Zinc Inhibition of Group I mGluR-Mediated Calcium Homeostasis in Auditory Neurons

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ABSTRACT

Zinc is widely distributed in the central nervous system (CNS), it functions normally as a synaptic modulator, and it contributes to neuronal death under pathologic conditions. Zinc colocalizes with glutamate in excitatory synapses, and the presence of zinc is well characterized in the synapses of the auditory system. Since chick cochlear nucleus neurons depend upon synaptic activation of metabotropic glutamate receptors (mGluRs) for maintenance and survival, the goal of this study was to determine (1) if zinc is released from the eighth nerve calyces onto nucleus magnocellularis (NM) neurons in the chick cochlear nucleus, and, if so, (2) what effect it has on group I mGluR-mediated calcium homeostasis of these neurons. Using *in vitro* slices and a fluorescent dye relatively specific to vesicularized zinc, we show that zinc is indeed localized to the presynaptic calyces and is released upon nerve stimulation or KCl depolarization. Experiments employing fura-2 calcium imaging show that zinc inhibits group I mGluR release of calcium from internal stores of NM neurons and disrupts activity-dependent calcium homeostasis in a manner identical to the mGluR5-specific antagonist 2-methyl-6-(phenylethynyl)pyridine. The mGluR1-specific antagonist 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester did not affect release of calcium from stores by the nonspecific mGluR agonist aminocyclopentane dicarboxylic acid, nor did it affect activity-dependent calcium homeostasis. We conclude that zinc is present in and released from the glutamatergic eighth nerve

calyces. The presence of zinc inhibits mGluR5, a major component of calcium homeostasis of NM neurons, and plays a modulatory role in the activity-dependent, mGluR-mediated calcium homeostasis of auditory neurons.

Keywords: chick nucleus magnocellularis, metabotropic glutamate receptor

INTRODUCTION

Neurons of the chick cochlear nucleus, nucleus magnocellularis (NM), require eighth nerve activation of metabotropic glutamate receptors (mGluRs) for maintenance and survival (Zirpel and Rubel 1996; Zirpel et al. 1998). Elimination of this activation results in an increase in intracellular calcium concentration ($[Ca^{2+}]_i$; Zirpel and Rubel 1996) that ultimately leads to the death of 20–40% of the neurons. The surviving neurons implement calcium-dependent compensatory mechanisms (Zirpel et al. 2000b) but show profound alterations in physiology, morphology, and metabolism (for review, see Rubel et al. 1990). The mechanism of this activity-dependent maintenance involves a complex interplay of multiple receptor types, second messenger systems, and calcium signaling.

Zinc has been shown to modulate synaptic transmission (Hollman et al. 1993; Nakazawa et al. 1995; Vogt et al. 2000) and to play a role in neuronal death (Canzoniero et al. 1999; Lee et al. 2000; Sheline et al. 2000). It is well known that synaptic endings in the cochlear nucleus contain zinc (Frederickson et al. 1988; Rubio and Ruiz 1998), and a preliminary study has shown

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that zinc translocates into the cytoplasm of the NM neurons which do not survive deafferentation (Wilson and Durham 1995). We hypothesized that the zinc in the eighth nerve calyces is released during synaptic activity and modulates the functioning of mGluRs, thus contributing to the dynamic equilibrium of calcium homeostasis in NM neurons. Consistent with this hypothesis, a dye that labels vesicularized zinc was localized to the calyces and showed significant reductions in fluorescence in response to nerve stimulation or KCl depolarization. Exogenously applied zinc inhibited the ability of the nonspecific mGluR agonist aminocyclopentane dicarboxylic acid (ACPD) to release calcium from intracellular stores of NM neurons and disrupted activity-dependent calcium homeostasis. The nonspecific mGluR antagonist methylcarboxyphenyl glycine (MCPG) also inhibited release of calcium from stores, similar to the effect of zinc, but more severely disrupted activity-dependent calcium homeostasis. The mGluR5-specific antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) paralleled the effects of zinc while the mGluR1-specific antagonist 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) was without effect. The effects of zinc and MPEP were not additive. These results suggest that calyceal zinc is coreleased with glutamate during synaptic transmission and plays a modulatory role in the activity-dependent calcium homeostasis of NM neurons by inhibiting mGluR5.

METHODS

HRP labeling

At embryonic day (E) 18, chick embryos were anesthetized with pentobarbital and perfused transcardially with 2% paraformaldehyde and 0.01% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and immersed in the same fixative for 1–2 hours at 4°C. Horseradish peroxidase (HRP; type VI, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and fine cactus (*Mammillaria elongata*) needles were dipped into this solution and allowed to dry at room temperature. An HRP-coated cactus needle was lowered into the eighth cranial nerve at the lateral margin of the brain stem and left there for 60 s. The brain was then immersed in 0.1 M phosphate buffer (pH 7.4) for 2 days at 4°C. The brain was then immersed for 4 hours in 4% glutaraldehyde in phosphate buffer at 4°C and placed in 30% sucrose in buffer overnight. Cryostat sections were cut at 25 μ m and reacted for HRP using the cobalt-intensified diaminobenzidine method. Sections were mounted on subbed slides from an alcohol–gelatin mixture, air

dried, counterstained with thionin, dehydrated, cleared, and coverslipped.

Slice preparation

Live *in vitro* tissue slices (300 μ m) were acquired from E18 embryos as previously described (Zirpel et al. 2000a,b) and maintained in oxygenated artificial cerebrospinal fluid (ACSF; 130 mM NaCl; 3 mM KCl; 2 mM CaCl_2 ; 2 mM MgCl_2 ; 26 mM NaHCO_3 ; 1.25 mM NaH_2PO_4 ; 10 mM glucose, pH 7.4). For pharmacology experiments, drug was added to ACSF.

Zinc imaging

Tissue slices were labeled with 5 μ M *N*-(6-methoxy-8-quinolyl)-p-carboxybenzoyl-sulphonamide (TFLZn) free acid (TefLabs, Austin, TX) in oxygenated ACSF for 20 min at room temperature. Imaging was performed with an inverted microscope using 340-nm excitation from a xenon lamp. Image acquisition and analysis were performed using IPLab (Scanalytics, Fairfax, VA). Outlines were drawn over the clearly labeled calyces (Fig. 1) and images acquired and analyzed online every 300 ms. A separate set of experiments was performed using a confocal microscope (CLSM) equipped with a UV laser (BioRad, Hercules, CA). The CLSM was operated in fast mode and 512-pixel $\times$ 512-pixel images were acquired at approximately 3 s intervals. Fluorescence measurements of confocal images were made using NIH Image (public domain software developed at the U.S. National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image>). Results were the same with both imaging techniques and have been pooled for presentation. Peak TFLZn fluorescence change was measured following eighth nerve stimulation or KCl depolarization. In 3 of 6 stimulated slices and 5 of 8 KCl-depolarized slices, there was a sharp artifact observed at the termination of the stimulus (see Fig. 1B). This artifact was present but much smaller in magnitude upon termination of other solution changes (e.g., from calcium-free ACSF back to normal ACSF), and no changes in fluorescence were observed. Therefore, we conclude that this inconsistent and transient drop in TFLZn fluorescence is, indeed, an artifact. Measurements were made so as to exclude this artifact.

Cochlea removals

Two days after hatching, four chicks underwent unilateral cochlea removal as previously described (Born and Rubel 1985; Zirpel et al. 2000b). Seventy-two hours after cochlea removal, chicks were sacrificed and *in*

FIG. 1. TFLZn labels the end-bulbs of Held and indicates zinc release by depolarization. **Aa.** End-bulbs labeled postfixation with HRP and counterstained with thionin. Arrowhead indicates calyx in which the unique morphology is readily apparent. **Ab.** End-bulbs labeled with TFLZn, a fluorescent dye specific for vesicularized zinc. Note same morphology as in Aa; especially in calyx denoted by arrowhead. **Ac.** NM labeled with TFLZn 72 hours after cochlea ablation, a time at which the eighth nerve axons and calyces have degenerated. Note staining pattern distinctly different than in normal, intact NM (b). Scale bar in Ab is 30 μ m for a, b, and c. **B.** TFLZn fluorescence decreases in response to eighth nerve stimulation and KCl depolarization. Top panel shows average TFLZn fluorescence from 8 neurons in a single slice that received 20 s of eighth nerve stimulation ($\diamond$) and 20 s of depolarization (both indicated by horizontal bar) with 80 mM KCl ($\circ$) following a 10-min recovery period. For comparison, both traces have been normalized to the same time abscissa. Lower panel shows the mean magnitude of TFLZn fluorescence decrease in response to eighth nerve stimulation ($n = 6$, $p = 0.013$, two-tailed t -test vs. 0) and depolarization with 80 mM KCl ($n = 8$, $p = 0.0007$, two-tailed t -test vs. 0). Asterisks denote a significant difference from zero.

vitro brain stem slices containing NM were acquired and labeled with TFLZn as described above.

Calcium imaging

Tissue slices were incubated in oxygenated ACSF containing 8 μ M Fura-2/AM (Molecular Probes, Eugene, OR), 1.7% anhydrous, DMSO, and 0.03% Pluronic for 30 min at room temperature. Slices were placed in the imaging chamber and bathed in normal, oxygenated ACSF for approximately 5 min before data acquisition. Ratiometric fluorescence imaging techniques used in this study were similar to those described previously (Zirpel et al. 2000a,b). Paired 350/380-nm excitation images were acquired approximately every 3–5 s or

every 60 s and ratioed on a pixel-by-pixel basis. Intracellular calcium concentration was estimated according to Grynkiewicz et al. (1985) using NIH Image. Five-point external calibrations were performed on the imaging system and the K_d of fura was calculated with each calibration. The range of K_d values was 75–172 nM. Up to 10 neurons were analyzed for any given experiment. However, each slice was considered $n = 1$ and the data from all neurons in a single slice were averaged. Cells were not included in the analysis if baseline $[Ca^{2+}]$ was greater than 250 nM, as this concentration is indicative of a dying cell (Zirpel et al. 1998). Cells were not added to or subtracted from the analysis of an experiment except when a complete loss of fluorescence was observed, indicating cell death (Johnson et al. 1994).

Intracellular calcium concentrations were plotted as a function of time using Excel (Microsoft Corp., Redmond, WA) and Cricket Graph (Cricket Software, Malvern, PA). Data are presented as means $\pm$ 1 SEM unless otherwise indicated. MPEP and CPCCOEt were from Tocris Cookson (Balwin, MO). TPEN was from Molecular Probes. All other pharmaceuticals, reagents, and chemicals were from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Zinc complexes with glutamate in a pH-dependent manner (Spiridon et al. 1998). The concentrations of zinc presented in this report reflect total added zinc and are not corrected for this functional chelation by glutamate or any other molecule. Thus, the concentrations reported for zinc effects are higher than the actual effective concentration due to this chelation.

Electrophysiology

Methods for stimulating and recording from *in vitro* chick brain stem slices have been previously described (Jackson and Parks 1982; Hyson and Rubel 1989; Zirpel and Rubel 1996; Zirpel et al. 1998). A concentric, bipolar, stimulating electrode (FHC, Bowdoinham, ME) was placed on the eighth nerve dorsolateral to NM. Stimulation pulses (50–100- μ s duration, 60–80 V) were delivered at a rate of 5 Hz. Field potentials were recorded with an ACSF-filled microelectrode ($\approx$ 1 M Ω) attached to an amplifier and monitored on an oscilloscope.

RESULTS AND DISCUSSION

Zinc in eighth nerve calyces

NM neurons receive excitatory glutamatergic input from the eighth nerve via specialized calyceal terminals called end-bulbs of Held (Jhaveri and Morest 1982; Carr and Boudreau 1991). These end-bulbs form

secure synapses that cover approximately two-thirds of the NM neurons (Parks et al. 1990). The morphology of these calyces can be seen in Figure 1A. Figure 1Aa shows eighth nerve terminals that have been orthogradely labeled with HRP. Figure 1Ab shows terminals that have been labeled with TFLZn, a fluorescent dye that binds to vesicularized zinc (Budde et al. 1997). Figure 1Ac shows NM labeled with TFLZn 72 hours following cochlea removal. At this time point the axons and calyces of the eighth nerve have degenerated and are no longer present (Parks and Rubel 1978). It is clear that the TFLZn staining pattern following calyx degeneration (Fig. 1Ac) is distinctly different than that seen in normal, intact NM (Fig. 1Ab). The staining is more diffuse, less intense, and evenly dispersed over the cell soma. The calyceal morphologies are not seen with TFLZn staining 72 hours after cochlea removal. These results are consistent with calyceal labeling of zinc with TFLZn in intact NM (Fig. 1Ab).

Action potentials elicited by stimulating the eighth nerve or depolarizing the terminals with KCl cause a transient decrease in TFLZn fluorescence (Fig. 1Ba) consistent with release of vesicles during synaptic transmission. The magnitude of the fluorescence change during nerve stimulation or KCl depolarization is shown in Figure 1Bb. These changes in TFLZn fluorescence were not observed in NM of chicks that had undergone cochlea removal 72 hours prior to slice acquisition ($n = 4$, data not shown). These results confirm the histochemical findings of Wilson and Durham (1995) showing that zinc is present in the eighth nerve calyces terminating on NM neurons. In addition, our results suggest that zinc is released during nerve stimulation and end-bulb depolarization.

Zinc inhibition of calcium release from intracellular stores

NM neurons express group I mGluRs that release calcium from intracellular stores (Zirpel et al. 1995) and contribute to the dynamic calcium homeostasis of these neurons during normal synaptic transmission (Zirpel and Rubel 1996). Therefore, we examined the ability of zinc to modulate the release of calcium by the nonspecific mGluR agonist aminocyclopentane dicarboxylic acid (ACPD, 1 mM) in nominally Ca^{2+} -free medium. The initial ACPD experiments were performed with 1 mM EGTA in the bathing medium. However, since EGTA also chelates zinc, and since previous studies have shown that ACPD elicits identical mGluR-mediated Ca^{2+} responses from NM neurons in the presence or absence of extracellular Ca^{2+} (Zirpel et al. 1995), Ca^{2+} was omitted from the medium during zinc experiments, but no chelator was added. ACPD consistently and repeatedly elicited a calcium response

under these conditions that is not affected by ionotropic glutamate receptor antagonists (Zirpel et al. 1995). Figure 2A shows a typical response. The presence of zinc in the superfusing ACSF attenuated the calcium response evoked by ACPD (Fig. 2A), second response) in a dose-dependent manner (Fig. 2B). NM neuron responses to ACPD do not show desensitization or attenuation in the time frame of the present experiments; repeated application of ACPD elicits consistent Ca^{2+} responses (Zirpel et al. 1995).

To determine if zinc affects one or both members of the group I mGluRs, specific and nonspecific antagonists were compared. Figure 2C shows that the mGluR5-specific antagonist MPEP (Gasparini et al. 1999; Santori et al. 1999) had a similar effect on ACPD-induced calcium release as did zinc, reducing the response by 60% (vs. 52.5% for 100 μM zinc). In a heterologous expression system, MPEP has been shown to specifically and potently inhibit chick mGluR5a (Storjohann et al. 1999). The combination of MPEP and zinc did not have an additive effect, inhibiting Ca^{2+} release to the same extent as either zinc or MPEP alone. In contrast, the mGluR1-specific antagonist 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) (Hermans et al. 1998; Litschig et al. 1999) was without effect on ACPD-evoked calcium release (Fig. 2C), suggesting that the release of calcium from stores (and zinc inhibition of this release) is mediated mainly through mGluR5. Consistent with this hypothesis, the nonspecific mGluR antagonist methylcarboxyphenyl glycine (MCPG) did not attenuate the ACPD-evoked calcium response to any greater extent than did zinc, MPEP, or the combination of zinc and MPEP (Fig. 2C; 79% vs. 52.5%, 60%, and 57.5%, respectively; $p > 0.05$ Fisher's PLSD *post hoc* analysis). Interestingly, the zinc chelator tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) caused a 60% potentiation of ACPD-evoked calcium release (Fig. 2C; $p = .0384$, Fisher's PLSD *post hoc* analysis). TPEN chelates zinc and other heavy metals both intracellularly and extracellularly, thus confounding interpretation of the localization of the zinc effect. To circumvent these problems with TPEN, the same experiments were performed using the more specific extracellular zinc chelator, calcium-saturated ethylenediaminetetraacetic acid (Ca-EDTA). The presence of Ca-EDTA in the superfusing medium also resulted in a potentiation of the ACPD-evoked calcium release that was not significantly different from the TPEN-induced potentiation (Fig. 2C), $p = 0.5264$, Fisher's PLSD *post hoc* analysis). This suggests leakage of zinc from the terminals in the absence of activity (e.g., via miniature excitatory postsynaptic currents; see Zirpel et al. 2000b). This leaked zinc appears to exert a low level of inhibition on the mGluRs that was relieved by chelating the zinc with TPEN or Ca-EDTA.

FIG. 2. Zinc inhibits the release of calcium from intracellular stores. **A.** Traces from 8 NM neurons showing $[Ca^{2+}]_i$ as a function of time. ACPD (1 mM) was applied in Ca^{2+} -free medium as indicated by the horizontal solid lines. Zinc was present in the superfusing solution as indicated by the horizontal dashed line. **B.** The effect of zinc inhibition is dose dependent. Zinc concentrations of 1, 10, 100 μM and 1 and 10 mM were applied to slices and a calcium response elicited by a 45 s application of 1 mM ACPD as shown in A. Four slices were tested for each concentration of zinc and error bars represent 1 S.D. **C.** Zinc inhibits mGluR5. Magnitude of average calcium response elicited by 1 mM ACPD (control; $n = 4$) in the presence of zinc ($n = 8$), MPEP (a mGluR5-specific antagonist; $n = 5$), the combination of MPEP (10 μM) and zinc (100 μM) ($n = 4$), CPCCOEt (a mGluR1-specific antagonist; $n = 9$), MCPG (a nonspecific mGluR antagonist; $n = 4$), TPEN (a zinc chelator; $n = 5$), and Ca-EDTA (a more specific zinc chelator; $n = 4$). Groups were compared by one-factor ANOVA that yielded a highly significant effect of treatment: $F_{(7,35)} = 9.011$, $p < 0.0001$. Pairwise comparisons were made using Fisher's PLSD *post hoc* analysis. Asterisks denote a significant difference ($p < 0.05$) from the control group.

This is consistent with the evidence that the end-bulbs "leak" glutamate in the absence of synaptic activity (Zirpel et al. 2000b).

Zinc can enter a cell by influx through NMDA receptors, Ca^{2+} -permeable AMPA receptors, and voltage-gated calcium channels (Sensi et al. 1997; Colvin et al. 2000), all of which are present on NM neurons. Influx of zinc into the NM neurons could confound the interpretation of these experiments by exerting an intracellular effect or by interacting directly with fura-2. The increased calcium response in the presence of zinc chelators in the present experiments would suggest that, under these experimental conditions, zinc is not entering the postsynaptic neuron. Since Ca-EDTA chelates extracellular zinc, the potentiation effect cannot be the result of an intracellular site of action following zinc influx. These results are consistent with zinc having an inhibitory effect on postsynaptic mGluRs.

Zinc disrupts activity-dependent calcium homeostasis

To determine if zinc inhibition of mGluR5 affected the activity-dependent calcium homeostasis in NM neurons, zinc was applied to a slice receiving eighth nerve stimulation while $[Ca^{2+}]_i$ was monitored. (Figure 3A) shows that during eighth nerve stimulation, NM neuron $[Ca^{2+}]_i$ remains stable near 100 nM (●; see also Zirpel and Rubel 1996). Application of 100 μM zinc under the same conditions caused an increase in $[Ca^{2+}]_i$ that increased rapidly between 20 and 30 min after application and then plateaued near 280 nM (Fig. 3A, Δ). If zinc is indeed being released during synaptic activity, the effect on activity-dependent calcium homeostasis seen in Figure 3A would be in addition to the effect mediated by endogenous zinc. Application of the zinc chelators TPEN (200 μM , $n = 3$ slices) or Ca-EDTA (1.5 mM, $n = 4$ slices) had no effect on the activity-dependent calcium homeostasis (data not shown). There are several reasonable explanations for this. First, the calcium homeostasis of NM neurons may be operating at peak efficiency even in the presence of the endogenously released zinc, and, thus, no further reduction in NM $[Ca^{2+}]_i$ would occur by removing zinc and increasing the efficiency of glutamate activation of mGluR5. The modulatory effect of zinc on mGluR5 may become significant at higher levels of stimulation. Similarly, the mGluR5-mediated component of calcium homeostasis may be less important at maintaining baseline calcium levels and more important for clearing calcium loads under different stimulation conditions. Thus, increasing mGluR5 activity by removing zinc inhibition with TPEN or Ca-EDTA would have no effect on the baseline $[Ca^{2+}]_i$ under these experimental conditions. Second, increased mGluR5 activity

FIG. 3. Zinc disrupts activity-dependent calcium homeostasis. **A.** Mean $[Ca^{2+}]_i$ of NM neurons receiving 5-Hz stimulation of the eighth nerve in normal ACSF (●, $n = 3$ slices) and in ACSF containing 10 μ M MPEP (□, $n = 3$ slices), 100 μ M zinc (△, $n = 4$ slices), 1 mM MCPG (◆, $n = 3$ slices), or the combination of 10 μ M MPEP and 100 μ M zinc (■, $n = 4$ slices). The abscissa has been normalized so that drugs were all added at $t = 0$. Error bars represent the standard error of the mean and are less than 50 nM where not visible. Inset shows field potential recorded during nerve stimulation. Horizontal scale bar = 1 ms; vertical scale bar = 0.5 mV. **B.** Mean $[Ca^{2+}]_i$ of NM neurons deprived of afferent activity in normal ACSF (○, $n = 3$ slices) and in ACSF containing 1.5 mM Ca-EDTA (▲, $n = 4$ slices), 100 μ M zinc (◇, $n = 5$ slices), and 100 μ M zinc but no added calcium (■, $n = 3$ slices). Error bars represented the standard error of the mean and are less than 20 nM where not visible.

resulting from removal of zinc inhibition may be undetectable with our present imaging system. Non-confocal imaging of *in vitro* slices yields relatively noisy signals and $[Ca^{2+}]_i$ changes of <20 nM are not reliably detected in this type of experiment, which has relatively low temporal resolution.

To determine if zinc was acting through mGluR5, as in the experiments presented in Figure 2, the mGluR5-specific antagonist MPEP was applied to slices receiv-

ing eighth nerve stimulation. Figure 3A (□) shows that MPEP application resulted in an increase in NM $[Ca^{2+}]_i$ that paralleled the increase seen with zinc application. However, the $[Ca^{2+}]_i$ increase induced by MPEP application was much more gradual than that of zinc but plateaued at the same level. The combination of zinc and MPEP (Fig. 3A, ■) was indistinguishable from that induced by zinc application alone. These results are consistent with zinc exerting its effect through mGluR5.

The nonspecific mGluR antagonist MCPG caused a rapid and large increase in NM neuron $[Ca^{2+}]_i$ when applied to slices receiving eighth nerve stimulation (Fig. 3A, ◆; see also Zirpel and Rubel 1996). The increase occurred immediately after application of MCPG and plateaued at approximately 750 nM. This indicates that there are other mGluR-mediated components contributing to the activity-dependent calcium homeostasis of NM neurons. NM neurons express high levels of mGluR1 (Zirpel et al. 2000a), but this group I mGluR does not appear to couple to calcium release from stores (Fig. 2C). Heterologously expressed mGluR1 has been shown to couple to an increase in adenylate cyclase activity (Sugiyama et al. 1989), and a component of NM neuron mGluR calcium homeostasis appears to depend on mGluR-mediated increases in cAMP levels (Lachica et al. 1995). Thus, NM neurons may express a mGluR1 that is linked to an increase in adenylate cyclase rather than release of calcium from stores. This mGluR1-mediated mechanism is likely to be a major component of the activity-dependent, mGluR-mediated calcium homeostasis. The application of MCPG would remove the mGluR-mediated regulation of calcium but leave intact and active the ionotropic AMPA receptors to depolarize the NM neurons. This depolarization would activate voltage-operated calcium channels and allow direct calcium influx through the Ca^{2+} -permeable AMPA receptors (Otis et al. 1995; Ravindranathan et al. 2000), resulting in the rapid and large increase in $[Ca^{2+}]_i$ seen in Figure 3A. It is possible, since fura-2 also binds zinc, that this increase reflects an influx and increase of cytosolic zinc, alone or in parallel with calcium. However, treatments which prevent calcium influx into NM neurons also attenuate the deafferentation-induced neuronal death (Zirpel et al. 1998, 2000b), whereas the presence of TPEN or Ca-EDTA in the bathing medium does not. Neither TPEN (Lu and Rubel, personal communication) nor Ca-EDTA (Fig. 3B, ▲) prevent the increase in $[Ca^{2+}]_i$ following activity deprivation. The presence of zinc in normal, Ca^{2+} -containing medium shows no effect on the increase in $[Ca^{2+}]_i$ in the absence of stimulation (Fig. 3B, ◇). Conversely, the absence of Ca^{2+} in the superfusing medium containing 100 μ M zinc prevents any increase in the fluorescence ratio of fura-2 (Fig. 3B, ■). While a contribution of zinc influx

to the increase of fura-2 fluorescence ratio cannot be ruled out entirely, these results strongly suggest that the fluorescence changes measured in the present experiments are predominantly changes in intracellular Ca^{2+} . However, Wilson and Durham (1995) reported cytosolic zinc in NM neurons 1 h after deafferentation. This level of zinc may be undetectable with the present imaging experiments or may be so small that, given the ≈ 20 nM resolution of our experiments, it is overshadowed by the large Ca^{2+} fluxes being measured by fura-2. Clearly, there is an influx of both Ca^{2+} and Zn^{2+} into NM neurons following deafferentation, but the experiments presented in Figure 2C suggest that Zn^{2+} is exerting an effect on postsynaptic mGluRs.

The inset of Figure 3A is an example of the field potentials used to monitor eighth nerve stimulation. The initial upward deflection is the shock artifact, the first downward deflection represents the depolarization of the calyces (the afferent volley), and the second downward deflection represents the depolarization of the NM neurons. None of the treatments used in this study consistently affected the latency or magnitude of this response. However, zinc modulation of AMPA receptors (Nakazawa et al. 1995; Easaw et al. 1999) would likely be undetectable with extracellular field potential recordings.

We conclude that zinc is released from eighth nerve terminals during synaptic activity. Zinc then inhibits mGluR5 receptors on NM neurons and contributes to the complex, activity-dependent, mGluR-mediated calcium homeostasis that is essential for NM neuron maintenance and survival. In addition to modulating ionotropic glutamate receptors, zinc has been shown to modulate GABA receptors (Berger et al. 1998; Fisher and MacDonald 1998) and glutamate transporters (Spiridon et al. 1998). Since NM neurons receive a GABAergic projection from the superior olivary complex (Yang et al. 1999) and undoubtedly rely on glutamate transport for controlling glutamate levels in the extracellular space, it would seem that synaptically released zinc from the eighth nerve is poised to play an important role in the overall functioning of NM, including auditory processing, that extends beyond the modulation of mGluR5 and calcium homeostasis.

The calcium-permeable AMPA receptors expressed by NM neurons are involved in deafferentation-induced neuronal death following activity deprivation (Zirpel et al. 2000b) and the NM neurons that do not survive deafferentation show increased levels of cytosolic zinc (Wilson and Durham 1995). One route of entry for zinc into neurons is through calcium-permeable AMPA receptors (Sensi et al. 1997), and activation of AMPA receptors potentiates zinc neurotoxicity (Weiss et al. 1993). Thus, zinc would appear likely to play an important role in NM neuron death

following activity deprivation. In the absence of synaptic activity, the calyces probably "leak" glutamate (Zirpel et al. 2000b) and zinc (TPEN and Ca-EDTA effect in Fig. 2C, this report). The calcium-permeable AMPA receptors would then be activated by the glutamate, which does not reach concentrations high enough to activate the mGluRs, allowing influx of calcium and zinc that would accumulate in the absence of the mGluR-mediated clearance mechanisms and lead to the death of 30% of the NM population. The role of zinc in NM neuron death following activity deprivation needs to be more fully investigated.

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REFERENCES

- BERGER T, SCHWARZ C, KRAUSHAAR U, MONYER H. Dentate gyrus basket cell GABA_A receptors are blocked by Zn^{2+} via changes of their desensitization kinetics: an *in situ* patch-clamp and single-cell PCR study. *J. Neurosci.* 18:2437–2448, 1998.
- BORN DE, RUBEL EW. Afferent influences on brain stem auditory nuclei of the chicken: Neuron number and size following cochlea removal. *J. Comp. Neurol.* 231:435–445, 1985.
- BUDDE T, MINTA A, WHITE JA, KAY AR. Imaging free zinc in synaptic terminals in live hippocampal slices. *Neuroscience.* 79:347–358, 1997.
- COLVIN RA, DAVIS N, NIPPER RW, CARTER PA. Zinc transport in the brain: routes of zinc influx and efflux in neurons. *J. Nutr.* 130:1484S–1487S, 2000.
- CANZONIERO LM, TURETSKY DM, CHOI DW. Measurement of intracellular free zinc concentrations accompanying zinc-induced neuronal death. *J. Neurosci.* 19:RC31, 1999.
- CARR CE, BOUDREAU RE. Central projections of auditory nerve fibers in the barn owl. *J. Comp. Neurol.* 314:306–318, 1991.
- EASAW JC, JASSAR BS, HARRIS KH, JHAMANDAS JH. Zinc modulation of ionic currents in the horizontal limb of the diagonal band of Broca. *Neuroscience.* 94:785–795, 1999.
- FISHER JL, MACDONALD RL. The role of an alpha subtype M2-M3 His in regulating inhibition of GABA_A receptor current by zinc and other divalent cations. *J. Neurosci.* 18:2944–2953, 1998.
- FREDERICKSON CJ, HOWELL GA, HAIGH MD, DANCHEV G. Zinc-containing fiber systems in the cochlear nuclei of the rat and mouse. *Hear. Res.* 36:203–212, 1988.
- GASPARINI F, LINGENHOHL K, STOEHR N, FLOR PJ, HEINRICH M, VRANESIC I, BIOLLAZ M, ALLGEIER H, HECKENDORN R, URWYLER S, VARNEY MA, JOHNSON EC, HESS SD, RAO SP, SACAAN AI, SANTORI EM, VELICELEBI G, KUHN R. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systematically active mGlu5 receptor antagonist. *Neuropharmacology.* 38:1493–1503, 1999.
- GRYNKIEWICZ G, POENIE M, TSIEN RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450, 1985.

- HERMANS E, NAHORSKI SR, CHALLISS RA. Reversible and non-competitive antagonist profile of CPCCOEt at the human type 1alpha metabotropic glutamate receptor. *Neuropharmacology*. 37:1645–1647, 1998.
- HOLLMANN M, BOULTER J, MARON C, BEASLEY L, SULLIVAN J, PECHT G, HEINEMANN S. Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron*. 10:943–954, 1993.
- HYSON RL, RUBEL EW. Transneuronal regulation of protein synthesis in the brain stem auditory system of the chick requires synaptic activation. *J. Neurosci*. 9:2835–2845, 1989.
- JACKSON H, PARKS TN. Functional synapse elimination in the developing avian cochlear nucleus with simultaneous reduction in cochlear nerve axon branching. *J. Neurosci*. 2:1736–1743, 1982.
- JHAVERI S, MOREST DK. Sequential alterations of neuronal architecture in the nucleus magnocellularis of the developing chicken: A golgi study. *Neuroscience*. 7:837–853, 1982.
- JOHNSON ME, GORES GJ, UHL CB, SILL JC. Cytosolic free calcium and cell death during metabolic inhibition in a neuronal cell line. *J. Neurosci*. 14:4040–4049, 1994.
- LACHICA EA, RÜBSAMEN R, ZIRPEL L, RUBEL EW. Glutamatergic inhibition of voltage-operated calcium channels in the avian cochlear nucleus. *J. Neurosci*. 15:1724–1734, 1995.
- LEE JY, COLE TB, PALMITER RD, KOH JY. Accumulation of zinc in degenerating hippocampal neurons of ZnT3-null mice after seizures: evidence against synaptic vesicle origin. *J. Neurosci*. 20:RC79, 2000.
- LITSCHIG S, GASPARINI F, RUEEGG D, STOEHR N, FLOR PJ, VRANESIC I, PREZEAU L, PIN JP, THOMSEN C, KUHN R. CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Mol. Pharmacol*. 55:453–461, 1999.
- NAKAZAWA K, INOUE K, WATANO T, KOIZUMI S, INOUE K. Zinc potentiation of neurotransmission and inhibition of background cationic conductance in rat cultured hippocampal neurones. *J. Physiol. (Lond.)* 484:447–462, 1995.
- OTIS TS, RAMAN IM, TRUSSELL LO. AMPA receptors with high Ca^{2+} permeability mediate synaptic transmission in the avian auditory pathway. *J. Physiol. (Lond.)* 482:309–315, 1995.
- PARKS TN, RUBEL EW. Organization and development of brain stem auditory nuclei of the chicken: Primary afferent projections. *J. Comp. Neurol*. 180:439–448, 1978.
- PARKS TN, TAYLOR DA, JACKSON H. Adaptations of synaptic form in an aberrant projection to the avian cochlear nucleus. *J. Neurosci*. 10:975–984, 1990.
- RAVINDRANATHAN A, DONEVAN SD, SUGDEN SG, GREIG A, RAO MS, PARKS TN. Contrasting molecular compositions and channel properties of AMPA receptors on chick auditory and brainstem motor neurons. *J. Physiol*. 523:667–684, 2000.
- RUBEL EW, HYSON RL, DURHAM D. Afferent regulation of neurons in the brain stem auditory system. *J. Neurobiol*. 21:169–196, 1990.
- RUBIO ME, RUIZ JM. Chemical anatomy of excitatory endings in the dorsal cochlear nucleus of the rat: differential synaptic distribution of aspartate aminotransferase, glutamate, and vesicular zinc. *J. Comp. Neurol*. 399:341–358, 1998.
- SENSI SL, CANZONIERO LM, YU SP, YING HS, KOH JY, KERCHNER GA, CHOI DW. Measurement of intracellular free zinc in living cortical neurons: routes of entry. *J. Neurosci*. 17:9554–9564, 1997.
- SHELLINE CT, BEHRENS MM, CHOI DW. Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD^{+} and inhibition of glycolysis. *J. Neurosci*. 20:3139–3146, 2000.
- SPIRIDON M, KAMM D, BILLUPS B, MOBBS P, ATTWELL D. Modulation by zinc of the glutamate transporters in glial cells and cones isolated from the tiger salamander retina. *J. Physiol. (Lond.)* 506:363–376, 1998.
- STORIOHANN L, LEVINTHAL C, STORMANN TM, PARKS TN. Molecular cloning and functional expression of chick metabotropic glutamate receptor 5 splice variants. *Soc. Neurosci. Abstr*. 25:977, 1999.
- SUGIYAMA H, ITO I, WATANABE M. Glutamate receptor subtypes may be classified into two major categories: a study on *Xenopus* oocytes injected with rat brain mRNA. *Neuron*. 3:129–132, 1989.
- VOGT K, MELLOR J, TONG G, NICOLL R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. *Neuron*. 26:187–196, 2000.
- WEISS JH, HARTLEY DM, KOH JY, CHOI DW. AMPA receptor activation potentiates zinc neurotoxicity. *Neuron*. 10:43–49, 1993.
- WILSON CJ, DURHAM D. Changes in the distribution of zinc in chick cochlear nucleus during deafferentation-induced neuronal death. *Assoc. Res. Otolaryngol. Abstr*. 18:36, 1995.
- YANG L, MONSIVAIS P, RUBEL EW. The superior olivary nucleus and its influence on nucleus laminaris: a source of inhibitory feedback for coincidence detection in the avian auditory brainstem. *J. Neurosci*. 19:2313–2325, 1999.
- ZIRPEL L, JANOWIAK MA, TAYLOR DA, PARKS TN. Developmental changes in metabotropic glutamate receptor-mediated calcium homeostasis. *J. Comp. Neurol*. 421:95–106, 2000a.
- ZIRPEL L, JANOWIAK MA, VELTRI CA, PARKS TN. AMPA receptor-mediated, calcium-dependent CREB phosphorylation in a subpopulation of auditory neurons surviving activity deprivation. *J. Neurosci*. 20(16):6267–6275, 2000b.
- ZIRPEL L, LACHICA EA, RUBEL EW. Activation of a metabotropic glutamate receptor increases intracellular calcium concentrations in neurons of the avian cochlear nucleus. *J. Neurosci*. 15:214–222, 1995.
- ZIRPEL L, LIPPE WR, RUBEL EW. Activity-dependent regulation of intracellular calcium in avian cochlear nucleus: Roles of protein kinases A and C and correlation with cell death. *J. Neurophys*. 79:2288–2302, 1998.
- ZIRPEL L, RUBEL EW. Eighth nerve activity regulates the intracellular calcium concentration of avian cochlear nucleus neurons via a metabotropic glutamate receptor. *J. Neurophys*. 76:4127–4139, 1996.