Context-Dependent Effects of NMDA Receptors on Precise Timing Information at the Endbulb of Held in the Cochlear Nucleus

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Pliss L, Yang H, Xu-Friedman MA. Context-dependent effects of NMDA receptors on precise timing information at the endbulb of Held in the cochlear nucleus. J Neurophysiol 102: 2627–2637, 2009. First published September 2, 2009; doi:10.1152/jn.00111.2009. Many synapses contain both AMPA receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR), but their different roles in synaptic computation are not clear. We address this issue at the auditory nerve fiber synapse (called the endbulb of Held), which is formed on bushy cells of the cochlear nucleus. The endbulb refines and relays precise temporal information to nuclei responsible for sound localization. The endbulb has a number of specializations that aid precise timing, including AMPAR-mediated excitatory postsynaptic currents (EPSCs) with fast kinetics. Voltage-clamp experiments in mouse brain slices revealed that slow NMDAR EPSCs are maintained at mature endbulbs, contributing a peak conductance of around 10% of the AMPAR-mediated EPSC. During repetitive synaptic activity, AMPAR EPSCs depressed and NMDAR EPSCs summed, thereby increasing the relative importance of NMDARs. This could impact temporal precision of bushy cells because of the slow kinetics of NMDARs. We tested this by blocking NMDARs and quantifying bushy cell spike timing in current clamp when single endbulbs were activated. These experiments showed that NMDARs contribute to an increased probability of firing, shorter latency, and reduced jitter. Dynamic-clamp experiments confirmed this effect and showed it was dose-dependent. Bushy cells can receive inputs from multiple endbulbs. When we applied multiple synaptic inputs in dynamic clamp, NMDARs had less impact on spike timing. NMDAR conductances much higher than mature levels could disrupt spiking, which may explain its downregulation during development. Thus mature NMDAR expression can support the conveying of precise temporal information at the endbulb, depending on the stimulus conditions.

INTRODUCTION

A critical component of how presynaptic inputs contribute to postsynaptic firing is the diverse set of ionotropic receptors on the postsynaptic membrane. This is particularly important at glutamatergic synapses, which contain both AMPA receptors (AMPARs) and NMDA receptors (NMDARs). These receptors have very different kinetics, which could affect how presynaptic activity influences postsynaptic firing, quite separate from the effects of NMDARs on longer-term plasticity. The specific consequences of this are not well understood.

We address this issue in the endbulb of Held, which is the synapse formed by auditory nerve (AN) fibers onto bushy cells (BC) in the anteroventral cochlear nucleus (AVCN) (Brawer and Morest 1975; Lorente de Nó 1981; Ryugo and Fekete 1982). AN fibers convey timing information about sounds in the precise timing of spikes. The endbulb and BCs have a number of specializations to preserve precise timing, including rapid AMPARs (Gardner et al. 2001; Isaacson and Walmsley 1995b) and low-threshold potassium conductances (Manis and Marx 1991; Oertel 1983; Rothman and Manis 2003). In vivo experiments indicate that BCs have lower variability in spike timing (“jitter”) compared with AN fibers, presumably through convergence of multiple inputs (Jorís et al. 1994; Louage et al. 2005; Paolini et al. 2001; Xu-Friedman and Regehr 2005a,b).

BCs express both AMPARs and NMDARs (Isaacson and Walmsley 1995a,b). Unlike AMPARs, NMDARs have slow kinetics, which in other systems is known to reduce the precision of spike timing. For example, in thalamic relay neurons, the AMPAR component of the EPSC is responsible for generating short-latency, temporally precise spikes, while NMDARs trigger longer-latency spikes with low temporal precision (Blitz and Regehr 2003; Miyata and Imoto 2006). Such effects of NMDARs seem at odds with the function of BCs in mature mice.

During development, NMDAR expression may be necessary for processes such as synaptic refinement. Mice begin hearing at P10-12 (Ehret 1976; Kamiya et al. 2001; Romand 2003), and the auditory system undergoes considerable anatomical and physiological changes around this time (Limb and Ryugo 2000; Taschenberger and von Gersdorff 2000). NMDAR expression is effectively eliminated from the developing calyx of Held (in the medial nucleus of the trapezoid body; MNTB) by 2–3 wk postnatally, which is consistent with the refinement of the temporal precision (Futai et al. 2001; Joshi and Wang 2002). Similar downregulation was found electrophysiologically in the rat endbulb by P18 (Bellingham et al. 1998). However, in situ hybridization and immunolabeling have suggested that NMDARs persist in older endbulbs and that NMDAR subunit composition matures by P21 (Joelson and Schwartz 1998; Sato et al. 1998).

To resolve these contradictory observations and to understand how NMDARs contribute to BC firing, we made patch-clamp recordings from brain slices of mice up to P30. Voltage-clamp recordings showed that NMDAR expression reaches a plateau after P21. In current clamp, blocking NMDARs with CPP reduced the probability of BC firing and increased the latency and jitter of firing. We tested these effects further using dynamic clamp (DC).

METHODS

All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory
Animals and were approved by Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Parasagittal slices of brainstems containing the AVCN were prepared from 14- to 30-day-old (P14-30) CBA/CaJ mice as described previously (Yang and Xu-Friedman 2008). Briefly, slices were cut in ice-cold, high-sucrose solution containing (in mM) 76 NaCl, 25 NaHCO3, 75 sucrose, 25 glucose, 1.25 NaH2PO4, 2.5 KCl, 7 MgCl2, and 0.5 CaCl2. Slices were incubated at 33°C for 20 min and transferred to normal recording solution containing (in mM) 125 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 20 glucose, 1 MgCl2, 1.5 CaCl2, 4 Na-L-lactate, 2 Na-pyruvate, and 0.4 Na-l-aspartate. Recordings were made at 32–34°C. Strychnine (10 μM) was present during all recordings. In some experiments, 10 μM 2,3-dioxo-6-nitro-1,2,3,4 tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) or 5 μM (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Harris et al. 1986; Lehmann et al. 1987) were added to block AMPAR or NMDAR currents, respectively. NBQX and CPP were from Tocris Bioscience (Ellisville, MO); all other chemicals were from Sigma (St. Louis, MO). For voltage-clamp recording, pipettes were filled with internal solution (in mM) 130 KMeSO3, 0 NaCl, 2 MgCl2, 0.16 CaCl2, 0.5 EGTA, 10 HEPES, 4 ATP, 0.4 GTP, and 14 di(tris) phosphocreatine (pH 7.3, osmolality: 300 mOsm). For current clamp, recording pipettes were filled with internal solution (in mM) 130 KMeSO3, 10 NaCl, 2 MgCl2, 0.16 CaCl2, 0.5 EGTA, 10 HEPES, 4 ATP, 0.4 GTP, and 14 di(tris) phosphocreatine (pH 7.3, osmolality: 300 mOsm). Electrode resistances were 1–2 MΩ. In voltage clamp, BCs were held at –70 mV to record AMPAR or at +40 mV for NMDA. Whole cell compensation parameters used were cell capacitance of 22.3 ± 0.9 pF, and series resistance of 4 ± 0.2 MΩ, which was compensated to 70%. In current clamp, holding current was applied to maintain resting membrane potential between trials at –60 mV. For current clamp, pipette capacitance neutralization was within ±1 pF and bridge balance was 4–10 MΩ. The cell input resistance at –60 mV was 37 ± 11 MΩ (n = 11 cells). Single presynaptic AN fibers were stimulated using a micropipette filled with standard external solution and placed in an area 30–50 μm away from the cell being patched. Stimulation of 5–15 μA were delivered through a stimulus isolator (A360, WPI, Saratoga, FL). Recordings were made using the Multiclamp 700B (Molecular Devices, Sunnyvale, CA) and digitized through an ITC-18 (Instrutech, Port Washington, NY) at 50 kHz controlled through custom-written software (mapPC) running in Igor (WaveMetrics, Lake Oswego, OR). Most recordings were made in the anterior part of the AVCN, where BCs receiving low-frequency input reside (Rouiller and Ryugo 1984). BCs were identified in voltage clamp by fast, depressing EPSCs with a large amplitude (2–20 nA) (Isaacson and Walmsley 1995b); in current clamp BCs were identified by their single, undershooting spikes in response to current injection (Oertel 1983). It is not possible to distinguish spherical and globular BCs based on their physiological properties (Ferragamo and Oertel 2001), so all recordings are grouped together.

In DC experiments, the AMPAR component was controlled by the ITC-18 DC interface. The nonlinearity of the NMDAR conductance was implemented using a slave computer running a custom-written Igor XOP and a National Instruments interface (PCI-6229). The voltage dependence of NMDAR conductance resulting from block by Mg2+ was measured using voltage-clamp experiments by holding BCs at potentials ranging from –70 to +40 mV and stimulating individual AN inputs in the presence of NBQX (Fig. 1A). NMDAR EPSC peak amplitudes were normalized to the amplitude measured at +40 mV and averaged over five experiments. There were no systematic changes in I–V curves for endbulbs from different aged mice (ages tested ranged from P14 to P26, average 20 ± 3 days). We fit the average data to a modified Woodhull equation, with form \( I = A [V_{m}(t) - V_{rev}]/[1 + [Mg] K_{0} exp(-z\delta F V_{m}/RT)] \), where \( K_{0} \) is the dissociation constant for Mg2+ with the channel at 0 mV, \( z \) is the valence of Mg2+, \( \delta \) is the fraction of the membrane potential affecting Mg2+ binding, \( F \) is Faraday’s constant, \( R \) is the gas constant, and \( T \) is the temperature. Using a least-squares approach, the key free parameters were \( K_{0} = 1.9 \text{ mM} \) and \( \delta = 0.73 \). To simplify the calculation for the dynamic-clamp interface, we also fit the data to a modified Boltzmann equation, with the form \( I = A [V_{m}(t) - V_{rev}]/[1 + exp((V_{1/2} - V_{max})/V_{scale})] \) using least-squares, yielding values of \( V_{1/2} = -1.2 \text{ mV} \) and \( V_{max} = 18.1 \text{ mV} \). These values were then used by the XOP on the slave computer to calculate the current through the NMDAR conductance as \( I(t) = (V_{m}(t) - V_{rev}) G_{NMDA} \frac{(1 + exp((V_{1/2} - V_{max}))}{(1 + exp((V_{1/2} - V_{max}))} \). The XOP ran at ~10 kHz. AMPAR and NMDAR currents were summed with a summing amplifier and fed into the command input on the Multiclamp 700B. Threshold was measured in DC for each BC as described previously (Xu-Friedman and Regehr 2005a). Thresholds were 10–35 nS for different cells (average: 20.4 nS ± 1.9, n = 22). Endbulb AMPA EPSCs had amplitudes of 2–20 nA in voltage-clamp experiments (average: 6.9 nA ± 3.6, n = 89). The relationship between threshold and AMPA EPSC amplitude could not be determined for individual cells, so AMPAR conductances in dynamic-clamp experiments were scaled to 10 times threshold. When single inputs were mimicked, the NMDAR conductance waveform was based on NMDAR EPSCs recorded in voltage clamp (Fig. 3E). When multiple inputs were mimicked, a constant NMDAR conductance was applied. The time course of AMPAR conductances was based on single EPSCs recorded in voltage clamp.

For current- and dynamic-clamp experiments with single inputs, latency was quantified as the duration between the start of the stimulus pulse and the point of maximum slope of the resulting spike (if any). For DC experiments with multiple jittery inputs, latency was quantified as the time difference between the average input time and the resulting spike. Jitter was the SD of the latency. All data are reported as means ± SE.
RESULTS

NMDARs persist at older endbulbs

We assessed the functional expression of AMPARs and NMDARs using voltage-clamp recordings in brain slices from CBA/CaJ mice aged P14 to P30. We stimulated individual AN fiber inputs and recorded AMPAR EPSCs at −70 mV. At this holding potential, NMDAR conductance is negligible. NMDAR currents were subsequently measured in the same cells holding at +40 mV. All endbulbs contained both AMPAR and NMDAR currents at all ages studied. The amplitude of the NMDAR current changed with age. Two representative cells are shown in Fig. 2A, that have matched AMPAR current amplitudes. The NMDAR current amplitude in the cell from the younger animal was larger than from the older animal (Fig. 2A, left vs. right). The amplitudes of both AMPAR and NMDAR currents were variable from cell to cell (Fig. 2, B and C), but they seemed to be highly correlated (D). This probably reflects the variability in endbulb size seen in anatomical studies (Limb and Ryugo 2000) and likely results from differing numbers of release sites between different endbulbs. To control for this variability, we used the ratio of the NMDAR conductance at +40 mV to the AMPAR conductance at −70 mV (NMDAR/AMPA ratio, NAR). NAR decreased significantly with age from 0.19 ± 0.02 for P14–17 (n = 41) to 0.09 ± 0.01 for P22–30 (n = 59; P < 0.001, Student’s t-test, Fig. 2E).

By contrast, neither AMPAR nor NMDAR currents showed changes in kinetics over this time period. We quantified the rise and decay of NMDAR currents by fitting to a single exponential: \( y = y_a + A \exp\left[-(t - t_0)/\tau\right] \). The rising phase was fit over the period from 10% of peak amplitude through the peak. The decay phase was fit starting 5 ms after the peak of the NMDAR current, through the end of the recording (usually >180 ms). A sample decay fit is shown in Fig. 1A. The time constants (\( \tau \)) did not change over the ages studied (Fig. 2, F and G) and were similar to those measured in the presence of NBQX (n = 49 cells), it was possible to measure the rate of rise (F). The rate of decay was measured in 117 cells (G). These measures show no significant changes with age. H and I: AMPAR current half-width (H) and NMDAR current half-width (I) over the ages studied (n = 122 cells). AMPAR and NMDAR half-widths do not change. Bold lines in B–I are the average values for all recordings at each postnatal day.

FIG. 2. Development of glutamatergic currents in the endbulb of Held following single stimuli. A: example traces of NMDAR and AMPA receptor (AMPAR) currents in juvenile (P14) and older (P22) mice. AMPAR currents throughout were measured at −70 mV, and NMDAR currents at +40 mV. Some NMDAR currents were recorded in the presence of NBQX. Traces are averages of 5–8 trials. Horizontal scale applies to both AMPA and NMDA traces. B and C: absolute conductance of AMPAR (B) and NMDAR (C) currents over the age studied (n = 122 cells). D: correlation between NMDAR and AMPAR conductances (n = 122 cells). E: NMDAR conductance measured at +40 mV normalized to the AMPAR conductance measured at −70 mV for each cell as a function of age (n = 122). This value is referred to as the NMDAR/AMPAR ratio, or NAR. NAR reaches a plateau after ~P18. F and G: kinetics of NMDAR currents over the ages studied, using single-exponential fits. For NMDAR currents measured in the presence of NBQX (n = 49 cells), it was possible to measure the rate of rise (F). The rate of decay was measured in 117 cells (G). These measures show no significant changes with age. H and I: AMPAR current half-width (H) and NMDAR current half-width (I) over the ages studied (n = 122 cells). AMPAR and NMDAR half-widths do not change. Bold lines in B–I are the average values for all recordings at each postnatal day.
reported before (Bellingham et al. 1998). We also quantified
half-width of AMPAR and NMDAR components, and neith-
er showed significant changes over the ages studied (Fig.
2, H and I). Changes in kinetics have been documented in
slices from animals younger than P14 (Bellingham et al.
1998). However, these changes appear to be largely con-
cluded in the older slices studied here, suggesting they are
mature in this respect.

While NMDAR conductance following isolated stimuli ap-
peared relatively minor compared with AMPAR, during normal
activity it could be different. AN fibers can fire at over 300 Hz
for extended periods during normal sounds (Joris et al.
1994; Kiang et al. 1965; Sachs and Abbas 1974; Taberner and
Liberman 2005); this can have significant effects on EPSC amplitude as a result
of short-term synaptic plasticity (Isaacson and Walmsley 1996;
Oleskevich et al. 2000; Wang and Manis 2008; Yang and
Xu-Friedman 2008). To match these firing rates, we studied NMDAR
and AMPAR currents during trains of 20 stimuli at 100, 200, or
333 Hz. As with single stimuli, the amplitude of the NMDAR
component during trains was lower in older mice compared with
younger mice but still persisted (Fig. 3A). Both AMPAR and
NMDAR components showed significant depression during
trains. We quantified the peak amplitude for each EPSC in the
train as the step increase in current after each stimulation (“∆” in
Fig. 3, A and B). This value represents the additional receptors that
are activated after each stimulus. Depression of the NMDAR
EPSC was even greater than depression of the AMPAR EPSC at
all frequencies used probably because of receptor saturation
(Yang and Xu-Friedman 2008).

However, a major effect of multiple stimulation was that
NMDAR currents showed considerable temporal summation
(TS). AMPAR currents showed no TS even at the highest fre-
cuencies because AMPAR currents at the endbulb decay partic-
ularly rapidly. We quantified the integrated NMDAR current for
each EPSC by measuring the peak current after each stimulation
relative to the baseline (Σ in Fig. 3, A and B). TS increased with
stimulation frequency even though depression was greater at
higher frequencies.

We used the integrated NMDAR current to calculate the NAR
during trains. Because of depression of AMPAR EPSCs and TS
of NMDAR EPSCs, the NAR changed throughout the train. We
assessed this change by comparing the NAR of the first pulse of
the train (NAR1) with the final pulse of the train (NAR20; Fig.
3C). As with single stimuli, NAR1 decreased with age from 0.2 ±
0.01 at P14–P17 (n = 9) to 0.1 ± 0.03 at P22–30 (n = 5). NAR20
depended highly on both age and stimulation rate. NAR20 was
greater than NAR1, particularly at higher stimulation rates (Fig.
3C). NAR20 was greatest over all stimulation rates in the youngest
mice examined (P14–17), reaching 2.4 ± 0.5 at 333 Hz. Even in
older animals (P22–30), NAR20 reached parity at 1.0 ± 0.1 at 333
Hz despite the fact that NAR1 was low at these ages. The
increases in NAR20 were quite similar across ages when normal-
ized to NAR1 (Fig. 3D); thus each age group showed similar
responses to stimulation frequency. This suggests that TS de-
velops at similar rates and follows similar mechanisms across all age
groups studied.

There was considerable cell-to-cell variability in the amount of
TS. There were differences in the rate of build-up as well as the
rate of decay. There were no obvious groupings that might
indicate the existence of different classes of synaptic inputs. To
illustrate the range of TS, we show NMDAR currents with

![Figure 3](http://jn.physiology.org/DownloadedFrom/http://jn.physiology.org/)

**FIG. 3.** Glutamatergic currents in the endbulb following trains of stimulation. A: example traces of NMDAR and AMPAR currents following 100 Hz stimulation in juvenile (P15) and older (P22) mice. Measurement of the relative (Δ) and integrated (Σ) amplitude is shown. Traces are averages of 15–17 trials. Horizontal scale applies to both AMPA and NMDA traces. B: relative amplitudes of NMDAR and AMPAR currents (Δ) and integrated amplitude of NMDAR current (Σ) following stimulation with 100-, 200-,
and 333-Hz trains (n = 20 cells). C: NAR at 1st (NAR1) and last (NAR20) pulse of the train in P14–17 (n = 9 cells), P18–21 (n = 6), and P22–30 (n = 5) mice following stimulation at 100, 200 and 333 Hz. Integrated (Σ) values were used for calculations. D: relative NAR for 100-, 200-,
and 333-Hz stimulation. Integrated (Σ) values were used for calculations. E: variation in temporal summation of NMDAR currents in different experiments. Traces are average NMDAR currents at 200-Hz stimulation in 3 cells, representing large, average, and small summation. Each response is normalized to the same amplitude for the 1st EPSC peak (arrow). The peak amplitudes were 845, 339, and 1,230 pA for the large, average, and small summation traces, respectively. Recordings from these cells were used to drive the DC experiments in Fig. 5C.
particularly large, close to average, and particularly small TS in Fig. 3E.

**Synaptically activated NMDARs affect BC firing**

Because NMDARs are present even into older endbulbs, they may modulate BC firing, such as by affecting the probability or timing of BC spiking. NMDARs activate and inactivate slowly, so one might expect that under conditions in which NMDARs are highly activated, BC spikes may be more likely yet less precise. By contrast, when NMDARs are blocked, BC spikes may be less likely but more precise.

To test this, we made current-clamp recordings from BCs in mice P15 to P20 (average age P17 ± 1), stimulating AN fibers in trains at the same rates as in the voltage-clamp experiments and quantifying characteristics of the BC spikes. In control conditions, the spike firing probability was near 1 at the beginning of the train but decreased later in the train especially at higher stimulation frequencies (Fig. 4, A and B). Both latency and jitter increased over the course of the train (Fig. 4B). This effect is consistent with depression of the AMPAR EPSC during the train (Fig. 3): as the EPSP amplitude decreases, the latency to spiking increases and the likelihood of spiking decreases. The AMPAR component appeared to be essential for firing in the endbulb. NMDARs alone were not able to support BC firing, as application of 10 μM NBQX abolished all BC firing following stimulation with 100-, 200-, or 333-Hz trains (n = 3, data not shown).

**FIG. 4.** Effects of changes in NMDAR conductance on bushy cells (BC) spiking in current clamp. A: example traces of 100- and 333-Hz trains before (Ctr) and after application of CPP in a P17 mouse. B: probability, latency, and jitter of control BC firing in regular recording solution and stimulation with 100, 200, and 333 Hz (n = 11). C: probability, latency, and jitter of BC firing in the presence of CPP (n = 9–11). Data were normalized to the control values (- - -). *, significant changes from control (P < 0.05, Student’s t-test). D: probability of BC firing at different holding potentials in the presence of CPP (n = 11). Data for VM = −58 mV (closed circles) and VM = −62 mV (open circles) were normalized to the control condition of normal resting potential (−60 mV; - - -). Asterisks indicate significant changes from control (P < 0.05, Student’s t-test).
To evaluate the impact of NMDARs on BC firing, we blocked them with 5 μM CPP. CPP application led to a decrease in the probability of firing for all stimulation frequencies (Fig. 4C). In addition, CPP caused a further increase in latency. Both these effects are consistent with the NMDAR component of the EPSC being generally excitatory, increasing the likelihood of spiking and causing BCs to reach threshold sooner and thereby shortening the latency. Thus the effects of CPP suggest that NMDAR currents do have a major impact on BCs firing under physiologically relevant conditions.

An important function of BCs is to relay and refine precise timing information. We quantified the relative change in jitter of BC spikes and found that where there were significant changes, jitter increased on application of CPP (Fig. 4C). Under no conditions did jitter decrease with CPP application. Thus NMDARs appear to support jitter reduction, and when CPP is applied, jitter increases. We had initially expected that the slow NMDAR current would increase jitter, but this was not the case.

The effects found in CPP experiments on probability and latency of BC firing were most prominent during 200- and 333-Hz stimulation. We consider this most likely results from major increases in NAR due to high TS at these frequencies (Fig. 3B). In sum, the effects observed when NMDA current was pharmacologically manipulated suggest that NMDARs play a computational role during normal activity, supporting a higher probability of BC firing, shorter latency, and lower jitter.

NMDARs could affect BC firing by depolarizing the cell. Such depolarization would bring the BC closer to threshold, thereby increasing the probability of response and shortening the latency. In addition, depolarization could activate other voltage-sensitive conductances, such as low-threshold potassium channels (Manis and Marx 1991; Rothman and Manis 2003), which could shorten the time constant of the cell. To test this, we examined BC firing at different membrane potentials in the presence of CPP. The appropriate amount of depolarization to apply was difficult to assess as the hyperpolarization caused by CPP application was not measurable during trains, suggesting that NMDAR activation triggers only slight depolarization. Therefore we tested deviations of ±2 mV from the normal resting potential of ~60 mV. Increasing holding potential to ~58 mV increased the probability of firing while decreasing it to ~62 mV reduced firing probability (Fig. 4D). However, these changes were much smaller than those resulting from NMDA block with CPP (Fig. 4C). This indicates that the depolarization caused by NMDA activation only partially accounts for the increase in BC spike probability and that it is necessary to take into account the more complex properties of NMDARs, such as their voltage-dependent conductance, which cannot be tested with simple current injection.

Amplitude and kinetics of NMDAR conductances affect BC firing

To test the role of NMDARs more fully, we turned to dynamic clamp (DC) (Robinson and Kawai 1993; Sharp et al. 1993). This technique allows us to mimic AMPAR and NMDAR conductances and to specify the amplitude, timing, and number of synaptic inputs, and then to quantify the effects on BC firing. The details of configuring the DC parameters are described more fully in Methods.

We began by considering single inputs. NMDAR and AMPAR conductances were based on the voltage-clamp recordings of the AMPAR and NMDAR currents (Fig. 5A). In this example, the first NMDAR conductance peak was scaled to 0.1 of the first AMPAR conductance (i.e., NAR = 0.1 or “1×”) to match the average obtained from voltage-clamp recordings. The initial AMPAR conductance was scaled to 10 times the minimum AMPAR conductance that elicited a BC spike (threshold). The DC interface calculated the appropriate AMPAR and NMDAR currents in response to the BC membrane potential, which were then summed and injected into the BC, which then fired a train of action potentials (Fig. 5A). Responses were qualitatively similar to those observed in current clamp. Most conspicuously, firing was less reliable in the second part of the train. This reliability depended on the size of the NMDAR component (Fig. 5B). When it was eliminated (“0” in Fig. 5B), spiking was even less reliable. This situation was similar to what was found when applying CPP to block NMDARs in current clamp. The DC also allowed us to explore a larger NMDAR contribution, NAR = 0.2 (twice normal or “2×”). In this condition, the firing probability was greater by the end of the train (Fig. 5B). To compensate for baseline differences between cells, we normalized all the values within each cell to those for NAR = 0. We tested different stimulation frequencies (100, 200, 333 Hz), NAR (0, 1×, 2× normal), and the position in the train (pulses 6–10, 11–15, and 16–20). In addition, we wanted to test the effect of TS. To do this, we drove the DC using NMDAR EPSCs that were recorded from cells that showed TS that was particularly large (“tall”), close to average (“middle”), or particularly small (“short”) (see Fig. 3E).

Considering firing probability (Fig. 5C, top), for 100-Hz trains, changing the NAR had no effect, because the probability of spiking was close to 100%. For 200- and 333-Hz trains, increasing the NAR to 1× or 2× normal significantly increased the probability of spiking. In addition, larger increases were seen with greater TS (tall > middle > short). Thus NMDAR currents enhance the probability of spiking in a dose-dependent manner, both by changes in NAR and TS.

The effects on latency paralleled the effects on the probability of firing (Fig. 5C, middle). Under conditions with larger NMDAR conductance (greater TS, larger NAR), the latency shift was more negative. These effects are consistent with NMDAR conductance generally contributing greater excitation, which increased the probability of firing and shortened the latency of firing. Also, similar to the results of synaptic stimulation, larger NMDAR conductance either reduced jitter or showed no significant effect, but never significantly increased it (red symbols, Fig. 5C, bottom).

Impact of NMDARs depends on the number of active inputs

The experiments conducted so far suggested that NMDAR activation actually supports transmission of precise temporal information at the endbulb. This raises the obvious question of why NMDARs are downregulated during development. There are several possible explanations for this. One is that deleterious effects of NMDARs on electrical properties of BCs come into play under different conditions from what we examined so far. We tested this possibility using DC.
The online version of this article contains supplemental data.

Fig. 5. Using dynamic clamp to study the effects of NMDARs on BC firing. A: examples of injected NMDAR (top) and AMPAR (middle) conductances and the BC response (bottom) for a 200-Hz train with 1× NMDAR/AMPAR ratio (NAR) and “middle” temporal summation. For NMDAR, the NAR scale, there were fewer spikes, particularly by the end of the train (Fig. 6, B and C).

Average results from several experiments indicated that the number of BC spikes per cycle was slightly >1 for inputs firing at 100 and 200 Hz and ~0.5 at 333 Hz (Fig. 7A, top). This probably happens because inputs at 100 and 200 Hz are above threshold but at 333 Hz are too small to trigger spikes unless multiple inputs occur simultaneously. Probability did not change much for NAR 0–2× normal but decreased at 4× for all frequencies tested (Figs. 6B and 7A, middle). This suggests that for normal NAR, multiple inputs provide sufficient excitation through AMPARs and NMDARs to ensure reliable firing of BCs but that 4× NAR is too much. Latency and jitter showed only small changes for NAR 0–2× normal but were greatly different for 4×.

To determine if these effects resulted primarily from the tonic depolarization induced by NMDAR activation, we repeated these experiments with no NMDA component but depolarizing the BC to different baseline membrane potentials (~30, ~40, or ~50 mV, Fig. 7B). Changes in the probability and latency of firing were qualitatively similar to those at differently scaled NAR (Fig. 7A). There were no major changes in firing at lower membrane potentials. However, firing was disrupted at ~30 mV with much lower probability of spiking and large shifts in latency.

These data suggest that increasing NAR can have adverse effects on firing resulting from the large shifts in baseline membrane potential. Thus increases in NAR over normal...
Williams et al. 1997). Therefore the subunit expression in each individual cell may explain the scatter we observed in NMDAR EPSC variability.

The downregulation of NMDAR conductance during maturation of the endbulb

NMDAR expression persisted in the endbulb in the oldest animals we studied. As endbulbs matured, the amplitude of the NMDAR component decreased to approximately P21, where it stabilized at a plateau. The mouse endbulb continues to show anatomical changes after that age (Limb and Ryugo 2000). However, smaller-scale structures such as vesicular density and mitochondrial volume appear to solidify before that (Ryugo et al. 2006). In addition, subunit composition of the endbulb receptors appears to be relatively stable over the ages studied here: mature subunit composition is achieved for AMPARs by P14 in rats (Koike-Tani et al. 2005) and for NMDARs by P21 in gerbils (Joelson and Schwartz 1998). We saw no systematic changes over development in AMPAR or NMDAR EPSC kinetics in the population of endbulbs studied, suggesting that any late changes in receptor composition do not significantly affect the electrophysiological properties. Therefore we consider the ages studied here in mice to be essentially mature.

This situation differs from the calyx of Held in the MNTB. There AMPAR kinetics show changes until P14–18, probably reflecting changes in subunit composition and synaptic structure (Joshi and Wang 2002; Taschenberger and von Gersdorff 2000; Taschenberger et al. 2002; Yousoufian et al. 2005). Furthermore, NMDARs appear to be greatly reduced or eliminated from the calyx by postnatal week 2–3 (Futai et al. 2001; Joshi and Wang 2002), which is quite different from the endbulb. In addition, the fidelity of firing in the MNTB was unaffected by blocking the NMDAR EPSC with APV in mature synapses (Futai et al.2001).

Our results emphasize that characterizing single EPSCs underestimates the importance of NMDARs during physiological activity. While AMPAR EPSCs depress (Isaacson and Walmsley 1995b; Oleskevich et al. 2000; Wang and Manis 2005; Xu-Friedman and Regehr 2005b; Yang and Xu-Friedman 2008), NMDAR EPSCs show significant temporal summation. At normal firing rates, NMDAR activation reached conductances equal to or greater than those of AMPARs. The relative build-up of the NMDAR/AMPAR ratio was similar for all ages studied (Fig. 2D). This suggests that the balance of NMDAR/AMPAR mainly depends on activity but not age.

TS of the NMDAR current showed cell-to-cell variability. The amount of TS did not appear to be age-dependent. This variability is likely to reflect differences in NMDAR subunit composition or localization. NMDARs in the mature endbulb contain about equal composition of NR2A-C (Joelson and Schwartz 1998). NR2A-expressing synapses are likely to have faster kinetics than synapses expressing other subunits (Flint et al. 1997). Therefore the subunit expression in each individual cell may explain the scatter we observed in NMDAR EPSC half-width and variability in TS.

**Discussion**

In this study, we show that synaptic currents mediated through NMDARs support the relaying of precise timing information in the auditory system. NMDARs are retained at the endbulb of Held late in development, suggesting they play a functional role in mature synapses. In current-clamp recordings, eliminating the NMDAR component pharmacologically reduced the probability of BC firing, increased the latency of firing, and increased jitter. DC experiments showed that these effects were dose-dependent over a physiologically-relevant range. Thus despite slow kinetics of NMDARs, they can play an important computational role in precise timing when single AN inputs are active. However, when multiple inputs are active, the NMDAR component did not play a major role. This indicates that the role of NMDARs in BC firing depends on the stimulus context. Furthermore, NMDARs could disrupt precise spiking, when presented at levels much higher than normal. This suggests there may be constraints on NMDAR expression at the endbulb.
Role of NMDAR in BC firing

Because the NMDAR component persists developmentally and becomes increasingly significant during extended activity, it is likely to play a functional role. Indeed pharmacological elimination of NMDAR reduced BC firing probability and increased latency in response to the activation of single AN inputs. These findings are consistent with NMDARs generally increasing excitation. This could happen in at least two ways. First, the small depolarizing conductance of NMDARs at rest may contribute sufficient depolarization to bring BCs closer to threshold. We tested this possibility and found that slight depolarization could only account for part of the change in response probability. Second, NMDARs could effectively amplify depressed AMPAR EPSPs if they are large enough to relieve Mg block. Endbulb NMDARs are insufficient by themselves to trigger firing because synaptic activation could not elicit spikes in the presence of NBQX. In addition, application of tonic NMDAR-like conductances in DC did not produce firing without the further addition of AMPAR-like conductances. NMDAR activation decreased the latency of BC spiking by as much as 0.2 ms. This is striking because this shift is much larger than the precise times that are used as cues for such behaviors as sound localization (e.g., 10 \( \mu \)s) (Klumpp and Eady 1956; Moiseff and Konishi 1981).

While other studies have found that NMDARs support higher response probability, this occurs in part by adding late or temporally imprecise spikes (retinal ganglion cell to lateral geniculate synapses: Augustinaite and Hegelund 2007; Blitz and Regehr 2003; visual cortex: Harsch and Robinson 2000; corticothalamic synapses: Miyata and Imoto 2006). This does not seem consistent with BC function. However, our results indicate that temporal precision at the endbulb is improved, and not degraded, by NMDARs. This is likely to result from increases in membrane conductance, either directly through the NMDARs themselves or indirectly through activation of potassium channels. Depolarization by NMDARs would activate voltage-gated potassium channels, which would decrease the input resistance of the BC and speed the time constant, which may enhance temporal precision. In addition, calcium influx through NMDARs could in principle activate calcium-activated potassium channels, which are present in the cochlear nucleus (Friedland et al. 2007) but have not yet been described in BCs. Another possibility is that the NMDARs amplify the AMPAR EPSPs (as suggested in the preceding text), making each AMPAR EPSP effectively larger and thereby increasing the precision of spike generation.

The effects of NMDAR conductance on spike probability, latency, and jitter were dose-dependent. We tested this in DC using NMDAR conductances with different amplitude or TS. Variability in TS has been shown to play an important role in other systems (Augustinaite and Hegelund 2007; Wu et al. 2004). Our experiments indicate that firing properties of BCs change with different degrees of TS. This raises the possibility that an individual cell could adjust its response by modifying the amplitude or temporal properties of NMDAR conductances, such as by changing receptor density, spatial distribution, or subunit composition. At the calyx of Held, Joshi et al. (Joshi and Wang 2007) found that paired stimulation induced an LTD-like phenomenon for summated NMDAR EPSCs in trains but not in single AMPAR and NMDAR EPSCs, which led to reductions of temporally imprecise spikes. Our results suggest that in the endbulb, such changes could have quite different effects. A reduction in NMDARs would be expected to reduce firing probability and increase latency and jitter. If long-term plasticity of NMDAR expression is also present in BCs, it could provide ways for BCs to optimize the timing properties of their responses, depending on their activity.

NMDARs and multiple inputs

We also investigated how NMDARs could influence BC firing when multiple inputs are active. BCs in mice appear to receive one to five inputs (Limb and Ryugo 2000; Oertel 1985;
Xu-Friedman and Regehr 2005a). During normal sounds, the number of inputs that are active from moment to moment is likely to vary depending on the thresholds of different AN inputs and the stochastic nature of their firing. In addition, individual firing histories may affect the contribution of each AN by short-term synaptic plasticity (Yang and Xu-Friedman 2008). We investigated a simplified case using DC for three identical inputs with NAR ranging from 0 to 4 times normal. Under these conditions, the NMDAR component made only a small contribution to the probability, latency, and jitter of BC firing over the physiological range of NAR. Based on this, it appears that the NMDAR component has greater importance under stimulus conditions when there is only one active input but would have less impact as more inputs are recruited, such as with louder sounds.

With very large NAR (4×), BCs showed highly depolarized baseline membrane potentials and disrupted firing. This suggests that under some conditions, NMDARs could negatively impact firing. However, we did not observe such extreme levels of NMDAR conductance even at the earliest ages examined, so our data do not explain the 50% downregulation from P14 to P21. It may be that other naturalistic conditions that we did not test can lead to extreme NMDAR activation, such as with yet larger numbers of active inputs. Alternatively, it may be other aspects of NMDAR physiology that are related to their downregulation, such as their role in intracellular signaling pathways for long-term changes in synaptic strength.

**Relevance to other time-coding systems**

Our findings may have implications for other systems where precise timing is important. Precise timing has been best explored in systems near the sensory or motor periphery. In the electroreceptive system, precise spike timing is important for the jamming-avoidance response as well as species recognition (Carr 1993; Carr and Friedman 1999; Heiligenberg 1991; Hopkins and Bass 1981; Kawasaki and Guo 1996). Precise spike timing also seems to be important in the visual system (Dan et al. 1998) and motor system (Hahnloser et al. 2002; Yu and Margoliash 1996). In some systems far from the periphery, such as in cortex, neurons do appear to have adaptations for generating precisely timed spikes (Mainen and Sejnowski 1995), although the behavioral significance is not yet clear. Experiments in visual thalamus have suggested that NMDARs increase the likelihood of spiking, but the additional spikes were not as precisely timed (Blitz and Regehr 2003). Our data indicate that in the AVCN, NMDARs can contribute to precise spike timing. It will be interesting to ascertain how NMDARs contribute to spiking in other systems and what other features are important in determining whether the spikes are more or less precisely timed.

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