; Smith et al. 1998; Thompson and Schofield 2000). To
assess the sum of inhibitory inputs independent of their source(s), we chose to stimulate inhibitory inputs in the direct
vicinity of the MNTB neuron from which we recorded (Fig.
1A). Because of the high-chloride intracellular solution (calcu-
lated $E_{Cl} = -5 \text{ mV}$), IPSCs recorded at a holding potential of
$-60 \text{ mV}$ were inward (downward). With this approach, we
were able to record inward currents at a holding potential of
$-60 \text{ mV}$ in all neurons ($n = 74$) that were tested.

Further analysis of the inward currents revealed that they consisted of two components, namely, a glycineergic and a
GABAergic component. Figure 2, A and B, show two examples of recordings from MNTB principal neurons. In Fig. 2A,
electrical stimulation in the vicinity of the neuron initially
revealed a total inhibitory current of $\sim 1 \text{ nA}$. Application of the
glycine antagonist strychnine reduced this current by $\sim 80\%$, and the resulting remaining current was $\sim 200 \text{ pA}$. The addi-
tional application of the GABA$_A$ antagonist SR 95531 com-
pletely eliminated all current, suggesting that the initial inward
current had two but not more components. In the case of the
neuron in Fig. 2B, the order of application of inhibitory blockers was reversed. In this neuron, the total inward current was $\sim 3.5 \text{ nA}$. Application of the GABA$_A$ blocker SR 95531
reduced the total amplitude of the remaining current by $\sim 400
\text{ pA}$ but, more importantly, eliminated a component with a slow
decay time constant from the total current. The additional
application of strychnine completely eliminated any current,
suggesting, again, the presence of exactly two components in
the inward current.

In 11 recordings in which both glycineergic and GABA$_A$ergic
inhibition were tested pharmacologically, the amplitude of the
glycinergic component was significantly larger than the ampli-
tude of the GABAergic component, accounting for $84.8 \pm
3.6\%$ of the total inhibitory current (Fig. 2C, top). In a sub-
sample of seven recordings, in which both glycineergic and
GABAergic blockers were applied consecutively, the glycine-
ergic component accounted for $1.63 \pm 0.32 \text{ nA}$ while the
GABAergic component accounted for $0.22 \pm 0.04 \text{ nA}$. In all
seven cases, inhibitory currents were completely eliminated by
a combination of strychnine and SR 95531. The same IPSC
traces were analyzed for synaptic charge rather than synaptic
amplitude by calculating the integral between the baseline and the
IPSCs. When analyzed for charge, the glycineergic compo-
nent was still larger than the GABAergic component, but the
GABAergic component now accounted for a larger proportion
of recordings from MNTB principal neurons. In Fig. 2,

Characterization of IPSCs Under in Vivo-Like Activity Levels

Recent work suggested that the main source of inhibition to
MNTB may originate from the VNTB (Albrecht et al. 2014).
VNTB neurons, like the majority of auditory brain stem neu-
rons, receive inputs that are highly active during the presenta-
tion of sound and additionally are spontaneously active during
acoustic silence. VNTB inputs originate from globular bushy
and/or stellate cells in the contralateral cochlear nucleus (Ku-
wabara et al. 1991; Thompson 1998; Warr 1969). Thus VNTB-
derived inhibitory inputs to MNTB likely respond to sound
stimulation with high firing rates and are likely characterized
by spontaneous activity. These activity patterns are well known

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to elicit short-term plasticity in synapses (Zucker and Regehr 2002). Thus inhibitory inputs to MNTB likely operate under conditions of chronic short-term plasticity, and one challenge with studying these inputs in vitro is that brain slices lack the activity levels observed in vivo and associated short-term plasticity (Hermann et al. 2007; Klug 2011; Wang et al. 2010; Wang and Manis 2008). Importantly, synaptic transmission at the excitatory input to MNTB has been shown to be significantly affected by chronic short-term plasticity, and synaptic currents under such conditions are significantly smaller than under standard conditions (Hermann et al. 2007).

To be able to compare inhibitory inputs to MNTB to their corresponding excitatory inputs under in vivo-like conditions, we stimulated these inputs for prolonged periods of time at various imitated “spontaneous” background rates with stimulus trains closely matching the statistical properties of spontaneous activity (Hermann et al. 2007, 2009). Specifically, we used stimulus trains with near-Poisson-distributed activity at frequencies of 5, 10, 20, 40, and 60 Hz to initially “condition” inhibitory synapses for at least 2 min. Subsequently, all test stimuli were embedded in the background activity. Since a typical set of voltage-clamp recordings from any given neuron at any one background activity lasted ~15 min, the entire stimulation protocol for this set of recordings could be looked at as one single gapless 15-min protocol with a total of ~4,500 stimulations (at 5 Hz) up to ~54,000 stimulations (at 60 Hz). We now consider the first 2 min of this protocol, which we termed the “conditioning period.”

Figure 3A shows the start and end sequences (first 3 s and last 3 s) of two IPSC traces that were recorded during the 2-min conditioning period. IPSCs recorded during the first 3 s of the conditioning period (left half of each train in Fig. 3A) represent a condition in which neurons in the brain slice lacked the typical in vivo spontaneous activity and had been incubated for a prolonged period of time without any stimulation, and synaptic responses presumably represent a state without the naturally present chronic short-term plasticity. IPSC amplitudes were large; the average of the first five IPSCs was 2.3 ± 1.1 nA (n = 37). By contrast, IPSCs recorded during the last 3 s (right half of the trains in Fig. 3A) of the same stimulus train were much smaller and represented a state in which synapses have been active for a prolonged period of time. The amount of depression depended on the background frequency used and tended to be larger for higher background frequencies (Fig. 3B).

The depression had especially large effects on synaptic amplitudes near the beginning of the stimulus train. The insets in Fig. 3A plot each IPSC amplitude measured in response to the 2-min conditioning train as one data point against time, and the red lines represent a fit of the data with a customized exponentially decaying function. IPSC amplitudes were more stable (although chronically depressed) during the second half

Fig. 2. Inhibitory inputs to MNTB neurons comprise both glycinergic and GABAergic components. Blockade of glutamate receptors isolates the IPSCs evoked by electrical stimulation. Subsequent block of glycine and GABA<sub>A</sub> receptors reveals the glycinergic and GABAergic components of the IPSCs. A: voltage-clamp recording of pharmacologically isolated inhibitory currents to a MNTB principal neuron, stimulated electrically in the direct vicinity of the neuron. Green trace: total inhibitory current, measured in the presence of the excitatory blockers DNQX and APV. Red trace: remaining current after application of strychnine and SR 95531 was reversed. Blue trace: combination of the glycine blocker strychnine and the GABA blocker SR 95531 eliminated all inhibitory current. B: same as A, except that the order of application of strychnine and SR 95531 was reversed. C: on average, the glycinergic component of the IPSC was greater than the GABAergic component. When IPSC peak amplitudes were compared (top), this discrepancy was larger than when IPSC charges were compared (bottom). All synaptic amplitudes reported in A–C were recorded with maximum stimulation.

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of the 2-min train and seemed to assume a new, depressed, steady-state value.

A mathematical fit was developed to describe magnitude and kinetics of depression and to determine whether 2 min of stimulation was sufficient to reach a steady state, defined as five time constants. Fitting allowed us to consider all data points recorded over the course of the 2 min and overcome the challenges with highly variable IPSC amplitudes measured in response to Poisson-distributed stimulus trains and provided the possibility to compare different frequencies. The function that was found to provide the best fit of the data set was an equation with an exponential decay and an exponentially decreasing rate change of the decay (see Methods).

Asymptotic amplitude values were significantly lower than initial amplitudes \( (P < 0.001) \) and ranged from 36% to 18% of initial amplitudes (Fig. 3B). These steady-state values were reached in <2 min in all cases except for 5-Hz stimulation; here the calculated stimulation time to reach steady state was after 442 ± 138 s. However, 2 min of simulated background activity of 5 Hz reduced amplitude size significantly to 46.9 ± 1.9% of the value of the initial amplitudes (Fig. 3B). The steady-state value assumed after five time constants was even lower, with a calculated value of 36% of initial amplitudes (Fig. 3B). By contrast, the 60-Hz stimulation protocol caused a number of failures, presumably fiber failures near the end of the 2-min duration, such that synapses were effectively stimulated at a frequency slightly less than 60 Hz. Because of this, we only include data from short high-frequency trains that were embedded into this background rate in this analysis, not the data from the background trains themselves.

Figure 3C describes the time course of synaptic amplitude depression over the 2-min conditioning period for the four rates of background stimulation that were included in the analysis (5–40 Hz). Depending on the background frequency, steady states varied between 0.47 (5 Hz) and 0.18 (40 Hz). We suggest that these steady-state amplitudes represent the synaptic strength of glycinergic inhibition to MNTB principal neurons much closer than values measured under more standard brain slice recording conditions. We also suggest that the situation shown in Fig. 3, B and C, represents a situation in which the inhibitory inputs to MNTB are firing at the spontaneous background rate but are not receiving any sound-related input.

**Sound Activity Embedded in Background Activity**

To imitate a situation in which sound-related activity is processed by MNTB inputs, we next embedded various high-

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**Fig. 3** Glycinergic currents persist after long-term stimulus train challenge. **A**: glycinergic IPSCs are large during the first 3 s of a 2-min Poisson-distributed stimulus train (left) and depressed but still substantial during the last 3 s of same train (center). The 2 pairs of traces show IPSCs in response to 2 different background stimulation frequencies, 5 Hz (top) and 20 Hz (bottom). Graphs on right show changes in IPSC amplitudes during the 2-min stimulus protocol. Each dot represents the amplitude of 1 IPSC plotted as a function of time within the stimulus protocol, and the red lines represent a custom exponential fit of the amplitudes. **B**: relative IPSC amplitudes after the 2-min stimulus protocol show apparent synaptic depression but are still substantial. IPSC amplitudes measured after 2 min of background stimulation were equivalent to the steady-state values, which were reached in <2 min in all cases except 5 Hz. In the case of 5 Hz steady state was not reached in 2 min, and thus both the value after 2 min of stimulation (5 Hz/2 min) and the steady-state value (5 Hz/asymp) are presented. **C**: in most cases, a steady-state value was reached by inhibitory synapses within the 2-min stimulus protocol, suggesting that this length of background stimulation was sufficient to transform synapses into an active state (fitting results). Note that a steady state was reached in each case within the first minute of the two 2-min stimulus protocol, with the exception of the 5-Hz stimulus train. All recordings were performed in the presence of DNQX, APV, and SR 95531 to block glutamate and GABAA receptors and used maximum stimulation. Significant difference: \(* P < 0.05\).
frequency trains into the ongoing background activity. Sound-evoked activity was imitated by short high-frequency trains of 50 pulses at 50, 100, and 300 Hz (which imitate putative neural activity in auditory brain stem centers caused by short tone bursts with duration times of 1, 0.5, and 0.17 s, respectively). Inhibitory inputs were stimulated with these trains while IPSC current trains from MNTB principal neurons were recorded in voltage-clamp mode. The same stimulus train was initially played at the beginning of a recording session before the start of the conditioning period (Fig. 4Ai) and then again after the conditioning period and seamlessly embedded in the background activity (Fig. 4Aii). The initial IPSC amplitude was \(~2.5 \text{ nA}\), which is equivalent to \(~42 \text{ nS}\). Over the course of the 50-pulse train, IPSCs depressed to \(~0.5–1 \text{ nA}\). When the same stimulus train was used on the same synaptic inputs after the 2-min conditioning period, most IPSC amplitudes in the train were smaller than in the unconditioned case (Fig. 4Aii, embedded train). However, note a significant synaptic facilitation between pulse 1 and pulse 4 of the embedded train. For a more detailed analysis of this phenomenon, see Fig. 6.

Figure 4B shows a second example of a high-frequency (100 Hz) IPSC train that was either recorded in isolation (Fig. 4B, top and bottom) or embedded in a train of background activity (Fig. 4B, middle). The initial IPSC amplitude was \(~4 \text{ nA}\). When the same stimulus train was used on the same synaptic inputs after the 2-min conditioning period, most IPSC amplitudes in the train were much smaller than in the unconditioned case (Fig. 4B, middle). After the conclusion of the experimental protocol on this recording, synaptic inputs were allowed to recover for \(~5 \text{ min}\), before the same 50-pulse train was tested again (Fig. 4B, bottom). Note that this recovery trace is very similar to the initial trace recorded before the start of the conditioning protocol, suggesting that the long-term stimulation protocol triggers specific and reversible mechanisms of short-term plasticity and causes no damage to the neurons (Fig. 4B, top vs. bottom).

Synaptic amplitudes in response to the first and last events of the 50-pulse IPSC trains are shown in Fig. 5A. Each group of bars represents one Poisson background frequency into which various 50-pulse trains were embedded, while each bar within a group represents one 50-pulse test train frequency. For each group of bars in Fig. 5A, the amplitude of the first three events is compared with the amplitude of the 48th–50th event of a 50-, 100-, or 300-Hz train. In the case of each conditioning frequency, the ratio of initial to last IPSCs in the train is highly reduced compared with “no conditioning” = “no background activity (no BA).” Note that for the 40-Hz and 60-Hz background activity, the 50-Hz test train was omitted, since its frequency was very similar to the two background rates. While the first group of bars (“no BA”) in Fig. 5A represents IPSCs recorded before the background activity was turned on, the last group of bars (“recov”) represents IPSCs recorded \(~5 \text{ min}\) after the completion of the background stimulation, after synapses were allowed to recover from the extended stimulation protocol. Synaptic amplitudes were not significantly different from amplitudes recorded before the start of the background stimulation, suggesting complete recovery.

The ratios for last to first IPSCs for each frequency combination are plotted in Fig. 5B. High ratios (i.e., values closer to 1) indicate that the last IPSCs in the train response were similar to the first IPSCs in amplitude, while smaller values indicate significant depression over the course of the 50-Hz test train. All ratios for all frequency combinations were significantly different from “no conditioning.” However, the frequency of the background activity did not influence the ratios significantly, indicating that background activity per se was more important than the exact frequency of the background activity.

Fig. 4. High-frequency stimulus trains embedded in low-frequency Poisson-distributed stimulus trains simulate sound-related neural activity embedded in spontaneous background firing. Ai: trace of a 100-Hz/50-pulse IPSC train recorded from a MNTB neuron before the start of the chronic long-term stimulation protocol. The brain slice was in a recovered state without any effects of short-term plasticity. Black bar indicates the train stimulation. Aii: the same train recorded from the same neuron after 2 min of 10-Hz background stimulation. The last few IPSCs recorded in response to the last second of the background stimulation (indicated by dots) are shown in the trace just before the start of the 50-Hz IPSC train (indicated by black bar), as well as the first few responses to background activity, which was resumed immediately after the end of the 50-Hz stimulus train (dots on top of the IPSCs). B: a voltage-clamp recording from a MNTB neuron whose inhibitory inputs were stimulated by a 100-Hz/50-pulse train before the start of the 2-min conditioning protocol (top), after the 2-min conditioning period (middle), and after the conclusion of the experimental protocol and a recovery time of \(~5 \text{ min}\) (bottom). All recordings were performed with maximum stimulation in the presence of DNQX, APV, and SR 95531 to block glutamate and GABA_\(A_\) receptors.
Prominent synaptic facilitation was observed in the majority of IPSC recordings and appeared to be one of the hallmarks of glycinergic afferents to MNTB. For inhibitory inputs to MNTB, facilitation seems to play a much more prominent role than for the corresponding excitation. Facilitation was measured within the first seven IPSC responses of high-frequency trains and could be observed in 106 of 146 (72.6%) of these trains (50, 100, 300 Hz). Figure 6A shows an example of an IPSC train clip that was recorded in response to a 50-Hz, 50-pulse stimulation train that was embedded in background activity (not shown). When the stimulus protocol was switched from the low-frequency background conditioning to the high-frequency test train, a first IPSC amplitude of ~1 nA was recorded. IPSC amplitudes in response to the second, third, and fourth IPSCs of the same train increased progressively, such that the amplitude in response to the fourth stimulus was almost four times the amplitude recorded in response to the first IPSC. For stimulations following the fourth pulse, IPSC amplitudes slowly decreased, presumably representing synaptic depression. Among the 106 recordings in which facilitation was observed, the largest IPSC occurred between the second and seventh IPSCs. The distribution is shown in Fig. 6B. The overall ratio of facilitation (ratio of largest IPSC at any position to first IPSC) was 4.44 ± 0.7. Conditioning with background activity or the frequency of the test trains had no significant influence on the position of the largest IPSC or the amount of facilitation (1-way ANOVA on ranks). Therefore, data were pooled.

Residual Charge

In some types of synapses, repeated stimulation results in a buildup of a tonic component through mechanisms such as asynchronous release or transmitter accumulation in the synaptic cleft (Eccles et al. 1966; Grabauskas and Bradley 2003; Lu and Trussell 2000; Scanziani et al. 1997). Such a residual charge would affect synaptic computation and spike train transformation at the MNTB in a different way than phasic, fast-decaying IPSPs would do. We therefore tested the amounts of residual charge in response to various types of repeated stimulation. Residual charge was measured during high-frequency trains (50, 100, and 300 Hz) without conditioning and after conditioning for 2 min with simulated background activity.
An example of a recording in which a buildup of residual charge can be observed during high-frequency stimulation is shown in Fig. 4Ai. This trace was recorded before the onset of background activity, and residual charge was substantial. The same train was tested on the same neuron again several minutes later when it was embedded in prolonged background activity (Fig. 4Aii). Note that now the residual charge in response to the same train was reduced substantially.

Conditioning synapses at any conditioning frequency significantly decreased residual charge for 50-Hz and 100-Hz high-frequency trains (Fig. 7B). In the case of 300-Hz high-frequency trains the residual charge tended to be reduced as well, but the reduction was significant only in the case of the 20-Hz background activity (P values: 10 Hz = 0.14, 20 Hz < 0.01, 40 Hz = 0.17, 60 Hz = 0.26). Residual charge increased significantly with the frequency of simulated sound inputs (residual charge for 50 Hz < 100 Hz < 300 Hz) in unconditioned and conditioned cells up to 40 Hz. In the case of the 60-Hz conditioning, there was trend toward a reduction (P = 0.09). However, and more importantly, when responses to equivalent high-frequency trains are compared between conditioned and unconditioned synapses, the same trains embedded in background activity produce less residual current.

Since conditioning influenced IPSC amplitudes, the ratio between instantaneous and residual charge was likely to be affected as well. Therefore the total residual charge of every high-frequency train was divided by the average amplitude of its IPSCs. Lower ratio values represent a stronger contribution of instantaneous charge to the total charge and more precisely timed inhibition. The ratio significantly increased with the frequency of the simulated sound stimuli, with 50 Hz (where available) < 100 Hz < 300 Hz under all conditions (P < 0.05, ANOVA) except for 60 Hz (P = 0.20, ANOVA).

**Number of Inhibitory Inputs**

The excitatory input into MNTB neurons, mostly mediated by the calyx of Held, is a single, giant synapse innervated by a single axon, which is either active or not active. By contrast, inhibitory inputs to MNTB are mediated by several fibers that can be differentially active during sound stimulation. A recording from such a set of inhibitory inputs to an MNTB neuron is shown in Fig. 8A. Glycinergic inputs to the principal neuron were pharmacologically isolated and stimulated in the same way as done for all experiments described here. Initially, stimulation voltage was low and was gradually increased in steps of ~2–3 V for each successive stimulation. Although the increase in stimulation voltage was gradual, the recorded IPSC amplitudes jumped from 0 to ~0.1 nA in a discrete step. Further increases in stimulation amplitude initially resulted in about the same IPSC amplitude, but at some point the observed amplitude increased, again in a discrete fashion, to ~1 nA. At least two more IPSC amplitude steps were observed when the stimulation amplitude was increased even further. Once an amplitude value of ~2 nA was reached, additional increases in the stimulus did not result in further IPSC amplitude increases, no matter how much the stimulus was increased. With maximal stimulation, an average IPSC amplitude of 3.07 ± 0.48 nA (n = 52) was observed.
A significantly when the assumed number of inputs is increased from 4 to 5, but then changes very little when 6 separate inputs are assumed. Thus, in this particular neuron, the result from the $k$-means cluster analysis suggests that 5 separate inputs were present. With this analysis, most cells showed relatively low numbers of current steps (Fig. 8C; mean $= 5.40 \pm 0.70$ steps, $n = 52$). These data suggest that a relatively low number of presynaptic neurons mediate the inhibitory input to MNTB.

**Comparison of Electrical Stimulation vs. Puff Stimulation**

Our employed method of electrically stimulating inhibitory inputs to MNTB neurons depends to some degree on the placement of the stimulation electrode. For each recording, we determined the optimal location for placement of the stimulation electrode, i.e., the location that yielded the largest inhibitory current (Fig. 1A). With this method it cannot be ruled out that some input fibers to MNTB remain unstimulated, and thus this method is likely to underestimate the true amplitude of inhibitory currents to MNTB neurons. Additionally, some input fibers may have been severed during the slicing process, adding to the underestimation of inhibitory currents.

To assess the possible effects of slice preparation and stimulation methods on the measured currents, we compared glycinergic currents activated by electrical stimulation with currents elicited by puffing glycine onto the cells via a puff electrode (Fig. 9A). By puffing glycine, receptor sites can be

In the example shown in Fig. 8A four or five apparent current steps were observed, suggesting four or five independent fibers providing inhibitory input to the MNTB neuron. A more objective and quantitative analysis to determine the number of input fibers is the $k$-means cluster analysis. For this analysis, amplitudes were assigned into clusters and the squared error was plotted against the number of clusters (Fig. 8B). The point in the curve where postulating additional clusters would not improve the squared error significantly is considered the actual number of clusters. In the particular example shown in Fig. 8B, the squared error decreases significantly when the assumed number of inputs is increased from 4 to 5, but then changes very little when 6 separate inputs are assumed. Thus, in this particular neuron, the result from the $k$-means cluster analysis suggests that 5 separate inputs were present. With this analysis, most cells showed relatively low numbers of current steps (Fig. 8C; mean $= 5.40 \pm 0.70$ steps, $n = 52$). These data suggest that a relatively low number of presynaptic neurons mediate the inhibitory input to MNTB.

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activated even if the corresponding presynaptic endings were damaged during slice preparation. Additionally, the glycine puff has a diameter of ~50–70 μm (determined by puffing dye onto the neurons, data not shown) and thus should be able to reach receptors even at more distant locations from the cell soma. Moreover, puff application of transmitter may also activate potential extrasynaptic receptors, thereby additionally increasing the measured currents.

In six neurons, puff application was compared directly to electrical stimulation. In these units, electrical stimulation was able to elicit 76.9 ± 5.7% of the current seen after glycine puffs onto the same cell (Fig. 9B; n = 6). There was a significant difference between the two stimulation methods (paired t-test, P = 0.012), and currents activated by glycine puffs were always larger than the currents elicited by electrical stimulation. However, the direct comparison between the two methods shows that the electrical stimulation approach recruits at least the majority of inhibitory inputs.

**Number of Glycinergic Synapses Observed on MNTB Neurons**

The approach presented in Fig. 8 attempted to quantify the number of glycinergeric inputs to MNTB principal neurons via an electrophysiological method. Alternatively, glycinergeric synapses to MNTB principal neurons can also be quantified with immunohistochemical methods. We used antibodies against both GlyT2 and gephyrin to label the presynaptic and postsynaptic aspects of glycinergeric synapses to MNTB neurons, respectively. The rationale of this approach was to label putative glycinergeric synapses, including those that could not be activated via either electrical stimulation or puff application.

GlyT2 antibodies label a transporter that moves glycine transmitter molecules into synaptic vesicles (Armsen et al. 2007; Atkinson et al. 2004; Liu et al. 2001) and thus labels presynaptic terminals but in some cases also afferent axons and somas of glycinergeric neurons, albeit with a weaker signal strength. Gephyrin, by contrast, is an anchor protein for both glycine and GABA receptors (Baer et al. 2003) and as such occurs in postsynaptic densities. A combination of juxtaposed GlyT2 and gephyrin labels indicates a potential glycinergeric synapse. Figure 10A shows a maximum projection of a confocal stack imaged from a section of MNTB neurons that were labeled with GlyT2 (green), gephyrin (red), and Nissl (blue); note that the red and green channels show a number of potential synaptic sites. To determine the number of glycinergeric synapses on MNTB neurons, we switched to a higher magnification (Fig. 10B, median projection of 3 single sections from the center of the soma). The MNTB principal neuron shown here was labeled with GlyT2 (green) and gephyrin (red) antibodies. The borders of the soma are indicated by the dashed line and were determined by analyzing both fluorescent Nissl label (not shown) and GlyT2 somatic label. Since a functional synapse consists of both a presynaptic and a postsynaptic terminal in close vicinity to each other, we only counted areas of juxtaposed red and green label as synapses. There are five areas near the cell membrane where juxtaposed GlyT2 (oriented toward the outside of the cell membrane, presumably marking presynaptic terminals) and gephyrin label (oriented toward the inside of the neuron, presumably marking postsynaptic terminals/receptor densities) can be detected, indicated by gray and white arrows/arrowheads. Note that these areas of juxtaposed label can be larger or smaller. The two white arrows near the left border of the image mark two large juxtaposed clusters that appear to consist of several smaller GlyT2/gephyrin clusters (Hruskova et al. 2012), while the arrowheads near the bottom and the right side of the image mark smaller clusters.

The data shown here do not address the question of whether each one of the five clusters received an independent afferent fiber input (in which case an electrophysiological experiment such as that described in Fig. 8A would yield 5 current steps) or whether one afferent fiber innervated more than one cluster (in which case the same experiment might yield fewer current steps). Therefore, in this particular example, five current steps would represent an upper limit for what would be expected from a synaptic stimulation experiment.

We counted synaptic clusters on 20 complete MNTB neurons that were immunolabeled and imaged with confocal microscopy as described above and found an average of 8.95 ± 0.76 synaptic clusters per neuron. The smallest number of clusters among 20 neurons was 4, and the largest number was 15 (Fig. 10C). Because of the specific methodological differences of the immunohistochemistry and the synaptic stimulation experiment described above, we propose that the data shown in Fig. 10C represent an upper limit for the number of glycinergeric inputs to MNTB, while the data presented in Fig. 8C represent a lower limit.

**DISCUSSION**

In this article we report on the physiological properties of glycinergeric and GABAergic inhibition to MNTB principal neurons. There are several main findings of this study.

Every MNTB neuron tested received a substantial amount of neural inhibition. Despite a low number of inhibitory input fibers, glycinergeric inhibition is large, even under conditions in which the synapses are repeatedly active for prolonged periods of time. Depressed synapses still produce substantial and largely phasic inhibitory postsynaptic conductances (IPSCs), which are in the same order of magnitude as conductances of the corresponding excitatory inputs when challenged with ongoing stimulation (Hermann et al. 2007, 2009). Synaptic facilitation is much more prominent in inhibitory inputs to MNTB neurons than it is for the corresponding calyceal excitatory inputs. Although the inhibition was mediated by both glycinergeric and GABAergic components, glycine makes a larger contribution.

While short-term plasticity is well studied in excitatory synapses, it is much less well understood in inhibitory synapses and has not been tested in inhibitory inputs to MNTB. Awatramani and Trussell (Awatramani et al. 2004) were the first to describe these inhibitory inputs to MNTB in brain slices of the rat. They found that the inhibition is comparable to the calyceal excitatory inputs in terms of conductance and could suppress spiking in MNTB neurons when active. Furthermore, they reported very fast kinetics of the glycinergeric inputs, producing postsynaptic phasic events even during short bursts of high-frequency stimulation. Finally, inhibitory inputs to MNTB consisted of a smaller GABAergic portion and a larger glycinergeric portion (Awatramani et al. 2004). Our data from the Mongolian gerbil presented here are consistent with these
earlier findings from the rat, suggesting that inhibitory inputs to MNTB neurons may be a general mammalian phenomenon. One discrepancy between our data from gerbils and Awatramani et al.’s data from rats is that they observed a significant age dependence of IPSC amplitudes while we did not. Species differences cannot be ruled out as a contributing factor, but we note that Awatramani et al. tested a much larger age range (P6–26) and observed a >10-fold increase in IPSC amplitudes over that range. We tested a much smaller age range and thus would expect to find a much smaller change in IPSC amplitudes, which might explain the discrepancy. However, both our study and the previous study report IPSC amplitudes on the order of several hundred picoamperes to several nanoamperes.

Awatramani and Trussell (Awatramani et al. 2004) did not challenge inhibitory fibers with long-term stimulus trains imitating the chronic background activity in the auditory brain stem. This is an important experiment because both the excitatory and inhibitory inputs to MNTB originate, directly or indirectly, in the contralateral cochlear nucleus, and therefore the firing patterns of these inputs are characterized by high activity levels during sound presentation and ongoing spontaneous activity in the absence of sound. In vivo, neurons of the auditory brain stem fire spontaneously, a property that results mainly from the dynamics of the transduction channels in the cochlear hair cells (Geisler et al. 1985; Hudspeth 1997; Kiang 1965; Liberman 1978; Roberts et al. 1988). Spontaneous firing can also be observed in the auditory nerve (Geisler et al. 1985; Liberman 1978) and many brain stem nuclei including the cochlear nucleus (Brownell 1975; Goldberg and Brownell 1973; Joris et al. 1994a; Schwarz and Puil 1997; Spirou et al. 1990, 2005) and MNTB (Kadner et al. 2006; Kopp-Scheinpflug et al. 2003; Smith et al. 1998; Sommer et al. 1993). The excitatory input to MNTB originates from globular bushy cells in the contralateral ventral cochlear nucleus (Held 1892, 1893; Kuwabara et al. 1991; Tolbert et al. 1982). One major source of inhibitory inputs to MNTB is the ipsilateral VNNTB (Albrecht et al. 2014; Kuwabara and Zook 1991; Thompson and Schofield 2000), which in turn receives excitatory input from globular bushy and/or stellate cells in the contralateral cochlear nucleus (Kuwabara et al. 1991; Thompson 1998; Warr 1969). Thus both inputs to MNTB originate, directly or indirectly, in the contralateral cochlear nucleus, are of auditory nature, respond to sound stimulation with ongoing firing patterns, have relatively high activity levels, and are very likely characterized by spontaneous activity. For the excitatory input, this ongoing background activity has major impacts on the dynamics of synaptic transmission (Hermann et al. 2007), but no information was available on the properties of the corresponding inhibitory input under similar conditions.

Specifically, we wanted to address the questions of 1) how severely synaptic inhibition would depress during ongoing

Fig. 10. Number of glycinergic synaptic clusters on MNTB principal neurons. A: confocal maximum projection of a section containing MNTB neurons that were labeled with glycine transporter 2 (GlyT2; green), gephyrin (red), and Nissl (blue). B: image of a medium projection of several single sections imaged from the center of a MNTB neuron. Red, gephyrin label; green, GlyT2 label. The outline of the principal neuron is indicated by the dashed line and was determined on the basis of both the fluorescent Nissl (not shown) and GlyT2 labels. Arrows and arrowheads mark clusters of juxtaposed GlyT2 and gephyrin labels, respectively, that mark putative glycinergic synapses. Note that there are 2 sizes of these clusters, larger ones (marked by arrows) and smaller ones (marked by arrowheads). All clusters can be observed directly near the cell membrane including that marked with the light gray arrowhead, which appeared near the cell membrane in a different section. C: the number of synaptic clusters observed on 20 MNTB neurons was higher than the number of inputs measured with electrical stimulation (Fig. 7) and represents an upper limit of the number of glycinergic inputs to MNTB neurons. Scale bars in A and B, 5 μm.
activity; 2) how that degree of depression compared with depression in the excitatory inputs under similar conditions, and 3) whether the phasic properties and fast decays of single IPSCs can still be observed during long-term activity.

Our findings suggest that IPSCs depress to a somewhat larger degree than the corresponding excitatory calyceal inputs do when both are chronically active (compare Fig. 3, B and C, of this report to Fig. 5D of Hermann et al. 2007). For example, when challenged with 20-Hz Poisson-distributed activity for 2 min, glycine mediated inputs depress to about one-fourth of their initial amplitudes; when challenged with 40 Hz, they depress to about one-sixth (this report, Fig. 3B). By comparison, the corresponding excitation depresses to \( \sim 46\% \) during 20-Hz stimulation and to 30% during 40-Hz stimulation of the same duration (Hermann et al. 2007, Fig. 3D). This comparison indicates that during responses to ongoing components of sound neural inhibition is still substantial but depresses to a slightly larger degree than the corresponding excitatory input. However, we also found that IPSCs facilitated to a much larger degree than the corresponding calyx of Held-mediated excitatory postsynaptic currents (EPSCs) when high-frequency trains were presented. While synaptic facilitation of EPSCs during train stimulation is typically very minor or completely absent both under typical slice conditions and under conditions when synapses are chronically active (Hermann et al. 2007, 2009; Taschenberger et al. 2005; Taschenberger and von Gersdorff 2000), IPSCs facilitated, on average, 4.4-fold at the onset of high-frequency trains (Fig. 6). We observed this facilitation primarily during the first few responses of a high-frequency train embedded in background activity, equivalent to the early part of a response to a sound stimulus embedded in spontaneous background activity, and then subsiding during the later part of the same response.

The specific temporal properties of facilitation in combination with the properties of depression during the later portion of responses to the same sound suggest dynamic and complex changes in the contributions of excitatory and inhibitory currents to the total synaptic current throughout auditory responses. Complex sounds such as speech sounds or vocalizations result in complex activity trains with changing interspike intervals at both the excitatory and inhibitory afferents to MNTB. Even if the firing patterns at the two inputs were identical, the differential properties in short-term plasticity would result in differential fluctuations in the strength of the two inputs.

We found that IPSCs have largely phasic properties and fast decays, especially during long-term stimulation. While fast kinetics are one of the hallmarks of many inhibitory projections in the auditory brain stem, one question generally not addressed by these studies was whether the chronic release of neurotransmitter over prolonged periods of time might eventually result in an accumulation of transmitter molecules in the synaptic cleft, which would lead to more tonic or residual components of the inhibition. In other words, do IPSCs become more and more tonic during ongoing stimulation mimicking in vivo activity? Our results suggest that this is not the case and, moreover, ongoing activity reduces residual current during high-frequency stimulation. The most likely reason for this decrease is that prolonged activity results in chronic synaptic depression, i.e., the release of fewer vesicles per unit time compared with control (resting) conditions. Thus, even after being challenged with long stimulus trains, glycine mediated inhibition to MNTB remains largely phasic and decays fast.

A recently published study by Kramer et al. (2014) investigated short-term plasticity at inhibitory synapses between MNTB and one of its upstream targets, the lateral superior olive (LSO). One of the findings of that study is that glycine mediated synapses at LSO can also be challenged with thousands of stimuli and respond to these ongoing stimulus trains reliably and with phasic currents. On the other hand, in contrast to MNTB glycine afferent synapses, MNTB glycine efferent synapses do not facilitate substantially (Kramer et al. 2014).

While calyx of Held-derived excitation is mediated by one single giant excitatory synapse driven by a single afferent axon, our data suggest that inhibitory inputs to MNTB neurons are mediated by, on average, five to nine input fibers. The lower number was determined by electrophysiology, while the higher number was determined by immunohistochemistry. Because of the experimental limitations of both techniques, one technique (electrophysiology) tends to underestimate the true number of physiological inputs while the other technique (immunohistochemistry) tends to overestimate the number of inputs. For example, electrical stimulation of inhibitory fibers in a brain slice may not stimulate all input fibers, or some input fibers may have been severed during the slicing process, and thus underestimate the true number of inputs. To address this caveat, we compared synaptic amplitudes evoked by electrical stimulation to amplitudes evoked by puff application of glycine in a number of neurons. The puff application yielded slightly higher currents, which might indicate that someafferent fibers were not activated by the electrical stimulation. However, note that the puff application might also have activated extrasynaptic glycine receptors, which would also lead to larger current measurements.

By comparison, our immunohistochemical analysis counted synapses without determining how many synapses may be connected to the same input fiber, thereby almost certainly overestimating the number of independent input fibers. Consistent with these limitations, one method yielded an average of five inputs while the other method yielded an average of nine inputs, suggesting that the true number of input fibers might be between these two values.

The relatively small number of input fibers, with each fiber contributing a substantial portion to the total inhibition, suggests that inhibitory inputs to MNTB are recruited from a small subset of source neurons, possibly restricted to a single tonotopic layer. At the same time, the observation that several fibers mediate glycine mediated inputs raises the question of whether all inputs are activated at the same time or whether different subsets of these fibers may be activated under certain behavioral or contextual conditions. The electrical stimulation protocol used for the majority of experiments was “maximal stimulation,” i.e., stimulus voltages were chosen large enough to stimulate all input fibers that can be stimulated electrically, with the goal to recruit the sum of all inhibitory inputs to MNTB. While we are confident that maximum stimulation was experimentally accomplished in all recordings, we cannot rule out the possibility that some input fibers might have failed after prolonged exposure to ongoing chronic stimulation. In particular, the prolonged stimulation with 60-Hz background activity seemed to be somewhat challenging to the input fibers, with
some stimulation failures occurring after prolonged stimulation with this protocol. By comparison, the excitatory calyceal input to the same neurons responds to prolonged stimulation of the same frequency with extremely high reliability (Hermann et al. 2007). If some fibers failed in response to a stimulus, our data might underestimate the true amplitude of inhibitory inputs to MNTB. Such intermittent fiber stimulation failures would also increase the variability of IPSC amplitudes beyond what would be expected as a result of short-term plasticity and vesicle release dynamics.

It is unclear whether sound stimulation recruits all input fibers simultaneously in vivo in the same way as the maximum stimulation protocol does, or only a subset of these fibers. If only a subset of inhibitory fibers are recruited in vivo, our data might overestimate the impact of inhibitory inputs on MNTB neurons. However, we speculate that at least the majority of these fibers must be recruited by sound stimulation, given that these inputs are of sensory nature and respond to external stimuli with high temporal precision, and given that blocking glycinergic inputs modifies spike trains in vivo (Tolnai et al. 2008).

In summary, the data presented here suggest that inhibitory inputs gain importance in shaping the responses of MNTB neurons when considered under physiologically relevant conditions and are therefore crucial for our understanding of mechanisms underlying processing of auditory information.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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