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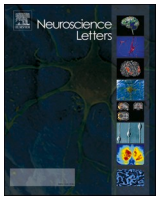

Intracellular zinc signaling influences NMDA receptor function by enhancing the interaction of ZnT1 with GluN2A

Rebecca Krall [a,c], Jenna R. Gale [a,b], Madeline M. Ross [a,b], Thanos Tzounopoulos [c,d], Elias Aizenman [a,b,c,*]

[a] Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[b] Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Pittsburgh, PA, USA
[c] Pittsburgh Hearing Research Center, University of Pittsburgh, PA, USA
[d] Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

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ABSTRACT

Zinc, loaded into glutamate-containing presynaptic vesicles and released into the synapse in an activity-dependent manner, modulates neurotransmission through its actions on postsynaptic targets, prominently via high-affinity inhibition of GluN2A-containing NMDA receptors. Recently, we identified a postsynaptic transport mechanism that regulates endogenous zinc inhibition of NMDARs. In this new model of zinc regulation, the postsynaptic transporter ZnT1 mediates zinc inhibition of NMDARs by binding to GluN2A. Through this interaction, ZnT1, a transporter that moves zinc from the cytoplasm to the extracellular domain, generates a zinc microdomain that modulates NMDAR-mediated neurotransmission. As ZnT1 expression is transcriptionally driven by the metal-responsive transcription factor 1 (MTF-1), we found that intracellular zinc strongly drives MTF-1 in cortical neurons *in vitro* and increases the number of GluN2A-ZnT1 interactions, thereby enhancing tonic zinc inhibition of NMDAR-mediated currents. Importantly, this effect is absent when the interaction between GluN2A and ZnT1 is disrupted by a cell-permeable peptide. These results suggest that zinc-regulated gene expression can dynamically regulate NMDAR-mediated synaptic processes.

1. Introduction

Zinc is a neuromodulator with diverse roles in the brain [1,2]. The majority of loosely bound or labile zinc is found in presynaptic vesicles in a subset of glutamatergic neurons throughout the cerebral cortex, hippocampus, amygdala, and auditory brainstem [3,4]. These zinc-containing neurons package zinc into vesicles using the transporter ZnT3 and synaptically release it in an activity-dependent manner [5,6]. The ion acts on multiple postsynaptic receptors to modulate both excitatory and inhibitory transmission [1]. Notably, zinc inhibits NMDA receptors through an allosteric binding site on the *N*-terminal domain of the GluN2 subunit [7,8]. GluN2A-containing NMDARs are highly sensitive to zinc, requiring nanomolar concentrations of the metal for inhibition [9].

Previously, it was assumed that ZnT3-dependent presynaptic release and diffusion across the cleft was sufficient to account for the modulatory actions of zinc on NMDARs [5,10]. However, recent work revealed that the postsynaptic transporter ZnT1 is necessary for NMDARs inhibition [11]. ZnT1, which transports zinc from the cytoplasm to the extracellular space, binds directly to the GluN2A subunit of NMDARs [12]. Disrupting either the interaction between GluN2A and ZnT1 or chelating postsynaptic intracellular zinc blocks endogenous zinc inhibition of NMDARs [11]. Therefore, ZnT1 and postsynaptic zinc critically regulate the localization and concentration of zinc driving endogenous NMDAR inhibition.

ZnT1 expression levels dynamically respond to intracellular zinc signaling through transcriptional regulation. When intracellular zinc increases, it binds to the metal regulatory element (MRE) transcription factor 1 (MTF-1) [13]. MTF-1 rapidly translocates to the nucleus, where it binds to the MRE on target genes to regulate transcription [14,15]. The ZnT1 gene, SLC30A1, contains two MRE tandem sequences in its promotor region, such that ZnT1 is rapidly upregulated following MTF-1 activation [16]. Thus, MTF-1 driven upregulation of ZnT1 may be a previously unrecognized mechanism regulating NMDAR-mediated neurotransmission. Here, we test the hypothesis that alterations in intracellular zinc can enhance inhibition of NMDARs via upregulation of

* Corresponding author at: Department of Neurobiology, University of Pittsburgh School of Medicine, 3501 Fifth Avenue, BST3-7035, Pittsburgh, PA 15260, USA.
E-mail address: redox@pitt.edu (E. Aizenman).

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ZnT1 and subsequent changes in GluN2A-ZnT1 interactions.

2. Material and methods

2.1. Neuronal cultures

All animal procedures were approved by the IACUC of the University of Pittsburgh School of Medicine. Cortical cultures were prepared from embryonic day 16 rats (Charles River Laboratory). Cortices were dissociated with trypsin and plated at 670,000 cells per well on glass coverslips in six-well plates. Cultures were utilized at 3–4 weeks *in vitro*.

2.2. MRE-Luciferase reporter & LDH assay

Cortical cultures were transfected with MRE-firefly luciferase reporter (pLuc-MCS/4MREa, 1 μg), *Renilla* luciferase reporter (pRLTK, 0.4 μg), plus blank vector (pBK-CMV, 0.1 μg) using Lipofectamine 2000. Twenty-four hours later, cells were treated with 250 nM pyrithione (a zinc ionophore) or 250 nM pyrithione with 10 μM $ZnCl_2$ in DMEM containing 2 % calf serum and 25 mM HEPES. Twenty-four hours later, luciferase expression was measured using the Dual-Glo Assay (Promega). Results were expressed as the ratio of firefly to *Renilla* luciferase activity [17]. To confirm that ZnPyr treatment did not significantly affect cell viability, a lactate dehydrogenase (LDH) release assay (Sigma) was run following treatment with 250 nM pyrithione alone, or in combination with $ZnCl_2$, or 1 mM kainate as a positive control. The concentration of "free" or labile zinc in these solutions is not known.

2.3. Western blot and qPCR

Cultures were exposed to 10 μM $ZnCl_2$ and 250 nM pyrithione overnight, then lysed in the presence of a protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride. For synaptosome-enriched lysates, we utilized the syn-PER reagent (Thermo Fisher). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated overnight with rabbit anti-GluN2A antibody (Cusabio, #CSB-PA14129A0Rb). Membranes with unfractionated protein were also probed with mouse anti-Beta-tubulin III (Tuj1) antibody (Covance, #MMS-435p); membranes with synaptosome-enriched were probed with mouse anti-Beta-actin (Sigma, A5441). Li-Cor IRDye-conjugated secondary antibodies labeled with IRDyes 700CW (685 nm) and 800CW (780 nm) were used and signals acquired using the Odyssey Infrared Imaging System (LI-COR), quantified using Fiji software.

Total RNA was isolated from cortical cultures exposed to 10 uM ZnCl2 and 250 nM pyrithione overnight using the TRIzol Plus RNA Purification Kit (Invitrogen). RNA samples were eluted in RNAse free H2O and were reversed transcribed into cDNA using iScript (Bio-Rad). Quantitative RT-PCR (qPCR) was performed with using the following primers: ZnT1 sense (FW): 5′-CACGCTAGTGGCTAACACCA-3′; ZnT1 antisense (RV): 5′-AGGAAAACACGGGTTCACAC-3′; β-actin sense (FW): 5′-ACTCTTCCAGCCTTCCTTC-3′; β-actin antisense (RV): 5′-ATCTCCTTCTGCATCCTGTC-3′. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX96 Touch Real-Time PCR detection system (Bio-Rad). The PCR reactions for each sample were run in triplicate and ZnT1 levels were normalized to β-actin.

2.4. Proximity ligation assay

Proximity ligation assays (PLA) were performed using Duolink PLA kit (Sigma). Cultures were treated overnight with either N2AZ (YGRKKRRQRRQRRNDSYLRSSL) or scN2AZ (YGRKKRRQRRQRRSNLSDSYLR) (3 μM) [11], and pyrithione (250 nM), or zinc pyrithione (10 μM $ZnCl_2$, 250 nM pyrithione). Coverslips were fixed in ice cold methanol and permeabilized with 0.1 % Triton-X in PBS. Coverslips were then incubated with rabbit anti-ZnT1 (Alomone Labs, #AZT-011), mouse anti-GluN2A (Sigma, #SAB5200888), and chicken anti-MAP2 (Abcam, #ab5392). A donkey anti-chicken fluorescent secondary antibody targeting MAP2 antibodies was used to visualize neuron morphology. Four random fields of view were imaged from each coverslip (60x) on a Nikon A1R laser scanning confocal. PLA puncta were counted automatically with Fiji ImageJ software. We used maximum intensity projection of 15 sequential images in the z plane. All images used Yen threshold setting.

2.5. Electrophysiology

Whole-cell recordings were obtained with glass micropipettes (3–6 MΩ) containing (in mM): 140 CsF, 10 CsEGTA, 1 CaCl2, 10 HEPES, pH = 7.2. Extracellular recording solution contained (in mM): 150 NaCl, 2.8 KCl, 1.0 CaCl2, 10 HEPES, 60 μM glycine, pH = ~7.2. Using *Ephus* [18] and a Multiclamp 700B amplifier (Molecular Devices), NMDAR EPSCs were recorded in voltage clamp (-70 mV) in the presence of TTX (Alomone Labs, 300 nM), DNQX (Hello Bio, 20 μM), and 4-Methoxy-7-nitroindolinyl (MNI)-caged glutamate (Tocris Biosciences, 40 μM). To evoke NMDAR EPSCs, we photolytically uncaged MNI-caged glutamate using 1 ms pulses of UV-laser light (355 nm, DPSS Lasers)m then ZX1 (Strem Chemicals, 100 μM) was applied to remove tonic or background zinc. The ZX1-mediated potentiation of the glutamate response for each cell was calculated as the percent increase in average response (10 sweeps, before and after ZX1) following application of the metal chelator.

2.6. Statistical analyses

Unpaired t-tests, one-sample t-tests, and ANOVAs were used to compare between treatments. Statistical analysis was completed in Prism 9 (GraphPad). Error bars in figures indicate mean ± SEM.

3. Results

3.1. Zinc pyrithione drives MRE-regulated gene expression in cortical neurons

We first identified a treatment that activates MTF-1 without itself causing neuronal injury [19], using the zinc ionophore pyrithione (Pyr) to increase intracellular zinc. No significant difference in cell viability was observed between untreated control cells and those treated with Pyr (250 nM) or 10 μM ZnPyr (10 μM $ZnCl_2$, 250 nM Pyr; Fig. 1A). Higher concentrations of ZnPyr (100 μM $ZnCl_2$, 250 nM Pyr) led to cell death, similar to the toxicity induced by 1 mM kainate (Fig. 1A).

To determine if ZnPyr treatment was sufficient to activate MTF-1 driven gene expression, we used an MRE-luciferase assay [17]. In this assay, neurons were transfected with a plasmid encoding a firefly luciferase with four tandem MRE repeats present in the promoter region. Firefly luciferase activity was then assayed to serve as a measure of MRE-driven gene upregulation. A non-inducible *Renilla* luciferase-expressing plasmid was used as control. One day after transfection, neurons were treated with Pyr (250 nM) alone or with zinc (10 μM, ZnPyr) overnight. Zinc-induced gene expression was quantified by measuring both firefly and *Renilla* luciferase activity and taking the ratio of Firefly/*Renilla* activity. We observed that ZnPyr led to a significant increase in MRE-driven transcription compared to Pyr and untreated controls (Fig. 1B).

As *ZnT1* is an MRE-regulated gene that is upregulated in response to increases in intracellular zinc [20], we assessed the relative mRNA expression of *ZnT1* following treatment with 10 μM ZnPyr (Fig. 1C), observing a significant increase in *ZnT1* mRNA expression following overnight treatment. We also treated neurons for 1 h with 10 μM ZnPyr and collected lysates immediately following treatment (0 hr), 3 hr post-treatment, and 24 hr post-treatment. In contrast to the results of the overnight incubation with ZnPyr, we did not observe a significant

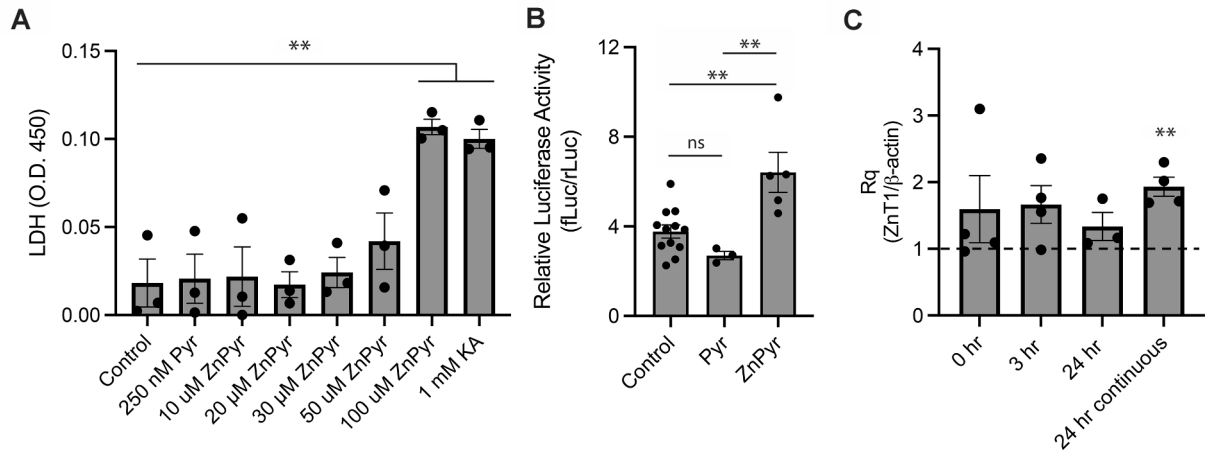

Fig. 1. ZnPyr treatment induces MRE-driven gene expression (**A**) LDH levels in phenol-free medium of cortical neurons treated with increasing concentration of ZnPyr (Pyr: 250 nM). Toxicity was observed following overnight treatment with 100 μM ZnPyr (One-way Anova, p = 0.0004) or 1 mM kainate (p = 0.0008) (n = 3) (**B**) MRE-driven firefly luciferase relative to *Renilla* luciferase in untreated, Pyr (250 nM), or ZnPyr (10 μM $ZnCl_2$, 250 nM Pyr) treated neurons (One-way ANOVA, p = 0.0012; Control, n = 12; Pyr, n = 3; ZnPyr, n = 5). ZnPyr treatment led to a significant increase in firefly/*Renilla* activity compared to control (Sidak multiple comparisons, p = 0.004) and Pyr (p = 0.003). (**C**) ZnPyr treatment led to a significant increase in *ZnT1* mRNA expression after 24 h of continuous exposure to ZnPyr (One sample, 2-tailed *t*-test vs 1, p = 0.007) but at no time point following a 1-hour exposure (0, 3 and 24 hr). Changes in mRNA expression were normalized to *β-actin* mRNA expression (n = 3–4).

increase in *ZnT1* mRNA expression at any of these time points (Fig. 1C). We must note, however, we did observe a trend towards enhanced ZnT1 expression, particularly at the 3 hr time point (p = 0.1), reminiscent of a previous study showing that *ZnT1* mRNA expression peaks following 3 h of continuous zinc treatment in non-neuronal cells [20],

As zinc has been shown to also upregulate NMDARs via Src-dependent phosphorylation of GluN2A and GluN2B [21,22], we determined whether increasing intracellular zinc led to an upregulation of GluN2A expression. We found neurons treated with 10 μM ZnPyr overnight exhibited no significant differences in GluN2A protein levels compared to untreated controls in both unfractionated and synapse-enriched samples (Fig. 2).

3.2. *ZnT1-GluN2A interactions are upregulated by ZnPyr*

We next determined whether intracellular zinc and MRE-driven gene expression led to an upregulation of GluN2A-ZnT1 interactions in neurons using PLA to fluorescently label locations where GluN2A and ZnT1 interact [11]. Neurons were treated overnight with either 10 μM ZnPyr or Pyr alone. We hypothesized that ZnPyr would increase the number of PLA puncta by increasing the instances of GluN2A-ZnT1 interactions, likely as a direct result of increased ZnT1 expression. Neurons were also treated overnight with a cell-permeant peptide that specifically disrupts GluN2A-ZnT1 interaction (N2AZ, 3 μM) or its scramble control (scN2AZ, 3 μM) [11].

We found that in scN2AZ treated cells, ZnPyr led to an average 2.4-fold increase in PLA puncta compared to cultures treated with Pyr (Fig. 3A,C), suggesting that ZnPyr upregulates ZnT1-GluN2A interactions. In contrast, N2AZ treated cells exhibited no increase with ZnPyr treatment compared to Pyr control (Fig. 3B,C). This result indicates that zinc treatment upregulates the number of GluN2A-ZnT1 interactions in neurons via an MTF-1/MRE-driven increase in ZnT1 expression.

3.3. *Increasing intracellular zinc leads to enhanced ZnT1-mediated zinc inhibition of NMDARs*

We hypothesized that upregulating the GluN2A-ZnT1 interaction would increase zinc inhibition of NMDARs. To test this, we used whole-

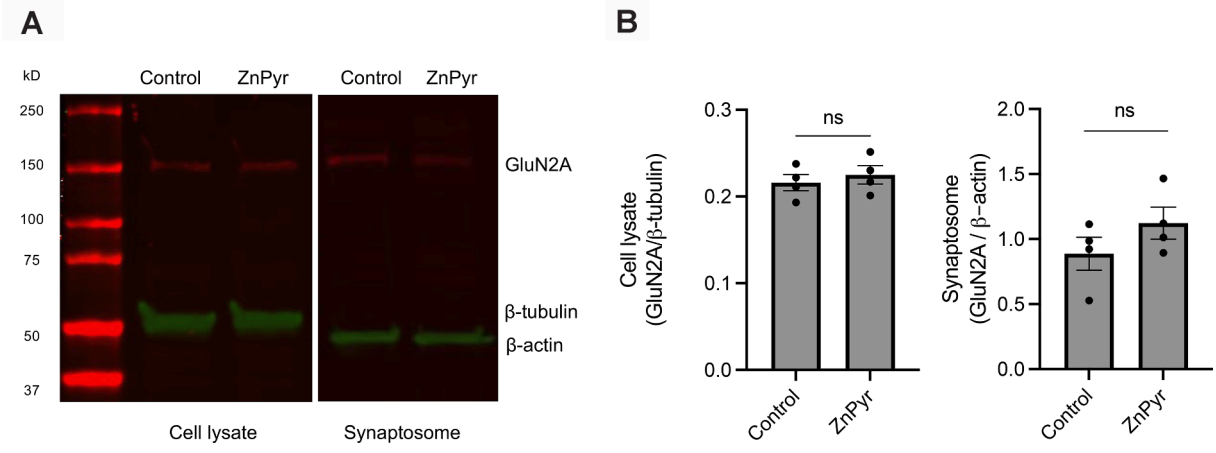

Fig. 2. ZnPyr treatment does not alter GluN2A protein levels. (**A**) Western blot of unfractionated and synapse-enriched samples taken from cortical neurons treated overnight in ZnPyr or control conditions. GluN2A protein was normalized to β-tubulin protein in unfractionated samples or β-actin protein in synapse-enriched samples. (**B**) Quantification of protein levels in control and treated cells (GluN2A/loading control) show no significant differences in GluN2A following ZnPyr treatment in either group (unfractionated: paired *t*-test, p = 0.18, n = 4, synapse-enriched: paired *t*-test, p = 0.42, n = 4).

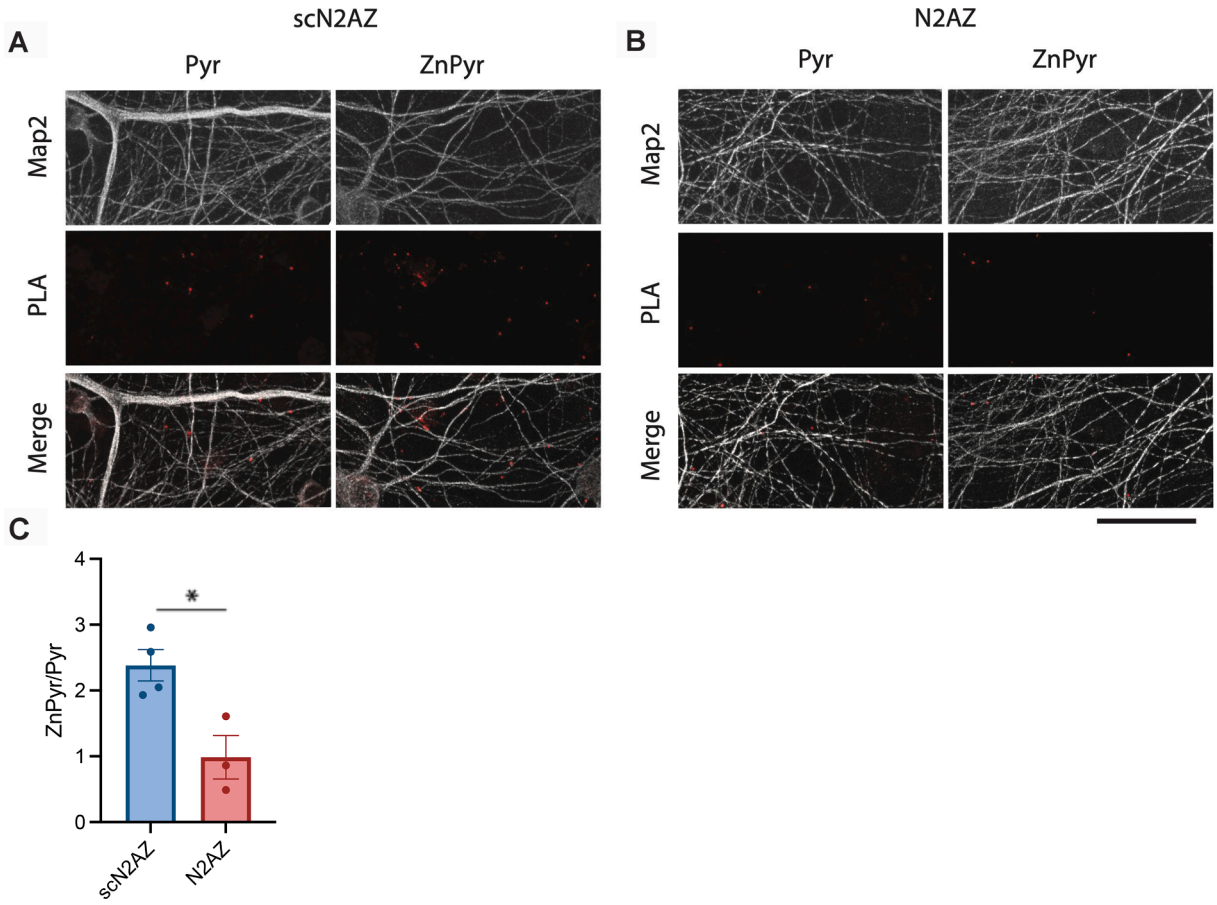

Fig. 3. ZnPyr increases GluN2A-ZnT1 interactions in scN2AZ but not N2AZ treated neurons. Representative images of rat cortical cultures following proximity ligation assay (PLA) of GluN2A and ZnT1 in scN2AZ (**A**) and N2AZ (**B**) treated cultures, comparing Pyr (left column) to ZnPyr (right column) treated cells. Top row shows MAP2 immunofluorescence. Middle row shows the PLA sites of interaction between GluN2A and ZnT1 (red puncta). Bottom row shows the merged images. Scale bar is 25 μm. (**C**) Quantification of the average ratio of ZnPyr to Pyr PLA puncta counts in scN2AZ (blue) and N2AZ (red) treated neurons. scN2AZ treated neurons exhibited a significantly higher ratio compared to N2AZ (unpaired *t*-test, p = 0.017, n = 4,3).

cell recordings in cultured neurons under voltage clamp, and evoked NMDAR currents by photolytically uncaging glutamate onto the cell. Neurons were treated overnight with either Pyr or ZnPyr, in the presence of scN2AZ or N2AZ to determine if increasing intracellular zinc increases ZnT1-dependent zinc inhibition of NMDARs. Tonic (background) extracellular zinc inhibition was determined by measuring the potentiation of NMDAR responses after the addition of the high affinity zinc chelator, ZX1 (100 μM). In scN2AZ treated neurons, we found that,

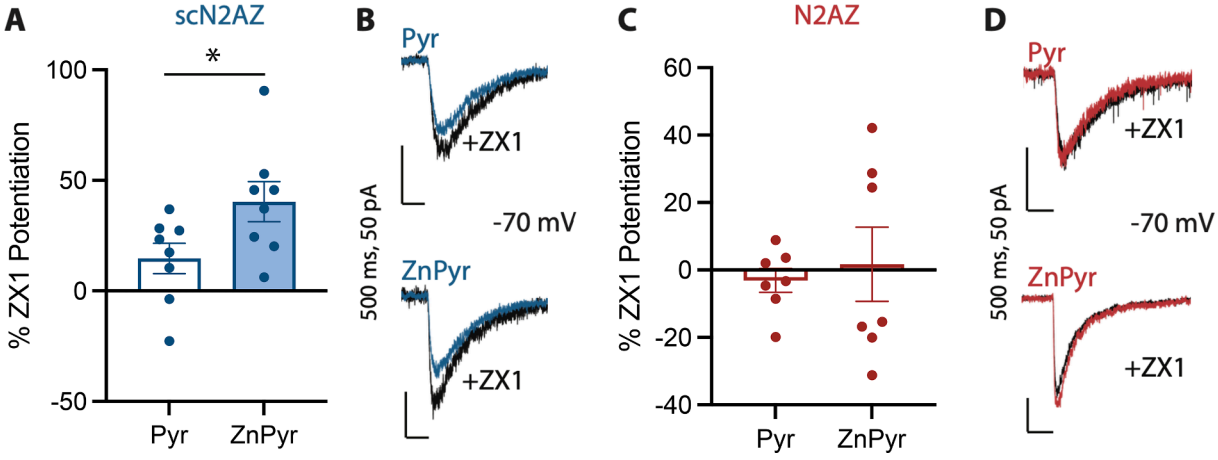

Fig. 4. ZnPyr increases ZnT1-dependent zinc inhibition of NMDARs (**A,C**) ZX1 potentiation of NMDAR currents was significantly increased between ZnPyr and Pyr groups in scN2AZ treated neurons (A, unpaired *t*-test, p = 0.04, n = 8). However, no significant differences were seen in N2AZ treated neurons (C, unpaired *t*-test, p = 0.068, n = 7). Bar graphs represent the average potentiation of responses 10 min after ZX1 application. (**B,D**) Sample traces of NMDAR currents from scN2AZ (B) and N2AZ (D) treated groups, averaged over 10 sweeps, evoked by photolysis of MNI-caged glutamate in cultured cortical neurons held at −70 mV in Mg^{2+} free solution, before (blue, scN2AZ; red, N2AZ, 3 μM) and after application of ZX1 (black; 100 μM).

ZnPyr led to a significant increase in ZX1-dependent NMDAR potentiation, when compared to Pyr control (Fig. 4A). In contrast, NMDAR-mediated currents in N2AZ treated cells were not potentiated following ZX1 treatment and there were no significant differences between Pyr- and ZnPyr-treated groups (Fiugure 4B). Together, these results indicate that ZnPyr treatment upregulates ZnT1-mediated tonic zinc inhibition of NMDARs.

4. Discussion

We describe a role of zinc-regulated gene expression in which intracellular zinc enhances inhibition of NMDARs via upregulation of the zinc transporter ZnT1 and increased interactions between ZnT1 and the GluN2A subunit. Previous studies have shown that increases in intracellular zinc can drive ZnT1 expression to protect against zinc toxicity [23–25], consistent with its documented role for maintenance of zinc homeostasis [26]. This study expands on the role of zinc-dependent regulation of ZnT1 to reveal an additional, previously unrecognized influence on NMDAR-mediated transmission.

Of note, recent evidence raises the possibility that ZnT1 expression may be co-regulated with that of the zinc importer ZIP4 [27]. Indeed, ZIP4 is localized in the post-synaptic density of hippocampal neurons, where its expression is responsive to changes in local zinc concentrations [28]. In non-neuronal cells overexpression of ZIP4 increases intracellular zinc in the absence of zinc supplementation [27]. Moreover, ZIP4 increased intracellular zinc as well as surface expression of ZnT1. Thus, the co-expression of these two zinc transporters would allow for the tight control of both intracellular zinc levels at the post-synapse as well as homeostatic regulation of NMDAR signaling.

This study used exogenous zinc to alter intracellular levels. However, multiple endogenous mechanisms can increase postsynaptic zinc. For instance, stimulation with either glutamate or KCl generates post-synaptic zinc transients [29–32]. These may occur either through uptake of synaptically released zinc or liberation of the ion from intracellular stores. In fact, intracellular zinc transients are induced by neuronal stimulation even in the presence of extracellular zinc chelators, suggesting an intracellular origin [29,31]. These transients can result from acidification driven by NMDAR-mediated calcium influxes, and subsequent proton-dependent release of zinc from intracellular ligands [33,34], or from NMDAR-dependent generation of reactive oxygen species triggering zinc release from metallothioneins [35,36]. Interestingly, zinc has also been shown to be released from thapsigargin-sensitive stores in an IP3 dependent manner [37].

Although the full consequences of changes in postsynaptic intracellular zinc are not yet fully understood, a variety of synaptic functions have been identified that are modulated by zinc. Zinc transients induced by neuronal depolarization triggers differential expression of 931 genes, including those implicated in synaptic structure and transmission [32]. This change in transcription occurs when intracellular zinc levels increase to just 220 pM, suggesting that even modest concentrations of zinc can have broad influence on neurons [31]. Intracellular zinc also influences synaptic plasticity, including modulation of long-term potentiation at both CA1 and CA3 neurons in the hippocampus [38,39]. Intracellular zinc has also been linked to structural organization and remodeling of the synapse. Notably, zinc stabilizes postsynaptic density scaffolding proteins critical for synapse maturation and plasticity [30,40]. Overexpression or knockdown of ZnT1 causes, respectively, increases or decreases in dendritic spine length and width [12]. Although the mechanism of ZnT1-driven alteration in morphology are unknown, NMDAR activation is known to be a critical modulator of synaptic strength and morphology [41]. Therefore, ZnT1 may mediate morphological changes through its regulation of NMDAR activation.

Beyond its role in zinc homeostasis, ZnT1 is associated with a variety of signaling functions. In addition to regulating inhibition of NMDARs, ZnT1 regulates Ras/Raf/MEK/ERK signaling [42] and voltage gated calcium channels [43,44]. ZnT1 interacts directly with the *N*-terminal regulatory domain of Raf-1 to promote its activity. ZnT1 enhancement of Ras-ERK signaling leads to upregulation of *T*-type calcium channel expression on the plasma membrane and subsequent increase in calcium currents [45]. ZnT1 also binds directly to L-type calcium channels to inhibit their activity [43,44]. Therefore, alterations in ZnT1 may influence multiple signaling pathways beyond zinc inhibition of NMDARs. Finally, decreases in intracellular zinc levels reduce ZnT1 expression through endocytosis and degradation of the transporter [20], allowing for bidirectional regulation of ZnT1-mediated signaling. As such, intracellular zinc may be a critical regulator of signaling pathways, including NMDAR function, through its impact on ZnT1 expression.

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CRediT authorship contribution statement

Rebecca Krall: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Jenna R. Gale:** Investigation, Formal analysis, Writing – review & editing. **Madeline M. Ross:** Investigation, Formal analysis. **Thanos Tzounopoulos:** Conceptualization, Writing – review & editing, Supervision. **Elias Aizenman:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Drs. Aizenman and Tzounopoulos have submitted a patent application for the peptide N2AZ: "PEPTIDES THAT ENHANCE NMDA RECEPTOR FUNCTION AND USE THEREOF;" Application No. PCT/US2020/063016, filed December 3, 2020.

Data availability

Data will be made available on request.

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