Different pools of glutamate receptors mediate sensitivity to ambient glutamate in the cochlear nucleus

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Yang Y, Xu-Friedman MA. Different pools of glutamate receptors mediate sensitivity to ambient glutamate in the cochlear nucleus. J Neurophysiol 113: 3634–3645, 2015. First published April 8, 2015; doi:10.1152/jn.00693.2014.—Ambient glutamate plays an important role in pathological conditions, such as stroke, but its role during normal activity is not clear. In addition, it is not clear how ambient glutamate acts on glutamate receptors with varying affinities or subcellular localizations. To address this, we studied “endbulb of Held” synapses, which are formed by auditory nerve fibers onto bushy cells (BCs) in the anteroventral cochlear nucleus. When ambient glutamate was increased by applying the glutamate reuptake inhibitor TFB-TBOA, BCs depolarized as a result of activation of N-methyl-D-aspartate receptors (NMDARs) and group I metabotropic glutamate receptors (mGluRs). Application of antagonists against NMDARs (in 0 Mg2+) or mGluRs caused hyperpolarization, indicating that these receptors were bound by a tonic source of glutamate. AMPA receptors did not show these effects, consistent with their lower glutamate affinity. We also evaluated the subcellular localization of the receptors activated by ambient glutamate. The mGluRs were not activated by synaptic stimulation and thus appear to be exclusively extrasynaptic. By contrast, NMDARs in both synaptic and extrasynaptic compartments were activated by ambient glutamate, as shown using the use-dependent antagonist MK-801. Levels of ambient glutamate appeared to be regulated in a spike-independent manner, and glia likely play a major role. These low levels of ambient glutamate likely have functional consequences, as even low concentrations of TBOA caused significant increases in BC spiking following synaptic stimulation. These results indicate that normal resting potential appears to be poised in the region of maximal sensitivity to small changes in ambient glutamate.

ambient glutamate; excitability; extrasynaptic

GLUTAMATE IS THE PRINCIPAL excitatory neurotransmitter in the central nervous system, and its level near synapses is under tight spatial and temporal regulation. Glutamate is maintained at levels in the nanomolar to micromolar range through diffusion and transporter activity (Le Meur et al. 2007). We refer to this as “ambient” glutamate, to differentiate from the glutamate transients near synapses. Ambient glutamate is important in disease, as increases are thought to trigger excitotoxicity, such as during stroke (Hazell 2007). However, little is known how ambient glutamate influences neuronal function under normal conditions.

One major question is whether there are receptor pools that are specifically sensitive to ambient glutamate. Ambient glutamate appears to be sufficient to activate metabotropic glutamate receptors (mGluRs) or extrasynaptic N-methyl-D-aspartate (NMDA) receptors (NMDARs) in hippocampus and cortex (Bandrowski et al. 2003; Fellin et al. 2004; Herman and Jahr 2007; Jabaudon et al. 1999; Kullmann et al. 1999; Sah et al. 1989). However, it is not clear if sensing ambient glutamate is the primary role of these receptors and whether synaptic NMDARs are shielded from ambient glutamate by reuptake mechanisms. This could be important because the different pools of receptors could play very different roles in controlling excitability. For example, synaptic receptors may be less accessible to ambient glutamate than extrasynaptic receptors. In addition, it has also been suggested that ambient glutamate could cause desensitization (Featherstone and Shippy 2008). So it is unclear whether the net effect of ambient glutamate would be excitatory or inhibitory.

To address these issues, we studied the glutamatergic synapses called “endbulbs of Held,” which are the synapses between auditory nerve fibers (ANFs) and bushy cells (BCs) in the anteroventral cochlear nucleus (AVCN). Ambient glutamate causes tonic activation of group I mGluRs in BCs, which is enhanced by the glutamate reuptake inhibitor threo-β-benzylxystaric acid (threo-β-BTX) (Chanda and Xu-Friedman 2011). However, it is not known whether additional glutamate receptors are affected, nor their cellular localization.

Our experiments indicate that both NMDARs and mGluRs are activated by an endogenous source of ambient glutamate, probably from glia. We used the glutamate reuptake inhibitor 3S)-3-[3-[4-((trifluoromethyl)benzoyl)amino]phenyl)methoxy]-(l-aspartic acid (TFB-TBOA) to manipulate ambient glutamate or synaptic stimulation to elevate glutamate near synaptic sites. These approaches revealed mGluRs are primarily extrasynaptic, while NMDARs are both synaptic and extrasynaptic. Ambient glutamate has effects that are specific for different cell types: synaptic NMDARs on BCs are activated by ambient glutamate, but those on neighboring T-stellate cells are not. Small changes in ambient glutamate had significant effects on the ability of BCs to respond to synaptic stimulation, especially near normal membrane potential, thereby maximizing the influence of ambient glutamate.

MATERIALS AND METHODS

Electrophysiology. Experimental procedures were approved by Institutional Animal Care and Use Committee. Slices were prepared from postnatal day (P) 14 to P21 and P35 to P40 CBA/CaJ mouse AVCN, as described previously (Chanda and Xu-Friedman 2010a; Pliss et al. 2009; Yang and Xu-Friedman 2008, 2009). Briefly, sagittal slices were cut in an ice-cold solution containing the following (in mM): 76 NaCl, 75 sucrose, 25 NaHCO3, 25 glucose, 2.5 KCl, 1.25 NaH2PO4, 7 MgCl2, 0.5 CaCl2. Then they were transferred to normal artificial cerebrospinal fluid until recording (in mM: 125 NaCl, 26 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 20 glucose, 1.5 CaCl2, 1 MgCl2, 4 Na-L-lactate, 2 Na-pyruvate, 0.4 Na-L-ascorbate, bubbled with 95%
O₂–5% CO₂). Cells were patched under an Olympus BX51 microscope with a Multiclamp 700B (Molecular Devices) controlled by a National Instruments PCI-6221, driven by custom-written software (matPC) running in Igor (WaveMetrics). The bath was perfused at 3–4 ml/min using a pump (403U/VM2; Watson-Marlow, Wilming-ton, MA), with saline running through an in-line heater to maintain the temperature at 33°C (SH-27B with TC-324B controller; Warner Instruments, Hamden, CT). Strychnine (10 μM) was present during all recordings.

Patch pipettes were pulled from borosilicate glass (outer diameter: 1.5 mm, inner diameter: 0.86 mm; Sutter Instrument, Novato, CA) to a resistance of 2.5 MΩ and filled with (in mM) 130 KMeSO₄ (current clamp) or CsMeSO₄ (voltage clamp), 10 NaCl, 10 HEPES, 2 MgCl₂, 0.5 EGTA, 0.16 CaCl₂, 4 Na₂ATP, 0.4 NaGTP, 14 Tris- creatine phosphate, 1 QX-314 (voltage clamp), pH 7.3, 310 mosM. Single ANF were stimulated using 6- to 20-μA pulses passed through a small glass micropipette placed in the neuropil. Single or paired pulses were applied every 10 s. Train stimuli were applied every 30 s. For voltage-clamp, BCs were held at −70 mV to record α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPAR)- mediated excitatory postsynaptic currents (EPSCs) or at +40 mV in the presence of 10 μM 2,3-dixo-6-nitro-1,3,4-tetrahydrobenzo- [f]quinoxaline-7-sulfonamide (NBQX) for NMDAR-mediated EPSCs with access resistance 3–14 MΩ, compensated to 70%; for current-clamp, we set the initial resting membrane potential (Vrest) to −60 mV using a small, constant holding current, which was not adjusted thereafter. BCs were identified in voltage clamp by EPSCs showing paired-pulse depression and having rapid decay kinetics (τ < 0.2 ms) (Chanda and Xu-Friedman 2010a), and in current clamp by their response to strong depolarizing current pulses with one to two under- shooting spikes (Wu and Oertel 1984). T-stellate cells were identified in voltage clamp by mild depression or facilitation in AMPA EPSCs with slower decay kinetics (τ > 0.2 ms), and in current clamp by multiple over-shooting spikes in response to depolarizing current pulses. Voltages were not corrected for liquid junction potential.

The pharmacological agents used were TFB-TBOA [selective excitatory amino acid transporter (EAAT) 1] and EAAT2 glutamate reuptake inhibitor, 1 μM), 2-methyl-d- (phenylethyl)npyridine hydrochloride (MPEP) (mGluR5-specific antagonist, 100 μM), (E)-ethyl 1,1a,7,7a- tetrahydro-7-(hydroxyimino)cyclopropa[β]chromene-1a-carboxylic acid (CPCCOEt) (mGluR1-specific antagonist, 100 μM), NBQX (AMPAR- type glutamate receptor antagonist, 10 μM), (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (NMDAR antagonist, 5 μM), 2-amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5- oxo-4H-chromene-3-carboxitrile (UCPH-101) (selective inhibitor of glutamate transporter EAAT1, 30 μM), dihydroxyacetic acid (DHK) (selective inhibitor of glutamate transporter EAAT2, 450 μM), ifenprodil (NR2B- specific NMDAR antagonist, 10 μM), MK-801 (activity-dependent NMDAR blocker, 4 μM), tetrodotoxin (TTX) (voltage-gated sodium channel antagonist, 0.5 μM), fluorescein (FC) (inhibitor of glial tricarboxylic acid cycle, 100 μM), bicuculline (GabAA receptor antagonist, 25 μM) and strychnine (glycine receptor antagonist, 10 μM). MPEP, CPCCOEt, NBQX, CPP, UCPH-101, DHK, ifenprodil and TTX were obtained from Abcam (Cambridge, MA), TBOA was from Tocris Bioscience (Ellisville, MO), and other chemicals from Sigma (St. Louis, MO).

Data are presented as means ± SE of the mean. Significance was determined using the paired, two-tailed, Student’s t-test, except where otherwise specified.

**Modeling.** Cleft glutamate was modeled for Fig. 4 using simple diffusion at the location of the instantaneous release of a disk of glutamate (i.e., approximating the release of multiple vesicles from one spot). Under these conditions, the glutamate concentration at time t is given by C(t) = C₀ [1 − exp (−D/R²)], where C₀ is the initial concentration (500 mM), D is the diffusion coefficient (0.4 μm²/s), and R is the disk radius (25 nm) (Crank 1956). The distance between receptor and the site of release was neglected for simplicity. For pairs of responses, identical amounts of glutamate were released on each pulse. Receptor models were based on published Markov models with multiple receptor states and the rates of transition between them (see RESULTS). These models were implemented in Igor, and solutions were derived using implicit or explicit Euler integration. Maximum step sizes were model dependent, and ranged from 0.2 to 10 μs. The models are identified more fully in the RESULTS.

**RESULTS**

**Influence of ambient glutamate on BC Vrest.** To evaluate the effects of ambient glutamate on BCs, we made current-clamp recordings from BCs and bath-applied the highly selective glial glutamate reuptake inhibitor TFB-TBOA (1 μM). TBOA caused BCs to depolarize by 12.8 ± 0.9 mV (N = 5) (Fig. 1A). This depolarization indicates glutamate receptors are highly sensitive to ambient glutamate, and that there are sources of ambient glutamate in the slice.

We isolated the contributions of different glutamate receptor subtypes using specific antagonists. In the presence of group I mGluR antagonists MPEP (100 μM) and CPCCOEt (100 μM), BCs depolarized by 5.8 ± 0.7 mV upon addition of TBOA (N = 5; Fig. 1, B and G). In the presence of the NMDAR antagonist CPP (5 μM), BCs depolarized by 6.4 ± 0.6 mV (N = 5; Fig. 1, C and G), which is consistent with the effects we have observed of TBOA in CPP (Chanda and Xu- Friedman 2011). Coapplication of NMDAR and mGluR antagonists completely blocked the depolarization by TBOA (N = 5, Fig. 1D). Analysis of variance confirmed that the co-administration of NMDAR and mGluR antagonists significantly reduced the TBOA-induced depolarization observed in the presence of either NMDAR or mGluR antagonist alone (F2,14 = 55.3, P < 0.001). When we added the individual depolarization effects of these two receptors, the sum was 12.2 ± 0.9 mV, which was similar to that by TBOA with no antagonist (P = 0.41, unpaired t-test). These results indicate that NMDAR and mGluR activation fully account for the depolarization caused by TBOA. Furthermore, in the absence of glutamate transporters, both NMDARs and mGluRs on BCs are sensitive to ambient glutamate.

TBOA blocks three types of glial glutamate transporters, EAAT1, EAAT2, and EAAT3 (Tsukada et al. 2005). To determine which specific type mediated the effects in Fig. 1, A–C, we applied subtype-specific inhibitors. Application of the EAAT1-specific inhibitor UCPH-101 (30 μM) caused BCs to depolarize by 11.8 ± 0.7 mV (N = 5; Fig. 1, E and G). Furthermore, this depolarization was not significantly different from the depolarization by TBOA alone (P = 0.33, unpaired t-test). By contrast, the EAAT2 inhibitor DHK (450 μM) caused no depolarization (0.08 ± 0.2 mV; N = 4; Fig. 1, F and G). These results indicate that EAAT1 is the primary transporter regulating glutamate at endbulbs, consistent with anatomical data (Josephson and Moret 2003).

We tested whether the normal regulation of extracellular glutamate was disrupted, such as by the preparation of brain slices. We applied exogenous glutamate (1 to 10 μM) to try to elevate ambient glutamate. However, there was no depolarization (0.05 ± 0.2 mV; N = 6; Fig. 1G), which indicated that normal reuptake mechanisms were robust and largely intact in these preparations, and that glutamate is regulated, even at these low concentrations.
Effects of ambient glutamate on AMPARs. In addition to the strong activation of NMDARs and mGluRs, we also considered whether these levels of ambient glutamate were sufficient to affect AMPARs. Activation of AMPARs is generally thought to require high glutamate concentrations (in the mM range), whereas AMPAR desensitization is affected by levels of glutamate in the micromolar range (Colquhoun et al. 1992; Zorumski et al. 1996), so we considered whether ambient glutamate might trigger significant desensitization. If AMPARs are desensitized by ambient glutamate, we would expect the EPSC amplitude to decrease as ambient glutamate levels rise. We tested this possibility by making voltage-clamp recordings from BCs and stimulating presynaptic ANFs. Application of TBOA had no significant effect on the amplitude ($P = 0.5$) or kinetics ($P = 0.6$) of the EPSC (8 cells; Fig. 2, A–C).

We further tested for desensitization by ambient glutamate in two ways. First, we looked for changes in EPSC decay rate, but this showed no change following TBOA application (Fig. 2C, $P = 0.6$, 8 cells). In addition, desensitization normally plays a role in short-term depression in endbulbs, when two pulses are delivered within 20 ms (Chanda and Xu-Friedman 2010a; Yang and Xu-Friedman 2008). We reasoned that pre-desensitizing AMPARs by ambient glutamate could occlude the desensitization during fast synaptic glutamate release, which should cause an increase in the paired pulse ratio (PPR). This would be expected to occur for intervals when desensitization is normally strong (3 ms), but not at intervals when
Effects of ambient glutamate on NMDARs. We wanted to determine whether ambient glutamate acts at synaptic or extrasynaptic NMDARs. If elevation in ambient glutamate by TBOA activated a significant proportion of synaptic NMDARs, then the amplitude of the evoked NMDAR EPSC would be expected to decrease. In addition, TBOA application could influence the NMDAR EPSC time course, either by interfering with glutamate clearance from the synaptic cleft, or by promoting its spillover to extrasynaptic receptors. We evaluated these possibilities by making voltage-clamp recordings from BCs, holding the voltage at +40 mV and stimulating a single presynaptic ANF in the presence of NBQX to block AMPARs. The NMDAR EPSC showed no significant changes during the application of TBOA, neither in amplitude (P = 0.4) nor kinetics (P = 0.3, 6 cells) (Fig. 3, A and E). This lack of occlusion means TBOA activates a negligible fraction of the synaptic NMDAR pool, so the depolarization caused by TBOA in Fig. 1B must result from activation of primarily extrasynaptic NMDARs. Therefore, we can largely independently assess synaptic NMDARs using evoked release vs. extrasynaptic NMDARs using TBOA.

It is possible that more intense synaptic activation could produce a build-up of glutamate to activate extrasynaptic NMDARs. To test this, we examined NMDAR EPSCs following train stimulation. As with single stimuli, TBOA did not significantly affect the amplitude of the first EPSC (EPSC1; P = 0.23, 6 cells) (Fig. 3C). Furthermore, the amplitude of the 20th EPSC (EPSC20) was also unchanged in TBOA (P = 0.18, 6 cells) (Fig. 3D), which supports our conclusion that a negligible number of synaptic NMDARs are activated by ambient glutamate. The decay time following train stimulation was significantly prolonged by TBOA (P = 0.004, N = 6 cells) (Fig. 3, B and E), unlike with single stimuli. This could have resulted from either the prolonged activation of synaptic NMDARs or the additional activation of extrasynaptic NMDARs. Under normal conditions in the absence of TBOA, the decay of the NMDAR EPSC was similar following single and train stimulation (P = 0.13, N = 6 cells, Fig. 3E), indicating that glutamate has little chance to accumulate or spill over during high levels of synaptic activation because of the efficient reuptake by glutamate transporters. This is consistent with anatomical data that show many endbulb release sites are near glial processes (Clarkson and Rubio 2012; Lauer et al. 2013) that likely express transporters.

These results indicate that activation of both synaptic and extrasynaptic NMDARs is tightly regulated by glutamate reuptake even during high levels of activation. Furthermore, when normal reuptake is disrupted by TBOA, few synaptic NMDARs appear to be activated by ambient glutamate at any given moment, so it is likely primarily extrasynaptic NMDARs that contribute to the TBOA-induced depolarization.

Estimation of ambient glutamate concentration based on receptor modeling. We wanted to understand why ambient glutamate had no measurable effect on AMPAR and NMDAR EPSCs. We used models of receptor activation to study this, and also to estimate the glutamate concentration near synaptic receptors. We used two AMPAR models, one based on mammalian Purkinje cells (Wadiche and Jahr 2001) and another on chick magnocellularis neurons, which have many similarities with BCs (Raman and Trussell 1992). We subjected both models to focal releases of glutamate, which caused large transient increases in open probability (Po) (Fig. 4, A and B). The response to the second pulse of glutamate was considerably reduced, because of desensitization. We modeled the effects of various concentrations of ambient glutamate. The Purkinje cell model showed little change in Po below 5 µM ambient glutamate (Fig. 4A), and the magnocellularis model showed little change below 0.5 µM. Neither model showed a change in PPR or decay rate except at very high glutamate concentrations (50 µM; Fig. 4, A and B, right). Therefore, given that AMPA EPSCs showed no detectable change in amplitude or PPR upon addition of TBOA, it suggests TBOA does not raise glutamate concentration above 0.5 to 5 µM.

We also considered models of NMDARs, both NR2A- and NR2B-containing (Amico-Ruvio and Popescu 2010; Kussius and Popescu 2010). The specific composition of BC NMDARs is unknown, so these models are appropriate as a first approx-
We varied the ambient glutamate concentration and quantified the NMDAR Po. Significant decreases in amplitude occurred for 0.5 mM for NR2A- and NR2B-containing NMDARs (Fig. 4, C and D). Thus the lack of change in NMDAR EPSC in the experiments of Fig. 3 suggests ambient glutamate does not rise above 0.5 mM, even in the presence of TBOA. We also wanted to determine if the NMDAR-dependent depolarization during TBOA application could give us insight into the ambient glutamate concentration. We quantified the steady-state Po of both NMDAR models in the absence of evoked neurotransmitter release and found that both receptor types were activated at glutamate concentrations as low as 200–300 nM (Fig. 4E). Thus these models suggest that there are ambient glutamate levels that would cause NMDAR-mediated depolarization, without significantly decreasing the peak NMDAR EPSC. It seems likely that, even in the presence of TBOA, glutamate remains at comparable levels.

Effects of ambient glutamate on synaptic vs. extrasynaptic NMDARs. The results so far suggest that the depolarization by TBOA primarily results from activation of extrasynaptic NMDARs, while ANF activation even for extended periods at high rates primarily drives synaptic NMDARs. We wanted to confirm this using pharmacological approaches to distinguish the two pools of NMDARs. It has been suggested that synaptic NMDARs are dominated by NR2A-containing, while extrasynaptic NMDARs are dominated by NR2B-containing (Papouin et al. 2012; Sheng and Kim 2002; Tovar and Westbrook 1999). To test this, we used the NR2B-specific NMDAR antagonist, ifenprodil (10 μM). BCs were held at +40 mV in the presence of NBQX to isolate NMDAR EPSCs. The single input was stimulated every 20 s. Ifenprodil reduced the amplitude of the evoked NMDAR EPSC by 50% (Fig. 5A), which

![Diagram of NMDAR models](http://jn.physiology.org/)
suggests synaptic NMDARs include both NR2A and NR2B subunits.

We verified that this mixed expression of NR2A- and NR2B-containing NMDARs was not a developmental artifact by repeating these experiments in older animals (P35–40). There was no significant difference between the amount of block by ifenprodil in slices from young vs. older mice (P = 0.2, unpaired t-test, N = 7 cells P14–21 vs. 6 cells P35–40; Fig. 5B). In addition, there was no significant difference in decay time between the two groups either in control (P = 0.8), or during ifenprodil application (P = 0.4, unpaired t-test, N = 7 vs. 6 cells; Fig. 5C). These results suggest that mature BCs express both NR2A- and NR2B-containing NMDARs at synapses.

We also tested whether the extrasynaptic NMDARs activated by TBOA application were NR2A- or NR2B-containing. In the presence of MPEP (100 μM) and CPCCOEt (100 μM) to block group I mGluRs, we quantified NMDAR activation in this experiment in voltage clamp by observing the change in holding current induced by TBOA (I_TBOA), while holding at +40 mV. We applied TBOA in the presence or absence of ifenprodil and compared the change in I_TBOA between first and second application. Ifenprodil blocked I_TBOA by 54 ± 1% (P < 0.001, N = 5 cells). This was nearly the same degree of block as for synaptic NMDARs. These results indicate that the NMDARs that are activated by TBOA application include NR2A and NR2B subunits and, furthermore, are in the same proportion as synaptic NMDARs. Thus it does not appear fruitful to try to isolate synaptic and extrasynaptic NMDARs based on receptor subtype. Instead we used physiological approaches to activate these different populations, that is synaptic stimulation or TBOA application.

To determine how ambient glutamate activates synaptic and extrasynaptic NMDARs, we used MK-801, which is an activity-dependent NMDAR blocker. For synaptic NMDARs, we first assessed baseline NMDAR EPSC amplitude by evoking EPSCs at +40 mV in the presence of NBQX every 30 s during a control period to obtain a stable response. Then synaptic stimulation was stopped, and 4 μM MK-801 was bath applied for 6 min, while the cell was maintained at +40 mV. After washout for another 6 min, synaptic stimulation was resumed. We found that the NMDAR EPSC was inhibited to 38 ± 16% (P < 0.001; Fig. 6, A and C). It is to be emphasized that there was no synaptic stimulation during the application of MK-801, yet the NMDAR EPSC was strongly inhibited. The remaining NMDARs were presumably capable of block by MK-801 had they been activated during the period that MK-801 was present: previous experiments using fiber stimulation and longer application of MK-801 showed complete block of NMDAR EPSCs (Yang and Xu-Friedman 2012). Control experiments without MK-801 showed no such decline (EPSC = 101 ± 26%, 5 cells, P = 0.95; Fig. 6, B and C), indicating it was not a nonspecific rundown effect. These results reflect that a significant proportion of synaptic NMDARs were activated at a low but consistent level by endogenous glutamate and subsequently blocked by MK-801 in the absence of any evoked activity. The population of activated NMDARs presumably changes constantly, so that MK-801 blocked a large fraction of NMDARs over 6 min of application.

We also used MK-801 to examine how ambient glutamate affected extrasynaptic NMDARs. We first measured the extra-
synaptic response by applying TBOA while holding at +40 mV, then thoroughly washed out TBOA, applied MK-801 for 6 min, and washed out MK-801. When we assayed the remaining NMDAR response with a second TBOA application, \( I_{\text{TBOA}} \) was greatly reduced (29 ± 3%, \( N = 5 \) control vs. 5 MK-801 cells, \( P < 0.001 \), Fig. 6, D and F), again in the absence of any synaptic stimulation. When MK-801 was not used, there was no decrease in \( I_{\text{TBOA}} \) (Fig. 6, E and F), indicating the effect in Fig. 6D was not a result of simple rundown. Thus the majority of extrasynaptic receptors appear to be exposed to and activated by ambient glutamate, even in the absence of evoked synaptic activity and with glutamate reuptake intact.

We also examined tonic activation of NMDARs using CPP. We did current-clamp recordings from BCs in 0 mM Mg\(^{2+} \) and 2.5 mM Ca\(^{2+} \) to relieve Mg\(^{2+} \) block of NMDARs at \( V_{\text{rest}} \). We observed a significant hyperpolarization (−1.8 ± 0.2 mV, \( N = 5 \) cells, \( P < 0.001 \), Fig. 6G), indicating that ambient glutamate can bind NMDARs. In the presence of normal Mg\(^{2+} \), however, there was no detectable hyperpolarization (0.35 ± 0.45 mV, \( N = 3 \) cells, \( P = 0.49 \)), suggesting there were no NMDARs open at normal resting potential.

Accessibility of T-stellate cells to ambient glutamate. The effects of MK-801 in Fig. 6A are surprising, because MK-801 is not expected to block synaptic NMDARs in the absence of synaptic activity. We performed a control for the effects of MK-801 using T-stellate cells, which are also located in the AVCN. For T-stellate cells, MK-801 had no effect on synaptic NMDARs in the absence of stimulation (Fig. 7A). T-stellate NMDAR EPSCs were fully capable of block by MK-801 when ANFs were stimulated in the presence of MK-801 (Fig. 7B and two additional similar experiments). This indicates that T-stellate NMDARs were not blocked in the experiment of Fig. 7A because they were not activated by ambient glutamate. In addition, the actions of MK-801 on BCs in the absence of stimulation (Fig. 6A) cannot be attributed to nonspecific block of unactivated NMDARs, because the same effect was not observed in T-stellate cells. Furthermore, it rules out that miniature EPSCs (mEPSCs) are sufficient to allow block by MK-801, because mEPSC frequency is similar between BCs and T-stellate cells (Lu et al. 2007), yet T-stellate cells are not blocked by MK-801. Finally, it indicates that the influence of ambient glutamate on BCs is highly specific, because synaptic NMDARs on T-stellate cells are not reached and activated by ambient glutamate.

We also assessed whether there was tonic activation of NMDARs on T-stellate cells. Applying CPP in the absence of Mg\(^{2+} \) had no effect on the membrane potential of T-stellate cells (0 ± 0.2 mV, \( N = 5 \) cells, \( P = 0.99 \), Fig. 7C), indicating no significant activation. We verified that T-stellates were sensitive to ambient glutamate by applying TBOA, which caused very strong depolarization (34.5 ± 1.6 mV, \( N = 4 \) cells, Fig. 7D).

The results of Figs. 6 and 7 taken together indicate that regulation of ambient glutamate is highly cell specific. Application of TBOA indicates that both cells are shielded from ambient glutamate by reuptake mechanisms. However, ambient glutamate is still able to access synaptic NMDARs on BCs but not T-stellate cells. There is tonic activation of NMDARs on BCs, but not T-stellate cells. Thus ambient glutamate specifically influences NMDARs in BCs.

The source of ambient glutamate. We also investigated the source of ambient glutamate that activates mGluRs or NMDARs. The known glutamatergic neurons in the AVCN include ANFs and BCs. In chick nucleus magnocellularis, glutamate release from ANFs activates mGluRs (Zirpel and Rubel 1996). To test whether ANFs in mammalian AVCN act similarly, we stimulated ANFs in long trains and recorded changes in membrane potential in current clamp. For this experiment, NMDAR activity was not used because of the inability to separate synaptic vs. extrasynaptic, so we focused instead on depolarization mediated by mGluRs. This experiment was conducted in the presence of 5 \( \mu M \) CPP, 25 \( \mu M \) bicuculline, and 5 \( \mu M \) strychnine to block NMDA, GABA and glycine receptors, respectively. We saw no depolarization following prolonged ANF stimulation (Fig. 8A, similar results in 7 cells). This indicates mGluRs are not driven by glutamate released from ANFs or the BC itself. Moreover, this result indicates that mGluRs are localized in extrasynaptic sites. We also stimulated in the neuropil strongly around the BCs in long trains and saw no depolarization. This strongly suggests that mGluRs are not driven by glutamate released from nearby neurons.

Another possibility is that glutamate is released following spontaneous action potentials. To test this, we used TTX (0.5 \( \mu M \)) to block spiking, and TBOA-induced depolarization was unchanged (Fig. 8, B and C). These results indicate that ambient glutamate is action-potential independent, suggesting it may come from a nonneuronal source, such as glia (Cavelier and Attwell 2005; Christian and Huguenard 2013; Han et al. 2013; Jourdain et al. 2007; Parpura and Haydon 2000; Woo et al. 2012).

Fig. 7. T-stellate cells in the anteroventral cochlear nucleus differ substantially in the effects of ambient glutamate. A: MK-801 had no effect on NMDAR EPSCs in the absence of stimulation. Open symbols are normalized EPSC amplitudes measured in voltage clamp at +40 mV from \( N = 5 \) cells. Solid symbols are averages. B: representative experiment showing block of NMDARs on T-stellate cells by MK-801 during synaptic stimulation. Similar effects were seen in \( N = 3 \) cells. Arrows indicate the representative evoked NMDA EPSCs shown in the inset. Inset scale bars are 10 ms and 0.1 nA. C: representative current-clamp experiment showing no tonic NMDAR activation on T-stellate cells. D: depolarization of representative T-cell following 1 \( \mu M \) TFB-TBOA application. Inset traces show cell activity at times indicated by arrows. Scale bars are 50 ms and 50 mV. Inset bar graph shows average depolarization for 4 similar experiments.
release of glutamate from glia (Paulsen et al. 1988; Rossi et al. 2007). We first performed control experiments that showed 100 μM FC had no effect on EPSC amplitude (EPSC in FC 95.8 ± 2.2% of control, P = 0.57, paired t-test, N = 3), mEPSC frequency (100 ± 0.8% of control, P = 0.74, paired t-test), or mEPSC amplitude (99.9 ± 0.3% of control, P = 0.80, paired t-test, N = 6) during bath application lasting over 50 min. Thus FC does not appear to have a direct effect on ANFs or BCs. However, bath application of FC (100 μM) caused a yet larger depolarization in TBOA (from 7.4 mV to 10.8 mV, N = 5, P < 0.001, Fig. 8, D and F). The additional depolarization in FC suggests that ambient glutamate is further elevated, implying a second regulatory role for glia, possibly as sources of glutamate. This depolarization was completely blocked by application of NMDAR and mGluR antagonists, indicating that it acted entirely through glutamate receptors (Fig. 8, E and F). These results indicate that FC increases the dysregulation of the release of ambient glutamate, reflecting an important role of glia.

Effects of ambient glutamate on spike generation in BCs. We wanted to understand the functional impact of ambient glutamate through its actions on both mGluRs and NMDARs, using TBOA application to modulate ambient glutamate levels. In control conditions, BCs initially fired reliably in response to ANF stimulation. However, as stimulation continued, spiking became less reliable later in the train (pulses 11–20; Fig. 9, A and B, left). This decrease in spiking was particularly acute at high, physiologically-relevant rates of activation when synaptic depression is strong (200–333 Hz) (Johnson 1980; Joris et al. 1994; Sachs and Abbas 1974; Taberner and Liberman 2005). In the example experiment in Fig. 9A, a nonsaturating concentration of TBOA (0.6 μM) was used to depolarize the BC by 2.5 mV. This small depolarization significantly increased the number of spikes (Fig. 9A, right), probably by bringing the BC closer to threshold. When we applied the saturating concentration of 1 μM TBOA, the example cell in Fig. 9B depolarized by 8 mV. This much larger depolarization did not increase the probability of spiking at later pulses, probably because of sodium channel inactivation.

We applied TBOA over a range of concentrations to yield different levels of depolarization in different cells. The spiking probability (P_spike) increased the most with depolarizations of 1.5 to 3 mV (for 200 Hz ΔP_spike = 0.35 ± 0.06, P = 0.002; for 333 Hz ΔP_spike = 0.25 ± 0.08, P = 0.006) (Fig. 9, C and D). The P_spike increased less with depolarization of 4–6 mV (for 200 Hz ΔP_spike = 0.10 ± 0.04, P = 0.01; for 333 Hz ΔP_spike = 0.06 ± 0.02, P = 0.044). By contrast, depolarization above 6 mV did not enhance spiking (for 200 Hz ΔP_spike = −0.01 ± 0.05, P = 0.89; for 333 Hz ΔP_spike = 0.07 ± 0.04, P = 0.19). In an earlier study (Chanda and Xu-Friedman 2011), reducing the effects of ambient glutamate by blocking mGluRs and NMDARs caused hyperpolarization and a decrease in the probability of spiking (for 200 Hz ΔP_spike = −0.13 ± 0.06, P < 0.05; for 333 Hz ΔP_spike = −0.08 ± 0.02, P < 0.05) (Fig. 9C). Together these results show that small shifts in V_rest have a significant impact on BC excitability. Furthermore, normal resting potential of −60 mV appears to be poised in the region of maximal sensitivity to small changes in ambient glutamate and resting potential.

To test the role of glia in regulating ambient glutamate, we used FC. FC has been used in a number of studies to inhibit the citric acid cycle selectively in glia, which thereby disrupts ATP-dependent activities of glia (Allen et al. 2004; Fonnum et al. 1997; Voloboueva et al. 2007) and promotes unregulated

Fig. 8. The source of ambient glutamate is likely nonneuronal. A: ANF stimulation causes no detectable activation of mGluRs. Current-clamp trace shows EPSPs and spikes in response to 50-Hz, 4-s activation of ANF in the presence of CPP to block synaptic NMDARs. Dashed line indicates resting potential before stimulation began. Similar effects were found in 7 experiments. B and C: TBOA-induced depolarization persists in 0.5 mM tetrodotoxin (TTX). Representative experiment is shown in B, and overall average responses are summarized in C. There was no significant difference between depolarizations in control and TTX (P = 0.29, N = 5 cells each). D: example experiment showing 100 μM fluorocitrate (FC) enhances the TBOA-induced depolarization. E: representative experiment of the effects of FC in the presence of glutamate receptor antagonists. NMDAR and mGluR antagonists completely block the depolarization by FC and TBOA. F: summary of 5 experiments showing TBOA-induced depolarization is elevated significantly after addition of FC (P < 0.001, N = 5 experiments) and blocked by antagonists (N = 4 experiments). V_m, membrane potential.
We show here that ambient glutamate plays an active role in modulating BC excitability, through tonic binding of both NMDARs and mGluRs. Furthermore, the source of glutamate is constitutively active, and even small changes in reuptake efficacy have a significant impact on membrane potential and spike probability. We also showed that both synaptic and extrasynaptic NMDARs were activated at a low but consistent level by endogenous glutamate. This is contrary to the idea that only extrasynaptic receptors are accessible to ambient glutamate when reuptake is intact.

These results have important implications. While the pathological role of ambient glutamate in stroke is highly emphasized, our results indicate that ambient glutamate may play a role in regulating normal excitability. Different cell types are regulated differently by ambient glutamate. Unlike BCs, synaptic NMDARs on T-stellate cells were not accessible to ambient glutamate, although T-stellates were sensitive to ambient glutamate when normal reuptake was blocked by TBOA. Thus activation of synaptic NMDARs by ambient glutamate is a cell-specific adaptation in BCs.

**NMDAR function in BCs.** It is striking that BCs utilize NMDARs, because the slow kinetics of NMDARs could be seen as incompatible with the role of BCs in relaying highly precise timing information about sounds. NMDARs are down-regulated over development (Bellingham et al. 1998), but they are not completely eliminated, and provide significant excitation on the fast timescale of synaptic transmission, particularly when the AMPA component is depressed (Pliss et al. 2009). The present experiments indicate an additional role for NMDARs in supporting BC excitability over longer time scales regulated by ambient glutamate.

The mGluR-activated conductance adds yet more to the depolarization through NMDARs. The role of mGluRs appears simpler, because they were insensitive to synaptic release, and so appear to strictly depolarize in response to ambient glutamate (Fig. 8A). The channel that actually mediates the depolarization has not yet been conclusively identified in BCs, but there are a number of candidates (Anwyl 1999). Our approach using TBOA activates extrasynaptic mGluRs and NMDARs together. Our laboratory showed previously that mGluR activation by ambient glutamate had a measurable effect on BC excitability (Chanda and Xu-Friedman 2011). The present results indicate the effect is even greater when NMDARs are also activated. However, it is possible that the two receptor types may lie in different parts of the cell and could be independently regulated by separate glutamate sources.

Our results indicate that both synaptic and extrasynaptic NMDARs respond to ambient glutamate, because they are both blocked by MK-801. This is important, because it means that the reuptake mechanisms that are thought to surround and insulate synapses do not shield synaptic NMDARs from the low levels of ambient glutamate. However, we also found that TBOA application has little effect on NMDA EPSCs, suggesting that it is primarily extrasynaptic receptors that are activated by ambient glutamate. These two results together indicate that there are many more extrasynaptic than synaptic NMDARs, and at any given moment, a low percentage of NMDARs are activated.

We can estimate the activation of these different NMDAR pools. For synaptic NMDARs, the average NMDAR EPSC was $197 \pm 71$ pA at $+40$ mV ($N = 10$ cells), which is equivalent to 4.9 nS on average, or 98 NMDARs assuming a single-channel conductance of 50 pS (Stern et al. 1992). A serial electron microscopic reconstruction of one rat endbulb had 155 release sites (Nicol and Walmsley 2002). Assuming mouse endbulbs have a similar number, then an EPSC activates less than one NMDAR per release site on average. This is consistent with the small size of NMDAR-mediated mEPSCs (Bellingham et al. 1998), and low NMDAR expression levels in...
electron microscopy (Rubio et al. 2014). TBOA application elicited an average current of 350 pA (N = 15 cells), which is equivalent to \( \sim 175 \), primarily extrasynaptic NMDARs. The total extrasynaptic pool is likely much larger, as the \( P_{0} \) of NMDAR is quite low at the estimated concentration of ambient glutamate.

The MK-801 results indicate that both synaptic and extrasynaptic NMDARs are activated even in a quiescent brain slice. Our experiments using CPP can give us an estimate of the actual number of activated NMDARs, using a simplified electrical equivalent of a BC: \( V_{\text{rest}} = (g_{\text{NMDA}} E_{\text{NMDA}} + g_{\text{other}} E_{\text{other}})/(g_{\text{NMDA}} + g_{\text{other}}) \), where \( g_{\text{NMDA}} \) and \( g_{\text{other}} \) are NMDA and other conductance, respectively; and \( E_{\text{NMDA}} \) and \( E_{\text{other}} \) are reversal potentials for NMDA and other conductances, respectively. For \( E_{\text{NMDA}} = 0 \) mV, we can rearrange this to \( g_{\text{NMDA}} = g_{\text{other}} V_{\text{CPP}}/V_{\text{rest}} \), where \( V_{\text{CPP}} \) is the change in \( V_{\text{rest}} \) upon adding CPP. We can approximate \( g_{\text{other}} \) from the input resistance, which was \( 52 \pm 11 \) MΩ at \( V_{\text{rest}} = -60 \) mV (\( N = 6 \) cells), similar to published values (Cao et al. 2007; Chanda and Xu-Friedman 2010b, 2011). We measured \( V_{\text{rest}} = -1.8 \) mV (Fig. 6G), so \( g_{\text{NMDA}} = 0.6 \) nS, or 12 NMDARs. Thus the low levels of ambient glutamate activate less than 4% of synaptic and extrasynaptic receptors at any given moment. The population of activated NMDARs presumably changes constantly, so that MK-801 can block a large fraction of NMDARs over 6 min of application. It is striking that so few receptors are still able to influence membrane potential and spiking, especially considering the low input resistance of BCs. For cells with higher input resistance, such influence could be even greater.

**NMDAR subunit switch.** Our results suggest that mature BCs include both NR2A- and NR2B-containing NMDARs at synaptic and extrasynaptic sites. These results differ from the idea that mature, synaptic NMDARs are primarily NR2A-containing, while immature and extrasynaptic NMDARs are NR2B-containing. Such a switch does appear to take place in NR2A and NR2B mRNA levels in chick nucleus magnocellularis and nucleus laminaris (Tang and Carr 2007). However, NR2A- and NR2B-containing NMDARs appear to be in both synaptic and extrasynaptic compartments in hippocampus (Harris and Pettit 2007; Petralia et al. 2010). Interestingly, another large mammalian auditory synapse, the calyx of Held in the medial nucleus of the trapezoid body, expresses primarily NR2A and NR2C subunits after the onset of hearing and lacks ifenprodil sensitivity (Steinert et al. 2010). This coincides with a dramatic acceleration in NMDAR EPSC kinetics. By contrast, ifenprodil sensitivity persists in the endbulb, and NMDAR EPSCs show little acceleration after the onset of hearing (Pliss et al. 2009). This presumably reflects differences in the functional role of NMDARs between the two synapses, despite their superficial similarity.

**Modulation of resting potential.** Our experiments indicate that both NMDARs and mGluRs are normally tonically bound by ambient glutamate and can, therefore, influence excitability and \( V_{\text{rest}} \). Furthermore, the mGluRs appear to be entirely insensitive to synaptically-released glutamate, suggesting they primarily contribute to \( V_{\text{rest}} \) in response to the BC’s local environment. This raises the possibility that tonic activation of both receptor types could be modulated. This could happen in at least two ways. First, as our experiments with TBOA show (Fig. 9), modulation could take place through changes in transporter efficacy. These transporters are expressed on glia (Huang and Bergles 2004; Rothstein et al. 1994), so glia could help set \( V_{\text{rest}} \) through regulating glutamate reuptake. It is not known whether this occurs normally, but we predict the consequences of even small changes in efficacy could lead to significant changes in spiking. BCs were extremely sensitive to small changes in ambient glutamate, especially under conditions of high ANF activity, when endbulbs depress and BC spiking normally decreases considerably. However, an increase in ambient glutamate significantly offsets such decreases (Fig. 9). Remarkably, this was at least partly as a result of recruitment of NMDARs, despite the presence of Mg\(^{2+}\), which normally blocks NMDARs at resting potentials. Glia could sense activity levels in the AVCN, such as through their own glutamate receptors, and could modulate transporter efficacy accordingly. We did not observe such an effect in response to focal stimulation of a limited set of ANFs (Fig. 8), perhaps because our stimulation was too limited, or because the extracellular signaling environment is disrupted in brain slices. This issue will require further investigation.

A second way that the level of NMDAR and mGluR activation could be modulated is through changing the amount of glutamate released at the source. Our experiments provide significant evidence that the source is nonneuronal. ANFs form the only known glutamatergic terminals onto BCs, but mGluRs could not be driven by evoked glutamate release from ANFs (Fig. 8). Therefore, spontaneous mEPSCs, which release much less glutamate, are unlikely to contribute significantly either. In addition, our MK-801 experiments suggest spontaneous quantal release is not the source of glutamate either, because the mEPSC rate in T-stellate cells is similar to BCs (Lu et al. 2007), yet MK-801 did not significantly block synaptic NMDARs on T-stellate cells. Moreover the TBOA-induced depolarization is unchanged in TTX, consistent with other reports (Herman and Jahr 2007; Jabaudon et al. 1999), indicating release of ambient glutamate is action-potential independent.

Our experiments using FC provide positive evidence implicating glia. FC appears to be specifically taken up by glia. Consistent with this, FC did not affect EPSCs or mEPSCs, indicating the release of neurotransmitter from ANFs and receptor responses on BCs were unchanged. However, the effects of TBOA were potentiated (Fig. 8D), indicating involvement of glia in this effect. It is notable that FC did not eliminate the depolarization induced by TBOA. This most likely means that there are at least two independent regulators of ambient glutamate, FC-sensitive mechanisms of glutamate release, as well as TBOA-sensitive glutamate transporters. When FC is applied, the release mechanisms appear to be dysregulated and ambient glutamate rises, but the transporters evidently remain effective and prevent depolarization until TBOA is applied. Other scenarios are also possible. It is most likely that the transporters are located on astrocytic glia, and additional investigation will be required to explain why these would continue to operate in the presence of FC. At this point, we conservatively conclude that glia are the most likely source of glutamate.

Estimates of ambient glutamate vary widely. In cerebrospinal fluid, glutamate is in the micromolar range (Lerm et al. 1986), while the concentration near synapses may be as low as 25 nM (Herman and Jahr 2007). Our TBOA experiments and modeling support the idea that glutamate concentration is less
than 1 μM. The increase in glutamate induced by TBOA is insufficient to desensitize AMPARs appreciably and does not occlude NMDARs involved in EPSCs. Our NMDAR models suggest the peak glutamate concentration is <500 nM, consistent with other estimates (Jabaudon et al. 1999). Resting levels of glutamate would be considerably less, but our experiments using the NMDAR antagonist CPP indicated that resting levels were sufficient to tonically activate some NMDARs, just as we had seen in similar experiments with group I mGluRs (Chanda and Xu-Friedman 2011). NMDARs have a small but measurable conductance at $V_{\text{rest}}$ (Pliss et al. 2009), and further depolarization arising from synaptic activity could amplify the effects of tonic NMDAR activation.

The ultimate effect of ambient glutamate is to depolarize BCs through NMDAR and mGluR activation. The mildest depolarization (<3 mV) by application of low concentrations of TBOA significantly increased BC spike probability. By contrast, blocking mGluRs and NMDARs causes hyperpolarization and a decrease in spike probability. This indicates that even small fluctuations of $V_{\text{rest}}$ can have a great impact on excitability. Furthermore, $V_{\text{rest}}$ lies near the region where spiking would be maximally sensitive to small changes in receptor activation caused by ambient glutamate. BCs relay temporal information in ANF spike trains to higher centers for sound localization (Grothe et al. 2010). Endbulbs show strong short-term depression, and BC spiking decreases at high rates of ANF activation, such as during prolonged intense sounds, which could affect perception. Restoration of spiking during high rates of activation may, therefore, be adaptively useful. Our results indicate that even small increases in ambient glutamate have large effects on BC excitability, which could provide a sensitive way for the cochlear nucleus to enhance BC responsiveness under different conditions.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: Y. Y. and M. A. X.-F. conception and design of research; Y. Y. performed experiments; Y. Y. and M. A. X.-F. interpreted results of experiments; Y. Y. and M. A. X.-F. prepared figures; Y. Y. and M. A. X.-F. drafted manuscript; Y. Y. and M. A. X.-F. edited and revised manuscript; Y. Y. and M. A. X.-F. approved final version of manuscript.

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