Regulated Expression of Surface AMPA Receptors Reduces Excitotoxicity in Auditory Neurons

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ABSTRACT

Dynamic regulation of the expression of surface AMPA receptors (AMPAR) is a key mechanism to modulate synaptic strength and efficacy in the central nervous system and also to regulate auditory sensitivity. Here we address the role of surface AMPAR expression in excitotoxicity by blocking clathrin-mediated AMPAR endocytosis in auditory neurons. We used a membrane permeable, dynamin-derived, myristoylated peptide (myr-Dyn) to inhibit surface AMPAR endocytosis induced by glutamate receptor agonists in culture and by noise exposure in vivo. Myr-Dyn infused into the mouse cochlea induced excitotoxic responses to acoustic stimuli that were normally not excitotoxic. These included vacuolization in the nerve terminals and spiral ganglion, as well as irreversible ABR threshold shifts. In cultured spiral ganglion neuronal cells, blockade of the reduction of surface AMPAR exacerbated neuronal death by incubation with NMDA and AMPA. This excitotoxic neuronal death could be prevented by calpeptin, a calpain specific inhibitor. These results suggest that the reduction of surface AMPAR by endocytosis during excitatory stimulation plays an important role in limiting the excitotoxic damage to the neuron.
INTRODUCTION

Dynamic changes in the expression of surface AMPA receptors (AMPARs) have been shown to regulate synaptic strength and efficacy, and are thought to underlie mechanisms of long-term potentiation and long-term depression in hippocampal neurons (Barry and Ziff 2002; Bredt and Nicoll 2003; Collingridge et al. 2004; Derkach et al. 2007; Malinow and Malenka 2002; Man et al. 2000; Newpher and Ehlers 2008; Sheng and Lee 2001; Song and Huganir 2002). In auditory neurons, we have shown that surface AMPAR expression is reversibly decreased both in response to acoustic overstimulation in vivo and with application of glutamate receptor agonists in culture (Chen et al. 2007). The reversible reduction of surface AMPARs following acoustic stimulation correlated with a decrease in the acoustic sensitivity of auditory neural responses.

Auditory neurons display excitotoxic responses to acoustic overstimulation, characterized by cellular swelling (vacuolization) in the spiral ganglion and vacuolization of dendritic endings near the inner hair cells (Pujol and Puel 1999; Wang et al. 2002). This process also can be seen in response to exogenous glutamate receptor agonists such as AMPA and kainate and is blocked by AMPAR antagonists (Khan et al. 2000; Le Prell et al. 2004; Ruel et al. 2000; Sun et al. 2001). It also can be exacerbated by drugs that block desensitization of AMPARs (Ruel et al. 1999). Thus the excitotoxic effect in the auditory neurons is thought to be mediated via activation of AMPARs. Recent evidence suggests that the excitotoxic response to acoustic overstimulation is followed by denervation of the inner hair cells and neuronal cell death over a period of days to months (Kujawa and Liberman 2006).

It is reasonable to hypothesize that the reduction in surface AMPA receptors with acoustic overstimulation serves not only to regulate auditory sensitivity, but may also
ameliorate potential excitotoxic effects of loud sounds. In the present study, we examined this hypothesis by blocking the removal of surface AMPARs during a normally non-excitotoxic acoustic exposure and observing signs of neuronal excitotoxicity. We blocked the sound-induced removal of surface AMPARs in vivo by infusing the mouse cochlea with a membrane permeable, dynamin-derived, myristoylated peptide (myr-Dyn), which inhibits clathrin-mediated AMPAR endocytosis (Marks and McMahon 1998; Nong et al. 2003). We specifically looked at the GluR2 subunit of the AMPAR. Myr-Dyn-infused ears demonstrated smaller reductions in surface GluR2 receptors and increased excitotoxic-like neuronal vacuolization. Noise-induced threshold shifts were exacerbated in myr-Dyn-infused ears, and, unlike noise-only ears, auditory sensitivity did not recover during a 6 h post-exposure period of monitoring. We also showed that normally non-excitotoxic concentrations of glutamate receptor agonists could induce excitotoxic responses in the presence of myr-Dyn in auditory neuronal culture. The excitotoxic response in auditory neurons was prevented by calpeptin, a specific calpain inhibitor. Therefore, we suggest that the decreased expression of surface AMPARs we observe in response to acoustic stimulation may serve to protect auditory neurons from the excitotoxic effects of acoustically mediated AMPAR activation.

METHODS

Cell culture

Auditory spiral ganglion neurons were cultured as described previously (Chen et al. 2007). The bulla was retrieved from postnatal day 3-5 CBA/CaJ mice and placed in ice-cold Hank’s balanced salt solution (HBSS, Gibco). The modiolus was isolated from the
surrounding tissue, cut into ~3 pieces, and transferred to an enzymatic solution containing collagenase type IV (0.5 mg/ml) and trypsin (2.5 mg/ml) at 37°C for 25 min. The enzymatic digestion was terminated by replacing the supernatant with culture medium (see below). The tissue was dissociated by gentle mechanical trituration with a pipette. The cell suspension was enriched for spiral ganglion cells by taking advantage of the observation that non-neuronal cells tend to stick to plastic Petri dishes while neuronal cells do not. The suspension was subjected to 3 sequential platings onto dry 35 ml dishes. After each plating, the suspension was poured off into another plate. The final plating was transferred into poly-L-ornithine coated 8-well-chamber coverglass (Nalge Nunc International, Naperville, IL) at a density of 2 cochleae per 8-well plate. Cultures were maintained in a humidified 5% CO2 incubator at 37°C for 15-18 h. Culture medium contained Dulbecco’s modified eagle’s medium (DMEM) and F-12 (1:1 v:v), 10% FBS, 5% horse serum, NT-3 (20 ng/ml), BDNF (5 ng/ml), 2% B-27 supplement, penicillin (100 U/ml), and streptomycin (100 µg/ml).

**Immunostaining of surface AMPA receptors**

Immediately prior to treatment, the cultures were gently washed 3 times with artificial perilymph (AP), which was composed of (in mM): NaCl, 120; KCl, 3.5; CaCl₂, 1.5; glucose, 5.5; HEPES, 20; titrated with NaOH to pH 7.5; total Na⁺=130 mM). The cultures were treated with 20 µM myr-Dyn for 10 min followed by myr-Dyn with 20 µM NMDA or with 20 µM AMPA for another 10 min at 37°C. As controls, the cells were treated with either 20 µM NMDA, 20 µM AMPA, 20 µM myr-Dyn or AP alone for 10 min. The cells were then washed 3 times with AP and fixed immediately with 4% formaldehyde/4% sucrose for 20 min and then blocked with 10% horse serum without permeabilization at room temperature.
Surface GluR2 was labeled by incubating for 2 h with mouse anti-GluR2 antibody recognizing the extracellular epitopes of GluR2 (1:200, Chemicon). Goat anti-mouse biotin (1:500, Jackson ImmunoResearch, West Grove, PA) was incubated with the culture for 1 h to amplify the GluR2 signal and the biotin-bound GluR2 was saturated with fluorescein-conjugated streptavidin (1:500, Jackson ImmunoResearch, West Grove, PA) for 1 h. The cells were then permeabilized with 0.15% Triton X-100 in 10% horse serum for 1 h and incubated with rabbit anti-neurofilament M antibody for 2 h. The neuronal marker was saturated with Cy3-conjugated goat anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA) for 1 h.

Fluorescence was visualized using a Zeiss Axiovert 200 inverted microscope and images were acquired with a 16-bit, cooled CCD camera (Princeton Instruments MicroMAX) driven by a LabView software package (National Instruments, Austin, TX). The exposure time was 0.3 sec for Cy3 and 1 sec for fluorescein.

For quantification of GluR2 receptors, wells were examined for clusters of neurons, based on neurofilament (Cy3) label. Filter sets were then switched and images taken of GluR2 (FITC) label. Five images, randomly taken based on the presence of neurons, were obtained from each well. The quantification of GluR2 labeling was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/) on the 16-bit images. Background values within the image were thresholded out, and GluR2 intensity per neuron was calculated as the mean pixel intensity multiplied by total pixel number and divided by the number of neurons.

Cellular viability assay and neuronal staining
To quantify excitotoxic cell death in auditory neuronal cultures, dead cells were labeled with ethidium homodimer-1 (EthD-1, Live/Dead Viability/Cytotoxicity kit; Invitrogen, Carlsbad, CA) and neurons identified by labeling with rabbit anti-neurofilament M. The cultures were pretreated with 20 µM myr-Dyn for 10 min followed by myr-Dyn with 20 µM NMDA or with 20 µM AMPA, or myr-Dyn alone for another 10 min at 37°C. In some experiments, calpeptin (50 µM) was added to myr-Dyn with NMDA or AMPA. Treatment with AP, 20 µM NMDA, 20 µM AMPA, or 50 µM calpeptin alone served as control. The cells were returned to the incubator with culture medium for 6 h and then incubated with 4 µM EthD-1 for 20 min at room temperature. Thereafter, the culture was fixed with 4% formaldehyde/4% sucrose for 20 min at room temperature. After permeabilization with 0.15% Triton X-100 in 10% horse serum for 1 h, the cells were incubated with rabbit anti-neurofilament M antibody overnight. The neuronal marker was saturated with FITC-conjugated goat anti-rabbit secondary antibody (1:100, Jackson ImmunoResearch, West Grove, PA) for 1 h. Images from 6-7 random regions were taken from each well based on the presence of neurons. Neuronal death was quantified as the number of EthD-1 positive neurons divided by total neurons. Incubation of the cells with 300 µM AMPA or 300 µM NMDA with or without 50 µM calpeptin for 24 h served as positive control and the staining was performed as above.

Auditory function measurements

CBA/CaJ mice (6-8 weeks) were randomly assigned to one of the following groups: myr-Dyn infusion only; noise exposure only; noise exposure with artificial perilymph infusion; and noise exposure with myr-Dyn infusion. In the myr-Dyn-only group, auditory function
was tested immediately prior to the infusion and at regular intervals for 7.5 h during the infusion. For the noise-exposure-only-group and the noise with AP infusion group, auditory function was tested immediately prior to noise exposure and at regular intervals for 60-80 min post-noise exposure (Data from these 2 groups have been previously published in a different format (Chen et al. 2007)). For the noise exposure with infusion of myr-Dyn group, auditory function was tested immediately prior to noise exposure and at regular intervals for ~6 h post-treatment.

The mice were anesthetized (ketamine, 100 mg/kg, i.p.; xylazine, 10 mg/kg, i.p.; boosters of 1/3 to 1/2 the original dose as needed) and kept in a warmed sound-attenuating chamber throughout the experiment. Auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) were recorded at the right ear using our standard methods (Chen et al. 2006). Briefly, ABRs were measured under computer control in response to tone pips (8, 20 and 45.2 kHz; 5-ms duration; 0.5-ms rise/fall; cos2 shaping; 30/s) with level adjusted in 5 dB steps over the range required to capture threshold. Responses were detected with subcutaneous stainless electrodes placed at the vertex and ventrolateral to the ipsilateral ear, with a ground near the tail. The response was amplified (10,000 times), filtered (0.1–3 kHz bandpass) and averaged (across 512 sweeps at each frequency-level combination; artifact reject = 15µV peak-to-peak). Threshold was defined as the lowest stimulus level at which response peaks were clearly and reproducibly present. These visual detection threshold judgments were confirmed following termination of the experiment by offline display and analysis of the stored waveforms.
The 2f1 – f2 DPOAEs were recorded as response amplitude vs. primary level functions 
(L1 = 10–75 dB SPL; L2 = L1 – 10; primaries incremented together in 5 dB steps) for f2 = 8, 
20, 45.2 kHz (f2/f1 = 1.2). Ear-canal sound pressure was amplified, digitally sampled, 
averaged (20 discrete spectra at each frequency-level combination) and Fast-Fourier 
transforms were computed from the averaged pressures. DPOAE level at 2f1 – f2 and 
surrounding noise floor values (±50 Hz of 2f1 – f2) were extracted and stored. Iso-response 
contours (L2 levels required to generate a DPOAE amplitude criterion of 0 dB SPL) were 
constructed from amplitude versus sound level data.

Noise exposure

The sound overexposure was a broadband noise (1-40 kHz), generated by a custom-built 
noise generator, filtered (Rockland Brickwall), amplified (Crown D-75), and presented, using 
a sound delivery tweeter (Radio Shack 40-1377), directly to the right ear of the animal at 93 
dB SPL for 10 min.

Intra-cochlear drug delivery

To test the role of removal of surface AMPAR in noise-induced neurotoxicity and 
hearing loss, we infused myr-Dyn into the mouse cochlea using a delivery method described 
previously (Chen et al. 2006). Briefly, animals were anesthetized as above and the surgical 
procedure was performed in the warmed chamber. A ventral skin incision was made 
longitudinally to expose the digastric muscle, which was cut to expose the underlying 
tympanic bulla and stapedial artery. The stapedial artery was cauterized at the entrance to the
bulla and the surrounding fat tissue was removed to expose the inferior-medial aspect of the 
bulla. A cochleostomy was drilled ~300 µm beneath the stapedial artery stump. The tip of a 
10 cm length of fused silica tubing (OD = 144 µm, ID = 75 µm; World Precision 
Instruments, Florida, USA) was then inserted into the cochleostomy. Dental cement was 
applied to seal the hole and to secure the tubing to the bulla.

An infusion/withdrawal syringe pump (Harvard Apparatus PHD 2000) was operated at an 
infusion flow rate of 1 µl/h. A three-way miniature manifold (Warner Instruments, now 
Harvard Apparatus MM-2) was placed in between the silica tubing and the pump to allow for 
changes of infusion solutions. Another three-way valve was placed between the manifold and 
the pump and an open/shut valve on another end of the drain line. When the drain open/shut 
valve was closed, the flow from the pump was directed into the cochlea. To change the 
 injection solution, the drain valve was opened and the new solution was loaded through the 
three-way valve into the line between the pump and manifold.

ABRs and DPOAEs were measured before and regularly during the infusion. The initial 
infusion was of AP (1 µl/h for 30 min), followed by myr-Dyn (200 µM for 90 min). Then the 
mouse was exposed to the broad-band noise as described above for 10 min and ABRs were 
tested regularly within 6 h following noise exposure until the animal was sacrificed for 
histological examination. In the control group, myr-Dyn (200 µM) was infused and ABRs 
and DPOAEs were monitored for 7.5 h.

Histological preparation

Anesthetized mice were perfused intracardially with 2.5% glutaraldehyde and 1.5% 
paraformaldehyde in a 0.1 M phosphate buffer. The petrous bone was extracted and the
round and oval windows opened to allow intralabyrinthine perfusion of fixative. The cochlea was kept in the same fixative at 4°C overnight and then osmicated (1% OsO₄ in dH₂O) for 60 min and decalcified (0.5 M EDTA with 1% glutaraldehyde) overnight. After decalcification, cochleae were dehydrated in ethanol and propylene oxide and then embedded in Araldite resins and sectioned in a horizontal plane parallel to the spiral axis of the upper turn at 20 μm with a carbide steel knife. Sections were mounted in Permount on microscope slides and coverslipped.

The cochlear spiral was reconstructed in 3D using Neurolucida® (MicroBrightField, Colchester, VT) software and tracking the heads of pillar cells as the reference point for cochlear lengths (Wang et al. 2002). Distance from base of the cochlea was computed for each section through the cochlear duct using custom software. The cochlear location was converted into characteristic frequency based on the place-frequency map of the mouse cochlea (Muller et al. 2005).

Spiral ganglion neurons at locations corresponding to frequencies of 6, 13, 27, 30, 33 and 60 kHz were visualized. The number of neurons with associated cellular swelling and total neurons were counted with 100X oil-immersion objectives at all focal depths through the cross section at chosen locations.

Vacuolization around the ganglion cell was identified as a distinctive clear area surrounding the spiral ganglion cells. These distinctive profiles have been examined in detail by electron microscopy (Wang et al. 2002) and have been shown to be associated with separation of the myelin lamina of the myelinating satellite cells with large fluid spaces. All ganglion cells in the mid-modiolar sections were counted, and all ganglion cells with associated cell swelling were counted.
All animal procedures were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary.

RESULTS

If, as we hypothesize, one of the functions of removal of surface GluR2 receptor is to prevent excitotoxic damage in the auditory neurons, then blockade of GluR2 removal should produce excitotoxic responses to even relatively low levels of acoustic stimulation. To test this hypothesis, we infused the mouse cochlea with myr-Dyn to inhibit the removal of surface AMPAR and then exposed the animal to a broadband noise for 10 min. We chose a noise exposure that does not generate excitotoxicity, but which we have previously shown to induce removal of surface GluR2 receptors, and we chose a concentration of myr-Dyn that we have previously demonstrated to block the removal of surface receptors in vivo (Chen et al. 2007).

The 10-min noise exposure alone produced a reversible loss in auditory sensitivity in the mouse (Fig. 1A). Neural-based ABR thresholds were elevated by ~15 dB at 8 kHz, ~20 dB at 20 kHz and ~30 dB at 45.2 kHz, when measured 2–6 min after noise and returned to near baseline levels within 60–80 min. When we infused the mouse cochlea with myr-Dyn before, during and after noise exposure, ABR thresholds did not recover; instead, they were up to 15-40 dB poorer, depending on frequency, than those recorded in noise-only ears at the 80 min post-exposure time point (Fig. 1C) and they did not recover over the 6 h period over which we monitored. Infusion of myr-Dyn (200 µM for 7.5 h, Fig. 1B) alone did not significantly elevate ABR thresholds (the ~5 dB maximum shifts across all frequencies were similar to changes we have recorded during extended AP infusion only). Thus an acoustic stimulus that
normally induced removal of surface GluR2 receptor to induce a temporary threshold shift,
became, in the presence of myr-Dyn, a potentially excitotoxic stimulus.

A robust and quantifiable consequence of exposure to intense acoustic stimuli or to
glutamate receptor agonist is the appearance of swelling around neurons in the spiral
ganglion (Fig. 2B), which, in transmission electron microscopy, appears as a clear space
between the neuron and the Schwann cell (Sun et al. 2001; Wang et al. 2002). Another
observation is the appearance of vacuolization at the nerve terminal near the inner hair cell
(Pujol and Puel 1999; Wang et al. 2002). Both phenomena are easily observed and quantified
with Nomarski optics (Fig. 2B, D). Because nerve terminal vacuolization can be seen as an
artifact of imperfect fixation via arterial perfusion, we only analyzed animals that, after
fixation, had a white liver and a hard brain, which are indicants of thorough fixation. To
assess excitotoxicity, we quantified the percentage of neurons exhibiting these phenomena 6
h after exposure to the 10 min noise stimulus described above. Control animals, exposed
either to myr-Dyn alone, or noise alone, showed no ganglion cell-associated vacuolization
(Fig. 2A) and minimal vacuolization in the terminals (Fig. 2C). However, significant
numbers of ganglion cell-associated vacuolizations were identified in ears exposed to noise
during myr-Dyn infusion (Fig. 2B, C). Vacuolization was greatest in the 30 kHz region (11%
of cells showed vacuolization) and decreased apically and basally. Similarly, vacuolization
in the nerve terminals mirrored the findings for ganglion cell-associated vacuolization.

To examine some of the mechanisms of excitotoxicity in auditory neurons, we turned to
cultured auditory neurons, where we have previously demonstrated that activation of AMPA
or NMDA receptors can induce removal of surface AMPA receptor (Chen et al. 2007). We
quantified surface and total AMPA receptors by measuring GluR2 labeling before (surface)
and after (total) permeabilizing the cells membrane. Fixation alone slightly increased permeability, as evidenced by an 11% increase in label for neurofilament (an intracellular protein). Consistent with our previous study, a 10 min incubation with NMDA (20 µM) or AMPA (20 µM) produced a ~ 55-60% decrease of surface GluR2 labeling. While myr-Dyn (20 µM, 10 min) alone did not significantly alter the presence of surface GluR2, myr-Dyn completely blocked NMDA- or AMPA- induced removal of surface GluR2 receptors from these cultured auditory neurons (Fig. 3A, B).

We also assessed the ability of myr-Dyn to enhance the excitotoxic effects of glutamate agonists with a viability assay using EthD-1 to label the nuclei of dead cells. Because calpain activation plays an important role in excitotoxic neuronal death, we determined if calpeptin, a calpain-specific inhibitor, could prevent neuronal death. Cultured auditory neurons were fixed and permeabilized to label neurons with anti-neurofilament M antibody. Live neurons were identified by positive neurofilament labeling with negative EthD-1 staining, while dead neurons were both neurofilament- and EthD-1 positive (Fig. 4A). We observed a relatively low neuronal death rate of ~2-3% in the control culture group 6 h after the treatment with artificial perilymph (AP) (Fig. 4B). This rate was not significantly altered by a 20-min application of myr-Dyn (20 µM) or by calpeptin (50 µM) alone (P>0.05).

When neurons were treated for 10 min with myr-Dyn followed by a 10-min incubation of myr-Dyn with AMPA or myr-Dyn with NMDA, the neuronal death rates were increased to 8.1% and 5.3%, respectively (Fig. 4B). Both of these increases could be prevented by the addition of 50 µM calpeptin. Thus normally non-lethal AMPA or NMDA concentrations became excitotoxic to auditory neurons when myr-Dyn blocked the removal of AMPARs on the cell surface. It was possible to induce neuronal death without myr-Dyn, but to do so
required very high concentrations of glutamate receptor agonists (300 µM) with incubation periods of 24 h. Under those conditions, AMPA induced death in 7.3% and NMDA in 5.3% of the neurons. Both could be prevented by 50 µM calpeptin (Fig. 4C).

DISCUSSION

We suggest that one role of regulated surface AMPAR expression is to limit excitotoxicity that might be induced during acoustic overstimulation. Intense acoustic stimulation can produce histological signs thought to be associated with excitotoxicity, which include vacuolization in the auditory nerve terminals and in the satellite cells near the ganglion cell bodies (Sun et al. 2001; Wang et al. 2002). The noise exposure we employed normally induces removal of surface AMPARs and does not induce signs of excitotoxicity. But when the removal of surface AMPARs was blocked with myr-Dyn, these same stimuli generated excitotoxic responses. In neuronal cultures from central nervous system, excitotoxicity can be observed as a calpain-mediated cell death (Lankiewicz et al. 2000; Bano et al. 2005). We showed that a brief exposure to glutamate receptor agonists at concentrations that induce surface AMPAR removal without generating cell death can induce cell death when removal of surface AMPARs is blocked by myr-Dyn.

The myristoylation of dynamin inhibitory peptide enables permeation of the peptide into the cell. Dynamin inhibitory peptide acts to block dynamin-dependent endocytosis by abolishing the recruitment of dynamin to clathrin-coated pits by amphiphysin (Grabs et al. 1997), a process involved in the endocytosis of AMPARs (Man et al. 2000). We cannot say what other cochlear processes are dynamin dependent, and thus potentially blocked by myr-dyn. We do show that fundamental indicators of cochlear function, such as the ABR and
DPOAEs are unaffected by myr-Dyn, suggesting limited roles for dynamin-dependent processes that are accessible to the infused drug. We have previously shown that infusion of myr-Dyn into the mouse cochlea can block the decrease of cochlear surface AMPARs induced by sound stimulation (Chen et al. 2007). In the present study, we demonstrated in cultured auditory neurons that myr-Dyn could inhibit the removal of surface AMPARs stimulated by agonists such as AMPA or NMDA.

Excitotoxicity following glutamate receptor activation is thought to result from excessive influx of extracellular ions, mainly Na\(^+\) and Ca\(^{2+}\), either directly via NMDA and AMPA receptors, or indirectly through voltage-gated Ca\(^{2+}\) channels or through Na\(^+\) loading and subsequent reversal of Ca\(^{2+}\)-Na\(^+\) exchange (Bano et al. 2005; Budd and Nicholls 1996; Choi 1988; Fryer et al. 1999; Lipton and Rosenberg 1994; Olney 1969; Rothman et al. 1987). A reduction in the number of AMPARs present on the synaptic surface should reduce the amount of ion current entering the auditory neuron for a given amount of transmitter released. We have suggested that regulation of GluR2 in auditory nerve fibers is important in regulating synaptic strength and dynamic range at this synapse. The current findings suggest that a consequence of this regulation is to reduce exposure of the neuron to damaging synaptic current that might induce excitotoxicity.

ABR threshold shifts observed after exposure to myr-Dyn plus noise are not likely due to an effect on the hair cells. First, DPOAEs, which are sensitive to outer hair cell dysfunction, were not different in the presence and absence of myr-Dyn following noise exposure, or for myr-Dyn alone. Second, while we did not specifically count hair cells, we did examine histological sections and saw no evidence of hair cell damage, or even stereociliary damage, in regions with vacuolization of nerve terminals.
If, as we have suggested (Chen et al. 2007), the reversible threshold shift following moderate noise exposure is due to removal and return of surface AMPARs, and if, as we have demonstrated, myr-Dyn blocks the removal of surface AMPARs, then we might expect myr-Dyn to prevent the noise induced removal and return of surface AMPARs and eliminate the threshold shift following moderate noise exposure. Instead, myr-Dyn had the opposite effect: Moderate noise exposure with myr-Dyn induced threshold shifts in the neurally-based ABRs that increased over the next 6 h instead of recovering. Our interpretation is that the threshold elevations in the presence of myr-Dyn are a consequence of excitotoxic insult resulting from the inability of the neurons to reduce surface AMPARs in face of prolonged neurotransmitter release. This interpretation is supported by the observation of anatomical signs of excitotoxicity (cell swelling around the spiral ganglion) and by the absence of an action of myr-Dyn on the outer hair cell-based DPOAEs. The time course of the delayed threshold elevation with noise in myr-Dyn is consistent with the finding that the delayed neuronal degeneration occurs over a period of hours due to excess entry of Ca\textsuperscript{2+} after excitotoxic stimuli (Budd and Nicholls 1996; Lipton and Rosenberg 1994; Rothman et al. 1987).

Up-regulation of the calpain pathway has been strongly implicated in the pathophysiology of excitotoxic neuronal death through its action to cleave cellular proteins (Goll et al. 2003; Ray and Banik 2003). We showed that a calpain-specific inhibitor prevented excitotoxic auditory neuronal death in cell culture, which is consistent with previous findings in the neurons from brain, retina and cochlea (Bartus et al. 1994; Cheng et al. 1999; Das et al. 2006; Kupina et al. 2001; Saatman et al. 1996).
In auditory neurons, cellular vacuolization in the spiral ganglion and vacuolization of auditory nerve terminals following noise exposure is thought to be an excitotoxic effect related to glutamatergic synaptic transmission (Pujol and Puel 1999; Sun et al. 2001; Wang et al. 2002). The afferent terminal vacuolization can be mimicked by cochlear perfusion of glutamate receptor agonists such as AMPA and kainate and this excitotoxicity can be blocked by antagonists to AMPARs (Ruel et al. 2000).

Noise exposure in the presence of myr-Dyn produced vacuolization around cochlear ganglion cells, with the greatest effects in the 20 to 30 kHz region, with less vacuolization apically and basally. This observation is similar to that reported by Wang et al (Wang et al. 2002) who used much more intense acoustic stimulation to induce excitotoxic responses in the auditory nerve. They suggested this was due to more severe damage to hair cells at higher frequencies because of their increased susceptibility, an outcome that could lower the amount of transmitter released at higher frequencies. An alternative explanation, based on Furness and Lawton’s (2003) observation of an increased density of glutamate receptors in the middle turn of the cochlea, is that the characteristic frequency regions most susceptible to excitotoxic responses are those with the greatest density of glutamate receptors.

In summary, we have shown that a moderate acoustic exposure, which is normally not excitotoxic, can be made excitotoxic if the auditory neuron is prevented from regulating surface AMPAR removal through application of myr-Dyn. Thus we demonstrate that a functional consequence of regulating synaptic strength by trafficking surface AMPARs is to limit excitotoxicity during acoustic exposure.

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FIG. 1. Blocking the removal of cochlear surface AMPARs with myr-Dyn increased and prolonged ABR threshold shifts. A: ABR threshold shifts in control animals infused with an artificial perilymph solution and exposed to noise. Ten mice were exposed to a 10-min broad-band noise (1-40 kHz, 93 dB sound pressure level) and ABR thresholds were measured up to 80 min after the noise. There were initial ABR threshold shifts of ~15 dB at 8 kHz, ~20 dB at 20 kHz and ~30 dB at 45.2 kHz, and the ABR thresholds were nearly recovered at the end of measurements. Means ± s.e.m. are plotted. We have previously published, in a different format, the data presented in panel A (Chen et al. 2007). B: ABR threshold shifts with myr-Dyn infused before, during and after the same noise exposure.
When the animals were exposed to the same noise in the presence of myr-Dyn, ABRs thresholds did not recover over the ~6 h-period monitored after noise. Myr-Dyn (200 µM) was infused 90 min before noise exposure and infusion with myr-Dyn continued throughout the experiment. Twelve mice were followed for up to 2.5 h after noise exposure, and 3 of them were followed up to 6 h after noise. 4 mice were exposed to myr-Dyn alone. Means ± s.e.m. are plotted. Myr-Dyn did not alter the DPOAEs, suggesting its effects on the outer hair cells (OHCs) were minimal. Myr-Dyn also did not alter noise-induced DPOAE shifts, suggesting that effects on ABRs were secondary to processes occurring beyond the level of the OHCs. C. Myr-Dyn (200µM) did not appreciatively alter the response of the DPOAEs to noise stimulation. Ten mice exposed to same stimuli as in panels A and B. D: myr-Dyn (200 µM alone, 7.5 h) in the absence of noise exposure elevated ABR and DPOAE thresholds only slightly (~5 dB).
FIG. 2. Myr-Dyn infusion before, during and after noise exposure increased vacuolization associated with auditory neurons. A: noise exposure alone (1-40 kHz, 93 dB sound pressure level) did not generate vacuolization associated with spiral ganglion cell bodies 6 h after exposure. The image is of ganglion cells in the 30 kHz region. B: vacuolization associated with the ganglion cells (arrows) was seen 6 h after the same noise exposure with myr-Dyn infusion. Again, the image is from the 30 kHz region. Three cochleae from each group were examined with similar findings. In the 3 cochleae examined, we observed 118 ganglion cell-associated vacuolization profiles out of 1763 neurons examined. In controls, we saw no vacuolizations in a similar number of ganglion cells examined. C: noise exposure alone did not induce vacuolization of the auditory nerve terminals at the inner hair cells. D: the same noise exposure with myr-Dyn induced vacuolization in auditory nerve terminals. The degree of vacuolization in afferent terminals and near ganglion cells was quantified for different cochlear regions. E: data were from collected from 12 animals – Ganglion cell-associated vacuolization was quantified in 3 control animals (noise only) and 3 experimental animals (noise + perfusion with myr-Dyn). Vacuolization in the nerve terminals were counted in 3 control animals (noise only) and 3 experimental animals (noise + myr-Dyn). Plotted are means ± s.e.m.
FIG. 3. Removal of surface AMPARs can be blocked by myr-Dyn in cultured auditory neurons. A: representative images of cultured auditory neurons with labeling of surface GluR2 and neurofilament (NF), and images from differential interference contrast light microscopy (LM). Auditory neurons in culture were identified by their appearance in DIC microscopy and by staining with an antibody against neurofilament M, a neuronal marker. Surface GluR2 was labeled by an antibody to an extracellular epitope on the receptor in the absence of permeabilization of membrane. B: myr-Dyn blocked the reduction of surface AMPARs induced by AMPA or NMDA. Incubation of the cultured neurons with AMPA (20 µM) or NMDA (20 µM) alone for 10 min decreased surface AMPARs to ~40-45%. Pretreatment of the culture with myr-Dyn (20 µM) for 10 min and during AMPA or NMDA incubation nearly completely blocked the removal of surface AMPARs. Each value represents the analysis of five images of neuronal clusters from each of three different
cultures. The number of neurons in each image ranged from 3-40. Plotted are means ± s.e.m. *: P<0.05, compared with control surface GluR2 without treatment as analyzed with a nonpaired Student’s t-test.
FIG. 4. Blockade of the removal of surface AMPARs with myr-Dyn increased excitotoxic ganglion cell death by AMPA or NMDA in culture. A: representative images of live and dead cells.
dead neurons. The dead cell has a shrunken or disrupted shape in light microscopy (LM).

Label with neurofilament (NF) was used to distinguish neurons from other cells in both live and dead cells. The dead cells were EthD-1 positive while the live cell were EthD-1 negative (L/D). B: blockade of surface AMPA receptor removal with myr-Dyn increased cell death following treatment with AMPA or NMDA. We chose doses of AMPA and NMDA (20 µM, 10 min) that we have previously demonstrated to produce reversible decreases in surface GluR2 (Chen et al. 2007). Treatment with 10 min of myr-Dyn (20 µM), AMPA (20 µM), NMDA (20 µM), or calpeptin (CP) alone, or no treatment resulted in ~2-3% neuronal death 6 h later. Pre-treatment of the culture with myr-Dyn (20 µM) for 10 min and during AMPA or NMDA incubation produced a significantly higher rate of neuronal death 6 h later, with 8.1% in the “AMPA with myr-Dyn” group and 5.3% in the “NMDA with myr-Dyn” group. The increase in neuronal death induced by myr-Dyn was prevented by coincubation with calpeptin (50 µM). C: in the absence of myr-Dyn, induction of ganglion cell death required very long exposure (24 h) at high concentrations (300 µM) of AMPA or NMDA. Treatment of the cultured ganglion cells for 24 h with AMPA (300 µM) alone or with NMDA (300 µM) alone resulted in neuronal cell death rates of 7.3% and 5.3% respectively. The significantly higher death rates with this treatment were nearly blocked by coincubation with CP (50 µM). Each group represents the analysis of 6-7 images of neuronal clusters from each of five different cultures. The number of neurons in each image ranged from 3-40. Plotted are means ± s.e.m. *: P<0.05, compared with control group without treatment as analyzed with a nonpaired Student’s t-test. There was ~3% neuronal cell death with treatment of the cells with CP (50 µM for 24 h) alone or without treatment.
FIG. 3.