Synaptic Transmission at the Calyx of Held Under In Vivo–Like Activity Levels

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Hermann J, Pecka M, von Gersdorff H, Grothe B, Klug A. Synaptic transmission at the calyx of Held under in vivo–like activity levels. J Neurophysiol 98: 807–820, 2007. First published May 16, 2007; doi:10.1152/jn.00355.2007. One of the hallmarks of auditory neurons in vivo is spontaneous activity that occurs even in the absence of any sensory stimuli. Sound-evoked bursts of discharges are thus embedded within this background of random firing. The calyx of Held synapse in the medial nucleus of the trapezoid body (MNTB) has been characterized in vitro as a fast relay that reliably fires at high stimulus frequencies (≤800 Hz). However, inherently due to the preparation method, spontaneous activity is absent in studies using brain stem slices. Here we first determine in vivo spontaneous firing rates of MNTB principal cells from Mongolian gerbils and then reintroduce this random firing to in vitro gerbil brain stem synapses at near-physiological temperature. After conditioning synapses with afferent fiber stimulation for 2 min at Poisson averaged rates of 20, 40, and 60 Hz, we observed a number of differences in the properties of synaptic transmission between conditioned and unconditioned synapses. Foremost, we observed reduced steady-state EPSC amplitudes that depressed even further during an embedded short-stimulation train of 100, 300, or 600 Hz (a protocol that thus simulates in vitro what probably occurs at the in vivo MNTB after a short sound stimulus in a silent background). Accordingly, current-clamp, dynamic-clamp, and loose-patch recordings revealed a number of action potential failures at the postsynaptic cell during high-frequency–stimulation trains, although the initial onset of evoked activity was still transmitted with higher fidelity. We thus propose that some in vivo auditory synapses are in a tonic state of reduced EPSC amplitudes as a consequence of high spontaneous spiking and this in vivo–like conditioning has important consequences for the encoding of signals throughout the auditory pathway.

INTRODUCTION

The calyx of Held is a large synaptic terminal innervating principal neurons of the medial nucleus of the trapezoid body (MNTB) (Forsythe 1994; Held 1893; Kuwabara et al. 1991; Smith et al. 1991). MNTB neurons sign-invert calyceal excitation into glycinergic inhibition to various nuclei in the auditory brain stem (Banks and Smith 1992; Bledsoe et al. 1990; Moore and Caspary 1983; Spangler et al. 1985; Thompson and Schofeld 2000). In vitro, the signal derived from the calyx generates large excitatory postsynaptic currents (EPSCs) with a short synaptic delay (Barnes-Davies and Forsythe 1995; Borst and Sakmann 1996; Sakaba and Neher 2001; Taschenberger et al. 2002). Speed and fidelity of synaptic transmission are considered very reliable up to several hundred Hertz in mature animals (Futai et al. 2001; Joshi et al. 2004; Taschenberger and von Gersdorff 2000; Wu and Kelly 1993), leading to a view of the calyx of Held as a very reliable relay synapse.

All the in vitro work mentioned earlier was performed in brain slices. Inherently, auditory brain slice preparations differ from intact brains in various parameters, including spontaneous activity. In vivo, neurons of the auditory brain stem fire spontaneously at frequencies that vary from >1 to ≥100 Hz, 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), resulting in spontaneous firing of the auditory nerve (Geisler et al. 1985; Liberman 1978). Spontaneous firing can also be observed in many brain stem nuclei including the cochlear nucleus (Brownell 1975; Goldberg and Brownell 1973; Joris et al. 1994; 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).

In an intact brain, MNTB neurons fire spontaneously at levels, which might be suitable to chronically induce some forms of short-term plasticity, such as synaptic depression or facilitation (Schneegans et al. 2002; von Gersdorff and Borst 2002). Sound stimuli, i.e., streams of high-frequency activity embedded in this spontaneous firing (Klyachko and Stevens 2006), would then be processed by the synapse on the background of chronic depression and/or facilitation (Fig. 1A). Because of the nature of the brain slice preparation, spontaneous activity and its potential effects on short-term plasticity might be lost in standard in vitro recordings (Fig. 1B). If that were the case, properties of synaptic transmission in the calyx of Held under in vivo conditions may be different from those commonly observed in vitro.

This study investigates synaptic transmission in the calyx of Held under in vivo–like spontaneous activity levels. We first measured the rates and statistical properties of spontaneous firing in the MNTB of Mongolian gerbils (Meriones unguiculatus) in vivo. Subsequently, we stimulated the afferent fibers that give rise to calyceal inputs in gerbil MNTB brain slices at physiological temperature for prolonged periods of time with stimuli that mimicked the random spontaneous activity as closely as possible (Fig. 1C). We assessed changes in synaptic transmission resulting from this long-term stimulation, such as synaptic currents, the degree of depression, recovery from depression, and finally the spiking properties of “spontaneously active” neurons.
SURGICAL PROCEDURES. Before surgery, animals were anesthetized by an initial intraperitoneal injection (0.5 ml/100 g body weight) of ketamine (20%) and xylazine (2%, both in physiological NaCl). During surgery and recordings, a dose of 0.05 ml of the same mixture was applied subcutaneously in scheduled intervals that were based on the animal’s body weight. Constant body temperature was maintained using a thermostatically controlled heating blanket. Skin and tissue covering the upper part of the skull were removed and a small metal rod was mounted to the frontal part of the skull using UV-sensitive dental-restorative material (Charisma, Heraeus Kulzer, Hanau, Germany). Custom-made earphone holders were attached to the gerbil head, allowing for the safe insertion of earphones or probe tube microphones into the ear canal. The animal was then transferred to a sound-attenuated chamber and mounted in a custom-made stereotaxic instrument (Schuller et al. 1986). The animal’s position in the recording chamber was standardized with reference to stereotaxic landmarks on the skull (Loskota et al. 1974). For electrode penetrations to the MNTB, a small hole of approximately 1 mm² was cut into the skull lateral to the lambdoid suture. Micromanipulators were used to position the recording electrode according to landmarks on the brain surface and a reference point, which was used for all penetrations. The meninges overlying the cortex were removed and saline was applied to the opening to prevent dehydration of the brain.

Typical recording sessions lasted 10–14 h. After successful recordings, the animal was killed by injection of an overdose of chloral hydrate (Sigma–Aldrich Chemie, Munich, Germany). The last electrode position was then marked with a current-induced lesion (20 µA for 80–120 s). The head was fixated in 4% paraformaldehyde and prepared for anatomical processing. Transverse sections were cut and Nissl-stained to verify the recording sites. An example of an anatomical verification is shown in Supplemental Fig. 1C. The lesion site can be clearly seen in the center of the left MNTB.

RECORDINGS OF NEURAL ACTIVITY. Single-unit responses were recorded extracellularly using 10-MΩ glass electrodes filled with 1 M NaCl. The recording electrode was advanced under remote control, using a piezodrive (Inchworm controller 8200, EXFO Burleigh Products Group, Victor, NY). Extracellular action potentials were recorded by an electrometer (npi electronics, Tamm, Germany or Electro 705, WPI, Berlin, Germany), a 50/60-Hz noise eliminator (Humbug, Quest Scientific Instruments, North Vancouver, BC, Canada), a band-pass filter (VBF/3, Bortolin Kemo, Porcia, Italy), and an amplifier (model 7607, Toeltnner Electronic Instrumente, Herdecke, Germany) and subsequently fed into the computer by an A/D-converter (RP2-1, Tucker-Davis Technologies (TDT), Alachua, FL). Clear isolation of action potentials from a single neuron (signal-to-noise ratio >5) was achieved by visual inspection on a spike-triggered oscilloscope and by off-line spike-cluster analysis (Brainware, TDT). Two examples of recorded single-cell spike waveforms are shown in Supplemental Fig. 1, A and B. The unit in supplemental Fig. 1A is an example of a neuron with a low spontaneous rate (10 Hz), whereas supplemental Fig. 1B shows an example of a neuron with a very high spontaneous rate (107 Hz). Both units were recorded from the same animal; histological verification of the recording site is shown in Supplemental Fig. 1C.

STIMULUS PRESENTATION AND RECORDING PROTOCOLS. Stimuli were generated at a 50-kHz sampling rate using TDT System III.

The online version of this article contains supplemental data.
Digitally generated stimuli were converted to analog signals (DA3-2/RP2-1, TDT), attenuated (PA5, TDT), and delivered to earphones (MDR-EX70LP, Sony, Berlin, Germany).

The standard stimulus was a 200-ms toneburst with a rise/fall time of 5 ms, presented at a repetition rate of 2 Hz. Stimulus presentation was randomized. To search for acoustically evoked responses, noise stimuli were delivered binaurally. When an auditory neuron was encountered, we first determined its best frequency (BF) and absolute threshold audiovisually to set stimulus parameters subsequently controlled by the computer. The frequency that elicited responses at the lowest sound intensity was defined as BF, the lowest sound intensity evoking a noticeable response at BF as threshold. These properties were confirmed by off-line analysis of the frequency versus level response areas. Monaural pure tones to each ear and binaural pure tones without interaural intensity or time differences were presented to define the aurality of the neuron. MNTB neurons responded to stimulation of the contralateral ear only, with a tonic/primary-like firing pattern, and were not affected by stimulation of the ipsilateral ear.

Spontaneous activity of a neuron was determined by recording action potentials in several 5-s-long intervals without sound stimulation and averaging the measured firing rate. All quantifications in this study are based on off-line analysis with the software packages Brainware (TDT), Matlab (The MathWorks, Natick, MA), and IGOR (WaveMetrics, Lake Oswego, OR).

In vitro recordings

Slices of brain stem were prepared from Mongolian gerbils (Meriones unguiculatus) aged 14 to 19 days (posthearing animals). Data from these different ages were pooled because no age-dependent variations in synaptic amplitudes, degree of depression, response to conditioning, or firing probability were observed (data not shown).

SLICE PREPARATION. Animals were briefly anesthetized by isoflurane inhalation (Isofluran Curamed, Curamed Pharma, Karlsruhe, Germany) and decapitated. The brain stem was dissected out under ice-cold dissection ringer (125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, NaHCO₃, 0.4 mM ascorbic acid, 3 mM myoinositol, 2 mM pyruvic acid; all chemicals from Sigma–Aldrich). Sections (200 – 250 μm) were cut with a vibratome (VT1000S, Leica, Wetzlar, Germany) and bubbled with 5% CO₂-95% O₂.

WHOLE CELL RECORDINGS. All recordings were performed at 36 – 37°C, after which the chamber was saturated with a 5-MΩ bipolar stimulation electrode (matrix electrodes with 270-μm distance; FHC, Bowdoinham, ME). Stimuli were 100-μs-long square pulses of 10 to 40 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 stimulus permits 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 20-, 40-, and 60-Hz Poisson-distributed stimulus trains (see Figs. 1 and 2, C and E). Sound-evoked activity was simulated by short trains consisting of 20 stimuli at 100, 300, or 600 Hz.

CONDUCTANCE-CLAMP EXPERIMENTS. Excitatory conductances were simulated with an SM-1 amplifier (Cambridge Conduction, Cambridge, UK). The 10–90% rise of the current output in response to a voltage change for this amplifier was 290 ns. Reversal potentials were set to 0 mV for the excitatory postsynaptic conductances (EPSCs). The conductance wavesforms used were previously recorded as EPSCs in voltage-clamp mode. After extrapolating the artifacts, EPSGs were normalized. All conductance values correspond to peak conductances. In experiments in which background leak was added, a constant step command was fed from the computer into the conductance-clamp amplifier by a separate channel and the reversal potential for this channel was set to ~60 mV. The separately calculated output of both channels was added together and fed to the HEKA amplifier.

STATISTICAL ANALYSIS. Data were analyzed in IGOR 5 (WaveMetrics), MS Excel 2004 (Microsoft, Redmond, WA), and Matlab 7 (The MathWorks). Unless otherwise noted, all errors are reported as standard error. Statistical significance was tested with a Student’s t-test, unless otherwise noted. Significant differences are marked with a single asterisk for values of P < 0.05 and with a double asterisk for P < 0.01.

RESULTS

In vivo spontaneous firing rates of MNTB cells

The first goal was to determine the spontaneous firing rates of MNTB neurons in the intact brain of Mongolian gerbils (Meriones unguiculatus). Neural activity was recorded in vivo from single cells in the MNTB with standard extracellular recording techniques. When a neuron was encountered and isolated, its basic response features such as aurality, auditory threshold, and frequency tuning were assessed. Among the 36 neurons from which activity was recorded, thresholds for sound stimulation ranged from 0 to 60 dB SPL (mean = 32 ±
2.8 dB SPL) and characteristic frequencies were between 486 Hz and 16.8 kHz. Consistent with known input patterns to the MNTB, all neurons could be excited when sound was presented to the ear contralateral to the recording site. None of the neurons showed any effects of ipsilateral stimulation.

After a neuron’s basic response properties to auditory stimulation were assessed, its spontaneous firing rate in the absence of sound stimulation was measured over 50 s and average discharge rates were calculated and defined as the neuron’s spontaneous firing rate. Among the 36 neurons, spontaneous firing rates ranged from 0.15 to 110 Hz (Fig. 2A). The mean spontaneous rate was 24.9 ± 5.5 Hz. Short clips of spike trains are shown in Fig. 2B. The spontaneous rates of these neurons were 16, 40, and 69 Hz, respectively. An analysis of the interspike intervals (ISIs) revealed that the spikes are near-Poisson distributed with the exception that very short ISIs (<1 ms) are underrepresented (three ISI histograms in Fig. 2C).

Introducing spontaneous rates into slice preparations of the MNTB

Based on these in vivo data, three representative frequencies of spontaneous rates were chosen for stimulation of the in vitro brain slice preparations: 20, 40, and 60 Hz (Fig. 2D). Distribution of the spike events in each one of these trains was chosen to be near-Poisson, to imitate the in vivo spontaneous activity as closely as possible (Fig. 2E). MNTB calyceal input fibers were stimulated with these spike trains for prolonged periods of time (≥2 min) and voltage-clamp recordings were performed from MNTB principal neurons. During the 2-min conditioning, 7,200 Poisson-distributed stimuli were presented in case of the 60-Hz train, 4,800 stimuli in the case of the 40-Hz train, and 2,400 stimuli in the case of the 20-Hz conditioning train.

Effects of “spontaneous” firing on excitatory synaptic currents in the calyx of Held

At the beginning of each experiment, a synapse was “rested” or completely recovered, i.e., no stimuli had been given to the input fibers for ≥5 min. During the 2-min conditioning period with Poisson-distributed activity, EPSCs depressed substantially with at least two exponential components. The three graphs in Fig. 3, A–C show EPSC amplitudes of three different neurons in response to 2-min conditioning stimuli at 20, 40, and 60 Hz (Fig. 3, A, B, and C, respectively). Each dot in the graphs represents the amplitude of one EPSC and the solid lines represent double-exponential fits.

The initial EPSC amplitudes in the three examples were between about 5 and 9 nA, fairly typical values of rested calyx of Held/MNTB recordings in animals of this age group (e.g., Taschenberger and von Gersdorff 2000; von Gersdorff and Borst 2002). We term this value the “initial amplitude” or “A₀.” The synaptic current then depressed substantially during the first few events of the stimulus train (insets in Fig. 3, A–C, initial steep declines of amplitudes), then declined much slower (later shallow decline of amplitudes), and then stabilized during the second half of the 2-min train to values between about 2 and 3 nA.

We were interested in these steady-state values measured during the second half of the conditioning period because

![FIG. 2.](9f2.png)
we hypothesize that these values represent the normal state of the "rested" synapse in vivo. The reason is that an in vivo gerbil calyx of Held presumably would fire spontaneously at frequencies similar to the conditioning frequencies used in this experiment and thus synaptic currents, even in the absence of any sound input, would be chronically depressed to values similar to those measured during the steady-state period of the conditioning phase. Therefore we term these steady-state current values the "in vivo initial amplitude" or "in vivo A_{0} ."

Figure 3D compares "rested A_{0} " values to "in vivo A_{0} " values for our sample of neurons, suggesting that typical synaptic amplitudes in the calyx of Held might be much smaller in vivo than those observed in standard in vitro experiments.

To assess the effects of the Poisson distribution, five neurons were tested with trains composed of Poisson-distributed stimuli...
versus trains with regularly spaced stimuli of identical frequencies. The type of stimulus train did not affect the time course of synaptic depression or the value of the observed in vivo $A_0$ (Fig. 3, E and F). The difference of $A_0$ values within each pair was not statistically different ($t$-test, $P = 0.74$). However, the variability of synaptic currents was much larger in the case of Poisson-distributed stimuli compared with evenly spaced stimuli (Fig. 3, E and F, Gaussian curve insets). On average, the standard deviation of synaptic currents was 0.04 when stimuli with regularly spaced intervals were used. Presumably, one physiological basis of this variability is the stochastic nature of vesicle release. In contrast, the SD of current amplitudes was 0.08 when stimuli were Poisson distributed. Most likely, the reason for this larger variability is the additional effect of changing interspike intervals, which is added to the variability caused by stochastic release. However, note that the type of stimulus train used does not appear to affect the final observed in vivo $A_0$.

**Effects of simulated tone bursts on “rested” versus “spontaneously” active synapses**

Our next goal was to determine the effects of the “spontaneous” activity on the processing of high-frequency trains by the calyx of Held synapse. The high-frequency trains attempt to simulate simple sound-evoked activity, such as short tones. We tested trains of 20 pulses at 100, 300, and 600 Hz, which simulated pure tones of 200-, 67-, and 33-ms duration, respectively. Effects of each stimulus train were tested before the conditioning period, i.e., on the “rested” synapse, and then after the conditioning period while the simulated tone activity was embedded in the “spontaneous activity,” and a third time ±5 min after the “spontaneous” stimulation was stopped. Figure 4A (black trace) shows an EPSC train recorded in response to a 300-Hz, 20-pulse-stimulus train from a “rested” neuron. The synaptic current measured in response to the first event was about 6.9 nA. Subsequently, the synaptic current depressed during the stimulus train, such that the current measured in response to stimulus number 20 was depressed to 1.6 nA, i.e., the EPSC was now only 23% of the initial current.

After the synapse had been conditioned with “spontaneous” activity of 60 Hz, the synaptic current in response to the first stimulus of the same 300-Hz train was about 2.1 nA and thus substantially lower than that in the “rested” condition (Fig. 4B, first event). More interestingly, the relative depression induced by the 300-Hz train was substantially less than it was under control conditions, such that the synaptic current at stimulus number 20 was still about 1.1 nA. Therefore in the precondi-

Synaptic amplitudes in response to the first and last events of the various 20-pulse-test trains are shown in Fig. 4C. For each group of bars, the amplitude of the first event (dark gray bar) is compared with the amplitude of the 20th event after a 100-

![FIG. 4.](image-url)
300-, and 600-Hz stimulus train. Note that the difference in amplitudes between event 1 and event 20 is greatest in unconditioned synapses and smallest in synapses that have been conditioned with 60 Hz of Poisson activity.

Figure 4D shows the amount of relative synaptic depression induced by the various 20-pulse trains. The bars represent the ratio of the 20th over the first postsynaptic current amplitude of the train; i.e., small values indicate that at event 20 only a small portion of the initial synaptic current was measured and therefore synaptic depression caused by the train was substantial. High values indicate that the high-frequency trains induced a much smaller amount of relative depression because the current measured at event 20 of the train was more similar to the initial current.

Overall, the 100-Hz trains induced the lowest amount of synaptic depression (dark gray bars) and the 600-Hz trains the highest amount (white bars). More interestingly, the same high-frequency train induced a much smaller amount of relative depression when the synapse was previously conditioned with spontaneous activity. In all cases the high-frequency trains induced a significantly smaller amount of relative synaptic depression when the synapses were previously conditioned with “spontaneous activity.”

Recovery of firing is very fast under in vivo–like conditions

Our next goal was to determine the recovery from synaptic depression in “spontaneously active” synapses. Recovery from depression is a critical property, especially in highly active auditory brain stem synapses, because the speed of recovery determines how well the neuron can respond to acoustic events that occur shortly after the first event. We first measured the recovery of firing patterns of MNTB neurons in vivo (Fig. 5A). Two identical tone bursts of 200-ms duration were presented to single MNTB units with a variable pause between them. The first tone burst elicited a certain firing rate and firing pattern in the neuron. When the second, identical tone burst was presented after only a very short pause, it elicited a lower response rate in the neuron, which was most apparent during the onset portion of the response (Fig. 5A, top). As the pause between the two tones was increased, neural responses to the second tone recovered progressively and at some point resembled the responses measured to the first tone (Fig. 5A, bottom). The in vivo recovery time course of six MNTB neurons is plotted in Fig. 5B. Among these six neurons, the average in vivo recovery time constant was 82 ± 23 ms, suggesting that recovery of neural responses in the MNTB to acoustic stimuli in vivo is typically very short.

This finding raises a dilemma because recovery from synaptic depression has been measured in the calyx of Held in vitro, with very different results. In these experiments, the presynaptic vesicle pool was depleted, either with a depleting high-frequency stimulus or by voltage clamping the presynaptic terminal to a positive potential. After this pool depletion, test stimuli were given at distinct time intervals to assess the degree of recovery. These experiments typically found recovery time constants at the order of several seconds, not milliseconds (e.g., von Gersdorff et al. 1997; Wang and Kaczmarek 1998).

The in vivo data and the in vitro data are not directly comparable because of additional recoveries at the level of the hair cells, auditory nerve (e.g., Spassova et al. 2004), and cochlear nucleus, as well as potential effects of inhibition. However, the in vivo recovery shown in Fig. 5B has to present an upper limit for the vesicular recovery at the level of the calyx of Held because the calyx of Held is one element of the network tested with the in vivo experiment. Therefore the in vivo data suggest that recovery from synaptic depression at the calyx of Held should occur with time constants of no longer than about 80 ms.
**Recovery from depression is very fast in spontaneously active synapses**

To test this, we measured recovery from depression in vitro. Recovery time constants were determined initially in unconditioned neurons by depleting the vesicle pool with a high-frequency train, then allowing the synapse to recover for a specified amount of time, and finally measuring the relative amplitude of a test EPSC. An example of a synapse in which the time course of recovery was determined with this method is shown in Fig. 6, A and B. Figure 6A shows data points on a magnified time axis, following a recovery time course that was best described with an exponential that had a time constant of 72 ms. The average fast time constant of our sample of neurons was $87 \pm 16$ ms. However, the fast time constant accounted for only about half of the recovery. Complete recovery to the average value presented in Fig. 6 is shown in Fig. 6A was $1.84$ s (Fig. 6B), whereas the average slow time constant of our sample of neurons was $1.59 \pm 0.17$ s. In each case tested, the two time constants together could account for the complete amplitude of the rested $A_0$ value.

Because of the nature of the experimental protocol, recovery from depression could not be measured in conditioned synapses with the same method as used earlier because the required time intervals (up to several seconds) would be far longer than the amount of time that a neuron is nonactive during “spontaneous activity”. Therefore the time course of recovery in conditioned neurons was measured by fitting an exponential function to the time course of EPSC amplitude recovery as a function of the preceding interpulse interval. The various interpulse intervals, which inherently occur during a Poisson-distribution of spikes, yield a suitable range of time periods to measure the fast recovery time constant. When this was done, we found fast time constants very similar to those found in unconditioned synapses. Figure 6C shows an example of a cell in which recovery from depression was measured with the described method. For events where the test stimulus followed shortly after a previous stimulus, the EPSC amplitude of the test EPSC was small. As the time interval before the test stimulus increased, the amplitude of the EPSC progressively increased, presumably as a result of recovery from depression. For this synapse, the time constant of recovery from depression was 74 ms. The average recovery time constant for our sample of neurons was $90 \pm 15$ ms. As for the unconditioned synapse, the relative contribution of the fast time constant to overall recovery accounted for about half of the rested $A_0$ amplitude. Therefore we assume that the (missing) slow component of recovery in conditioned synapses might be similar to that of unconditioned synapses, although we were unable to measure this parameter for the reason described earlier.

The fast recovery time constants of both unconditioned and conditioned synapses are very similar to the recovery time constants measured in vivo shown in Fig. 5, suggesting that recovery from activity in the calyx of Held occurs at a time course similar to that of the recovery of other components of the circuit. Although we also found a slow component of recovery that was in a range similar to that described previously by other groups, our in vivo data suggest that the

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** A and B: recovery from depression in unconditioned synapses. Data were obtained with a protocol that depletes the vesicle pool with a 300-Hz, 20-pulse-stimulus train, followed by a pause of variable time to allow for pool refilling, followed by a test stimulus. Amplitudes plotted in graphs refer to the amplitudes measured in response to the test stimulus. Recovery could be best described with double-exponential fits that had fast time constants of $87 \pm 16$ ms and slow time constants of $1.59 \pm 0.17$ s. C: recovery from depression in conditioned synapses. In contrast to A and B, recovery was assessed by analyzing the variable ISIs inherently contained in the Poisson-distributed trains. Only the fast component was measured here and was found to be very similar to the fast component of unconditioned synapses. D: illustration of recovery from depression in active synapses, which happens within milliseconds. In vivo $A_0$ point is indicated at time = 0; the amplitude of the bar is based on data in Fig. 3D. This amplitude represents the state of a synapse after it has been conditioned with a 60-Hz Poisson train for several minutes, but has not received a high-frequency stimulation. Presumably, this situation compares to the state of a synapse in an intact brain, while the animal is not receiving sound stimulation. Typical values for synaptic currents after 100-, 300-, and 600-Hz 20-pulse stimulus trains are presented in the graph. These amplitudes presumably compare to the state of a synapse after a short tone burst has just been played to the animal. Naturally, synaptic amplitudes are lower than those in the in vivo $A_0$ state, as a result of the recent high-frequency activity. Position of the bars along the x-axis is chosen such that the respective amplitudes correspond to the value of the exponential curve at the same time. Time of recovery indicates the time it would take for the synapse to recover in vivo from one of the mentioned 20-pulse-stimulus trains back to the in vivo $A_0$ point. Time constant of exponential = 90 ms, corresponding to the average value presented in Fig. 6C; values of bars correspond to last group of bars in Fig. 4C.

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short time constant might be the dominant one for in vivo recovery.

This point is further illustrated in Fig. 6D. The general idea of this cartoon is that recovery in vivo does not proceed up to the point of a completely full pool, i.e., the rested A_0 seen in silenced synapses in brain slices. Rather, in vivo, recovery of the calyx progresses up to the in vivo A_0, the value of synaptic current that is typically available to a spontaneously active and thus chronically depressed synapse for the processing of high-frequency sound stimuli. The graph in Fig. 6D plots a typical exponential time course of recovery with a time constant of 90 ms, as determined earlier. The typical “in vivo A_0” point of a synapse, which is spontaneously active at 60 Hz without acoustic input, is marked at time 0 (value = 29% of the rested A_0; see Fig. 3D). When the synapse now processes a tone burst of 20 pulses at 100, 300, or 600 Hz, the synaptic amplitude depresses even further, typically to the values indicated by the respective bars (values of amplitudes based on data from Fig. 4D). At the end of the tone burst, the synapse recovers back to the in vivo A_0 value, which takes longer or less long, depending on the frequency of the tested stimulus and the resulting depression. In this example, which assumes an exponential time course of 90 ms for vesicle pool refilling, the recovery of the synapse back to the in vivo A_0 point would take between 25 and 74 ms. In other words, a spontaneously active synapse might need only 25–74 ms to recover from a high-frequency sound input because the synapse is chronically depressed by the spontaneous activity and thus recovers only partially back to the steady-state level.

**Reduced synaptic reliability in active calyces**

The data presented so far suggest that synaptic currents produced by the calyx of Held under simulated in vivo conditions are considerably smaller than those typically measured in vitro in “rested” or silent brain slices. The very large synaptic currents produced by rested calyces in older animals are known to bring postsynaptic neurons well above threshold for firing action potentials in response to each synaptic event—when the slice was rested. However, after the neuron was conditioned with 60 Hz of spontaneous activity, the number of failures during the 300-Hz train increased (Fig. 7H, black solid line and closed circles, n = 5). However, when synapses were conditioned with 60 Hz of spontaneous activity, the number of failures during the 300-Hz train increased (Fig. 7H, black solid line and closed circles, n = 5). In most cases, postsynaptic neurons still answered reliably during the initial three or four events of the train, although the reliability declined afterward.

The probability of a failure to occur increased with the number of the event in the 20-pulse train. Events early in the train were less likely to fail than events in the latter part of the stimulus train.

Among our sample of neurons tested with this method, we observed some variability in the number of failures as well as other response characteristics. For example, some neurons showed fewer failures, whereas others showed a higher number. Also, in some neurons stimulation of the fiber pathway with 300 Hz resulted in a small plateau from which action potentials to the stimuli were fired (Fig. 7A), whereas the plateau was absent in other neurons. Also, we observed some variation in the height of the action potential. Differences in channel complement or best frequency to which the neuron was tuned might account for this variability.

For our sample of neurons, the probability of a postsynaptic spike was tested as a function of the position of the event in the train. Firing probability was defined as the number of spikes fired in response to a given stimulus in the train over the number of repetitions presented. In the unconditioned synapse, the firing probability was almost 100% throughout the 300-Hz stimulus train; very few failures occurred toward the end of the stimulus train (Fig. 7H, black dotted line and open circles, n = 5). Among our sample of neurons tested with this method, we observed some variability in the number of failures as well as other response characteristics. For example, some neurons showed fewer failures, whereas others showed a higher number. Also, in some neurons stimulation of the fiber pathway with 300 Hz resulted in a small plateau from which action potentials to the stimuli were fired (Fig. 7A), whereas the plateau was absent in other neurons. Also, we observed some variation in the height of the action potential. Differences in channel complement or best frequency to which the neuron was tuned might account for this variability.

The reliability of synaptic transmission was also tested with conductance-clamp recordings. The advantage of conductance clamp was twofold: First, this method does not rely on the presynaptic axons and the calyx to follow the intense stimulation protocol. Therefore synaptic failures arising from axonal failures can be ruled out more reliably than with the technique presented earlier. Second, conductance clamp offers the possibility of combining simulated synaptic currents with added background leaks to reduce the neuron’s input resistance (see following text). In our recordings, EPSG waveforms derived from 300-Hz EPSC trains were used to simulate calyceal synaptic currents in response to a 300-Hz stimulus train. Two waveforms were used in these experiments: a 300-Hz, 20-pulse EPSG waveform that simulated synaptic currents of a rested calyx (Fig. 7C, bottom) and an EPSG waveform that simulated the response of a calyx to the same stimulus train embedded in spontaneous activity (Fig. 7D, bottom). Both waveforms were previously recorded as EPSG waveforms from an MNTB neuron under voltage-clamp conditions, while electrically stimulating the input fibers to the calyx. The waveforms were chosen to reflect the observation that peak EPSG currents are larger in rested than in conditioned synapses (Fig. 3D) and that synaptic depression within a high-frequency train is reduced in active synapses (Fig. 4, A and B). Conductance-clamp recordings with these two waveforms were performed on seven MNTB neurons. The neurons reliably fired action potentials when excitatory synaptic currents typical for a rested calyx were injected (Fig. 7C, top). In the rare case that failures could be observed, they occurred toward the end of the train (Fig. 7H, red dotted line and open squares, n = 7). However, when currents typical for a spontaneously active synapse were used, failures in the neuron’s response to the 300-Hz, 20-pulse EPSG
FIG. 7. Reliability of synaptic transmission in spontaneously active synapses. A: current-clamp recording of MNTB neuron while the calyceal input fibers were stimulated with a 300-Hz, 20-pulse train; the neuron responded to each stimulation with one action potential. B: after the slice was conditioned with 60 Hz of spontaneous activity, a number of failures could be observed during the same 300-Hz train. For each failure, an excitatory postsynaptic potential can be observed in the place of the missing action potential, suggesting that the failure was postsynaptic. C, top: responses of MNTB neuron when a 300-Hz conductance waveform was used to simulate currents of a rested synapse; the neuron responded to each event with one action potential, similar to that observed when the calyceal fibers were stimulated. Conductance waveform is shown in the bottom panel. Peak conductance was 232 nS. D, top: responses of MNTB neuron when a 300-Hz conductance waveform was used to simulate currents of a synapse that was conditioned with 60 Hz of spontaneous activity; the neuron failed to respond to a number of events. Corresponding conductance waveform is shown in the bottom panel. Peak conductance was 56 nS. E: various levels of background leak were added to the EPSG waveforms under conductance-clamp conditions, effectively reducing the input resistance of the neurons to values that are closer to input resistances observed in neurons under in vivo conditions. F and G: example of a loose-patch recording of a MNTB neuron while calyceal input fibers were...
train could be observed (Fig. 7D, top). These failures tended to occur more frequently toward the end of the EPSG waveform, but could sometimes also be observed early in the train (Fig. 7H, red solid line and closed squares, \( n = 7 \)).

Interestingly, the firing probabilities and frequencies for conditioned synapses shown in Fig. 7E closely match those observed in vivo. For both current-clamp and conductance-clamp experiments, the firing probability of the first few events in the train was 0.7 to 1.0, based on the calculation method described earlier. For a train frequency of 300 Hz, this corresponds to a firing frequency of about 200 to 300 Hz. The onset portion of the in vivo spike trains shown in Fig. 5A had a firing frequency of about 260 Hz (considered is the response to first tone of the pair only). The average firing frequency of the neuron in response to the latter part of the 200-ms tone was about 80 Hz. This corresponds well to the in vitro data shown in Fig. 7E, where the firing probability of the neuron to the latter portion of the train is about 1/3, i.e., about 100 Hz.

A number of studies have observed that a neuron’s input resistance is substantially higher in brain slices than in the intact brain (e.g., Bernander et al. 1991; Paré et al. 1998). The main reason for this observation is probably that neurons in the intact brain receive a large number and variety of synaptic inputs. When these inputs are activated at different points in time, postsynaptic receptors open and thus decrease the neuron’s input resistance. In brain slices, many of these inputs are silent and/or cut, with the result that the neuron’s input resistance increases. It is unknown whether and how much the input resistance of an MNTB neuron differs in brain slices compared with the intact brain because the projection pattern to MNTB neurons is much simpler than in the case of, say, cortical neurons. However, prominent glycine receptor inhibition to MNTB has been described in vitro (Awatramani et al. 2004). These inputs, when activated, will decrease the input resistance of postsynaptic neurons. An artificially high-input resistance in neurons of MNTB brain slices would facilitate the neuron’s responses to synaptic events. In this case, the synaptic failures shown in Fig. 7, A–E would be an underestimate of the true in vivo failure rates. We attempted to address this issue by adding a background leak to MNTB neurons during presentation of the EPSG waveforms. The background leak had a constant amplitude of 10–90 nS with a reversal potential of −60 mV and effectively reduced the input resistance of the postsynaptic neuron up to fivefold. Figure 7F shows the overall firing probability of six MNTB neurons, when EPSG waveforms of 300-Hz trains plus various amounts of background leak were tested. As expected, the firing probability decreased with increasing background leak, although the effect was minor.

The last approach to test the reliability of synaptic transmission was to use “loose-patch” extracellular recordings. For these experiments, an MNTB neuron in a brain slice was only loosely patched without obtaining a gigaseal and no break-in into the neuron was performed, such that the recordings were effectively extracellular. In some recordings, action potentials of both the calyx of Held (termed “prepotential”) and the postsynaptic principal neuron could be observed. An example is shown in Fig. 7, F and G. In this recording, stimulation of the calyceal input fibers produced three peaks in response to each event. The first one was the stimulation artifact (labeled “artifact”), followed by the prepotential (labeled “pre”), and then followed by the postsynaptic action potential (labeled “post”). The advantage of this method is that the interior environment of the postsynaptic cell is left undisturbed. For whole cell recordings, a common concern is that the perfusion of the neuron with artificial intracellular fluid might change the firing properties of the neuron, which would result in inaccurate measurements of failure rates. However, even when the postsynaptic neuron was left intact, transmission failures could be observed in the calyx of Held synapse when the slice was driven at in vivo–type activity levels. Figure 7F shows a loose-patch recording of a rested brain slice. Consistent with the data shown earlier, the synapse was appreciably fail-safe when a 300-Hz, 20-pulse train was tested, i.e., each prepotential was followed by a postsynaptic action potential (Fig. 7F).

Furthermore, the latency of synaptic transmission was increased when synapses were spontaneously active (Fig. 7, F and G). In this cell, the synaptic latency increased by 0.19 ms when the synapse was conditioned. For all 11 neurons from which spike-latency data were available, the average latency increase was 0.4 ± 0.13 ms. This discrepancy matches well with the discrepancy between published values for in vitro synaptic latency (Taschenberger et al. 2002; von Gersdorff and Borst 2002) versus in vivo latency (Guinan and Li 1990; Kopp-Scheinpflug et al. 2003) at the calyx of Held. These data suggest that highly active calyces have a longer synaptic latency than previously reported in vitro, but also that our conditioning protocol might be suited to transform calyces into a functional state that more closely resembles the functional state of an active in vivo calyx of Held.

In summary, the data presented in Fig. 7 suggest that the calyx of Held synapse shows a substantial amount of synaptic
failures, after cells were stimulated for several minutes with Poisson-distributed activity. One possible interpretation of these results is that in vivo, the MNTB might not be the simple and reliable relay that is commonly observed under standard in vitro conditions.

**DISCUSSION**

The main question addressed in this study is the question of how synaptic transmission in the calyx of Held synapse changes when synapses are stimulated for prolonged periods of time with Poisson-distributed activity, which, we hypothesize, imitates naturally occurring spontaneous activity. There are four main findings. First, the introduction of “spontaneous” activity into in vitro preparations of the calyx of Held considerably depresses synaptic currents, even at relatively low spontaneous frequencies of 20 Hz. Second, in these “spontaneously active” synapses, the degree of additional depression induced by high-frequency trains (i.e., simulated sound inputs) is reduced considerably. Third, recovery from synaptic depression is very fast. Data from corresponding in vivo extracellular recordings also show fast recovery of firing and are consistent with these in vitro findings. Fourth, in chronically active synapses with reduced synaptic currents, the reliability of transmission is reduced during high-frequency bursts of afferent input.

*Background firing in MNTB neurons*

Spontaneous activity in the lower auditory system is a widespread phenomenon. It is assumed that this activity is explained by the probabilistic behavior of the transduction channels of the inner hair cells and the resulting chronic transmitter release at the hair cell synapse. The spontaneous activity is still present at the level of the cochlear nucleus (Brownell 1975; Goldberg and Brownell 1973; Joris et al. 1994; Schwarz and Puil 1997; Spirou et al. 1990, 2005) and most auditory brain stem nuclei, such as MSO in bats (Grothe 1994), MNTB in cats (Smith et al. 1998), MNTB in gerbils (Kopp-Scheinpflug et al. 2003), and MNTB in rats (Sommer et al. 1993). Consistent with our data presented here, studies of spontaneous activity in the lower auditory system typically report a large variability of rates among neurons, even within the same species or the same nucleus. One possible explanation for this large variability is that different neurons receive inputs from different classes of auditory nerve fibers with low, medium, or high spontaneous rates (Liberman 1978), which would give rise to auditory brain stem neurons with very diverse spontaneous firing rates.

For the experiments presented here, three frequencies of Poisson-distributed activity were chosen for stimulation of brain slices: 20, 40, and 60 Hz. Although the mean spontaneous firing rate in our sample of neurons was 24.9 Hz and thus closer to the lowest of these frequencies, the three frequencies chosen for stimulation successfully cover the spectrum of observed in vivo spontaneous rates (see Fig. 2A; see also Kopp-Scheinpflug et al. 2003). However, because of the nature of the brain slice preparation, the original in vivo spontaneous firing rate of a given neuron is unknown. Therefore it is possible or even likely that an originally low spontaneously active neuron was stimulated with a high-frequency stimulus train and vice versa. However, all neurons in our in vitro sample responded stereotypically and in a very similar fashion to our various stimulus protocols and no responses were observed that could be explained by the use of an inappropriate background stimulation rate.

Measurements of spontaneous activity presented in this study were performed under anesthesia. As with almost every type of anesthesia, the ketamine—xylazine mixture used in this study might have depressed the neurons’ spontaneous activity to a certain degree (Destexhe et al. 2003). Therefore the actual spontaneous firing rates in MNTB neurons of behaving gerbils might be higher than those shown in this study. On the other hand, the values for spontaneous activity determined here match closely with findings of other studies using various species and various types of anesthesia or, in some cases, no anesthesia at all (e.g., Irvine 1992; Kiang 1965; Ryan and Miller 1978). We therefore conclude that the values presented here are representative or, at worst, a conservative lower limit of the true effects induced by spontaneous activity.

We also note that double-walled sound-attenuated rooms by themselves create the biologically unnatural situation of complete absence of sound. Natural auditory environments always contain a certain level of background noise, which contributes to the background activity of auditory neurons. Therefore the effects of chronic activity on synaptic transmission in behaving animals might be even larger, but are not likely to be smaller than presented here.

*Prolonged spontaneous spiking changes properties of synaptic transmission*

Our data show that prolonged stimulation even at a frequency of 20 Hz decreases synaptic currents to less than half of the original value, whereas stimulation with frequencies of 60 Hz reduces currents to about one third. It might be surprising to find that such low frequencies cause such a high degree of depression because all brain slices were prepared from animals well past the onset of hearing and recordings were performed at physiological temperature. α-Amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptor desensitization as well as N-methyl-d-aspartate (NMDA) currents, although playing a substantial role in preparations from young animals (Neher and Sakaba 2001; Sakaba and Neher 2001), play only a very minor role in animals past the age of hearing onset (Futai et al. 2001; Renden et al. 2005; Taschenberger et al. 2005). Under these conditions, MNTB neurons can follow stimulation frequencies of ≥600 Hz for at least short periods (Futai et al. 2001; Taschenberger and von Gersdorff 2000; Wu and Kelly 1993). The depression we observed in response to long-term stimulation progressed with at least two time constants: an initial, fast time constant, which can be seen during the first few stimulus pulses, and a much slower time constant. The mechanisms for the slow time constant are unclear, but are likely to be multiple and will be studied in the future. When the processing of high-frequency trains was tested in rested versus spontaneously active synapses, the observed relative degree of synaptic depression caused by the high-frequency train was much larger in the rested than in the active synapse. This might have important functional implications at the calyx of Held, which sustains high levels of activity.
Our findings also suggest that recovery from synaptic depression is very fast under biologically relevant activity levels. These time constants of about 90 ms are an order of magnitude shorter than previously reported values for rested synapses, which are in the range of several seconds (e.g., von Gersdorff et al. 1997: 4.2 s; see also Ishikawa and Takahashi 2001; Schneggenburger et al. 2002; Wang and Kaczmarek 1998; Wu and Borst 1999). Age, temperature, and species differences might account for some of this discrepancy. However, there is strong evidence suggesting that calcium accumulation in the presynaptic terminal through high-frequency firing may play a role in speeding up the recovery from depression (Wang and Kaczmarek 1998). It appears that this faster recovery plays a dominant role in active synapses.

Reliability of synaptic transmission

In vitro studies of the calyx of Held in animals past hearing onset have reported very reliable synaptic transmission and a number of cellular specializations to increase synaptic reliability. Our data are consistent with the view that “rested” calyces produce large synaptic currents and have a high transmission reliability. However, we show that in chronically active synapses, the synaptic currents are much smaller. Our current-clamp, conductance-clamp, and extracellular action potential recordings all suggest that spontaneously active synapses may show synaptic failures during periods of high-frequency activity. Therefore the calyx of Held may not always show the reliable 1:1 transmission postulated from in vitro experiments in rested synapses. This finding is consistent with previous in vivo results from the MNTB. Among the in vivo studies performed in the MNTB only those where both presynaptic and postsynaptic activity have been recorded simultaneously can address the question of transmission failures at the calyx of Held synapse. To our knowledge, two studies report simultaneous pre- and postsynaptic recordings at the MNTB and both agree on the occurrence of postsynaptic failures (Guinan and Li 1990; Kopp-Scheinflug et al. 2003) in vivo. However, the two studies differ in the number of failures observed. Guinan and Li (1990) found failures mainly with prolonged high-frequency stimulation of the afferent fiber bundle and only occasionally with sound stimulation, whereas Kopp-Scheinflug et al. (2003) found a substantial number of failures with sound stimulation. Species differences might account for some of this discrepancy, but note that these recordings were performed with intact synaptic inhibition and under anesthesia. Because MNTB neurons are known to receive a substantial amount of glycinergic inhibition (Awatramani et al. 2004), it is possible that some of these failures are the result of spike suppression by inhibition and that some of the difference observed in the two studies arises from differential recruitment of synaptic inhibition. Nevertheless, the presence of synaptic inhibition alone also questions the interpretation of the calyx of Held as a fail-safe “relay” synapse. This view has been formed by previous in vitro studies performed in slices from animals past hearing onset, where EPSCs well above action potential threshold have been measured even in response to stimulus frequencies of several hundred Hertz. However, these stimulus trains typically consisted of no more than 20–50 stimuli, with considerable recovery time of several seconds between trials. Our experimental setup avoided these periods of silence because they do not occur under in vivo conditions. Presumably the lack of prolonged periods of recovery keeps calyces in a chronic state of synaptic depression and causes transmission to fail during periods of embedded high-frequency activity.

In conclusion, the aim of this study was to perform a first description of the effects of prolonged “spontaneous activity” on synaptic transmission at the calyx of Held synapse. We conclude from our data that synaptic transmission in the calyx of Held differs in a number of significant ways when synapses are stimulated with a Poisson-distributed stimulus train for prolonged periods of time. Future studies will determine the specific contribution of multiple modulators, receptors, or channel types in the calyx of Held synapse to the “rested” or the “spontaneously active” synaptic state.

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