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Mechanisms of zinc modulation of olfactory bulb AMPA receptors

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Abstract

The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptors mediates most fast excitatory transmission. Glutamate binding to AMPA receptors (AMPA receptors) causes most AMPARs to rapidly and completely desensitize, and their desensitization kinetics influence synaptic timing. Thus, factors that alter AMPAR desensitization influence synaptic transmission. Synaptically released zinc is such a factor. Zinc is a neuromodulator with effects on amino acid receptors and synaptic transmission in many brain regions, including the olfactory bulb (OB). We have previously shown in the OB that zinc potentiates AMPAR-mediated currents at low concentrations (30 μ M, 100 μ M) and inhibits them at a higher concentration (1 mM). It has been hypothesized that zinc potentiates AMPARs by decreasing receptor desensitization. Here, we used cyclothiazide (CTZ), a drug that blocks AMPAR desensitization, to determine whether zinc-mediated potentiation and/or inhibition of AMPA-evoked currents reflect(s) changes in AMPAR desensitization. Zinc largely had biphasic concentration-dependent effects at OB AMPARs. CTZ completely blocked potentiation by zinc but had no significant effect on inhibition. There was a significant negative correlation between the degree of potentiation of AMPAR-mediated currents by 100 μ M zinc and a quantitative measure of the degree of AMPAR desensitization (the steady-state to peak [S:P] ratio of AMPA-evoked currents), but no correlation between the degree of current inhibition by 1 mM zinc and the S:P ratio. Together, these findings suggest that low zinc concentrations potentiate rat OB AMPARs by decreasing receptor desensitization, but that the inhibitory effects of higher zinc concentrations are mediated by a separate mechanism.

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AUTHOR CONTRIBUTIONS

LJB and PQT designed the experiments. LJB performed the experiments and LJB and PQT analyzed the data. LJB wrote the first draft of manuscript. Both authors contributed to further drafts of the manuscript and agreed on the final version.

DECLARATION OF INTERESTS

None.

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Keywords

glutamate receptors; olfaction; cyclothiazide; desensitization; rat

INTRODUCTION

Of the different subtypes of ionotropic glutamate receptors, the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype mediates the fast component of excitatory transmission at central synapses. AMPA receptors (AMPA receptors) are tetramers composed of four subunits (Rosenmund et al., 1998; Mano and Teichberg, 1998), which were originally known as GluR1-4 (or GluRA-D). In 2009, the GluR1-4 subunits were renamed GluA1-4 based on new nomenclature (Collingridge et al., 2009).

Each AMPAR subunit contains a single agonist binding site (Rosenmund et al., 1998; Smith and Howe, 2000). Binding and activation by glutamate causes most AMPARs to rapidly and essentially completely desensitize, and the desensitization kinetics influence the timing of synaptic events (Jonas and Spruston, 1994; Jones and Westbrook, 1996; Otis et al., 1996a; Robert et al., 2001). Fast desensitization limits the duration of the current and the frequency at which AMPARs can produce a full-amplitude response to glutamate (Jones and Westbrook, 1996). At some synapses, desensitization may regulate both the decay of single excitatory postsynaptic currents (EPSCs) and EPSC amplitude during repetitive firing (Trussell et al., 1993; Barbour et al., 1994; Takahashi et al., 1995; Otis et al., 1996b; Otis et al., 1996a). Thus, factors that influence AMPAR desensitization have the potential to significantly influence synaptic transmission.

Alterations to various regions of AMPAR subunits affect the rate and extent of receptor desensitization. For example, alternative splicing of the flip/flop site produces flip and flop splice variants, which have substantially different kinetic profiles (Sommer et al., 1990; Mosbacher et al., 1994). Inclusion of the flip isoform in an AMPAR generally causes it to desensitize more slowly (slower desensitization time constants) and less completely (higher steady-state: peak [S:P] ratio of AMPA current) than inclusion of the flop isoform (Mosbacher et al., 1994; Dingledine et al., 1999). RNA editing at the close by R/G site alters recovery rates from desensitization (Lomeli et al., 1994), and a point mutation in the glutamate binding site has been shown to block AMPAR desensitization (Stern-Bach et al., 1998).

Synaptically released zinc is a substance with the potential to influence AMPAR desensitization. Located in many brain regions, including the olfactory bulb (OB) (Donaldson et al., 1973; Gulya et al., 1991; Ono and Cherian, 1999), zinc is a trace element with catalytic, structural, and regulatory functions that also appears to serve as a neuromodulator (Bitanirwe and Cunningham, 2009; Nakashima and Dyck, 2009; Paoletti et al., 2009; McAllister and Dyck, 2017; Blakemore and Trombley, 2017). Zinc-mediated modulation of amino acid receptors and synaptic transmission has been widely described (Smart et al., 1994; Bitanirwe and Cunningham, 2009; Nakashima and Dyck, 2009; Paoletti et al., 2009; McAllister and Dyck, 2017; Blakemore and Trombley, 2017). We and others have shown that zinc modulates AMPARs expressed in *Xenopus* oocytes (Rassendren

et al., 1990) or on cells from different brain areas (Mayer et al., 1989; Bresink et al., 1996; Blakemore and Trombley, 2004) in a biphasic concentration-dependent manner. In general, AMPAR-mediated currents are potentiated by low concentrations of zinc (30–300 μ M) and inhibited by higher concentrations of zinc (1–3 mM) (Mayer et al., 1989; Bresink et al., 1996; Blakemore and Trombley, 2004).

Smart et al. (1994) initially suggested that zinc could potentiate AMPA-induced responses by affecting receptor desensitization (Smart et al., 1994). Consistent with this notion, Bresink et al. (1996) subsequently suggested that zinc decreases/inhibits AMPAR desensitization and high-affinity [3 H] AMPA binding in a manner similar to cyclothiazide (CTZ) (Bresink et al., 1996), a drug that strongly and reversibly reduces rapid glutamate receptor (AMPA) desensitization in a concentration-dependent manner (Yamada and Tang, 1993; Yamada, 1998). Several groups of investigators have since used CTZ as a means of exploring the mechanisms underlying zinc-mediated potentiation (Lin et al., 2001; Sun et al., 2010) and inhibition (Zhang et al., 2002; Sun et al., 2010) of AMPARs in a variety of brain areas (e.g., hippocampus, retina).

Our previous investigation of N-methyl-D aspartate (NMDA), gamma-aminobutyric acid (GABA), and glycine receptors in the OB suggest that in addition to the type of amino acid receptor, the state of the (glycine) receptor (open versus desensitized) may influence zinc's modulatory effects (Trombley and Shepherd, 1996). We have also previously shown that zinc has diverse effects on AMPARs expressed by OB neurons, including mitral/tufted (M/T) cells and interneurons (Blakemore and Trombley, 2004). In addition, we have used CTZ to characterize the desensitization kinetics of AMPARs on OB neuron subtypes (Blakemore and Trombley, 2003). In the present study, we used CTZ to determine whether zinc-mediated potentiation and/or inhibition of AMPA-evoked currents mediated by AMPARs on OB neurons (M/T cells) reflect(s) changes in AMPAR desensitization.

The present study advances findings of previous studies in several ways. First, previous studies have largely explored the effects of zinc and CTZ on the peak component of AMPAR-mediated currents. Here, we explored these effects on the steady-state component of AMPAR-mediated currents, which is the most important component of the current in regard to AMPAR desensitization because the peak current does not reflect an equilibrium condition between open and desensitized states (Yamada and Tang, 1993). Second, because concentration-response curves for CTZ-mediated enhancement of quisqualate-activated (AMPA-mediated) currents revealed half maximal effective concentrations (EC₅₀) for CTZ of 12–14 μ M (Yamada and Tang, 1993), we used a higher concentration of CTZ (100 μ M) than used in some previous studies (10–20 μ M) to maximize the effects of CTZ on AMPARs.

Our findings suggest that zinc largely has biphasic concentration-dependent effects at AMPARs on M/T cells. Coapplication of CTZ with AMPA and zinc completely blocked the potentiating effects of 100 μ M zinc on AMPAR-mediated currents in most cells but had no significant effect on the degree of inhibition of AMPAR-mediated currents by 1 mM zinc. There was a significant negative correlation between the degree of potentiation of AMPAR-mediated currents by 100 μ M zinc and a quantitative measure of the degree of AMPAR

desensitization (the S:P ratio of AMPA-evoked currents). However, there was no correlation between the degree of inhibition of AMPAR-mediated current by 1 mM zinc and the S:P ratio. Together, these findings suggest that low concentrations of zinc potentiate AMPARs expressed by rat OB neurons by decreasing receptor desensitization, but that the inhibitory effects of higher concentrations of zinc are mediated by a separate mechanism.

EXPERIMENTAL PROCEDURES

Animals

All procedures were approved by the Florida State University Institutional Animal Care and Use Committee. In addition, all experiments were carried out in accordance with the current (8th edition) of the National Institute of Health Guide for the Care and Use of Laboratory Animals. The total number of animals (Sprague–Dawley P0–P4 rat pups) used for culture experiments was 72 (6 rat pups per set of OB cultures).

Tissue Culture

The methods for preparing primary cultures of OB neurons have been previously described (Trombley and Blakemore, 1999; Trombley et al., 1999; Blakemore et al., 2006; Blakemore et al., 2018). Briefly, the OBs were dissected from male and female postnatal days 0–4 (P0 to P4) Sprague–Dawley rat pups (Charles River, Wilmington, MA, USA), stripped of their meninges, cut into small pieces, and incubated for 1 hr at 37° C in a calcium–buffered papain solution (Worthington Biochemical, Lakewood, NJ, USA). Gentle trituration was used to dissociate the tissue into a single-cell suspension, and cells were plated in 35-mm culture dishes at varying cell densities (250,000–350,000 cells per dish) onto a layer of OB astrocytes. The neuronal growth media consisted of 95% Minimal Essential Medium (MEM; Gibco/Thermo Fisher Scientific, Waltham, MA, USA), 5% horse serum (Gibco/Thermo Fisher Scientific), 6 gm/L glucose, and a nutrient supplement (Serum Extender, Collaborative Research, Inc., Bedford, MA, USA). One day after plating, cytosine-B- β -arabinofuranoside (10^{-5} M) was added to culture dishes to prevent glial cell overgrowth.

Neuronal Identity

The OB contains several subtypes of glutamatergic projection neurons – mitral cells and tufted cells, – which synapse with various subtypes of interneurons (Nagayama et al., 2014). In the present study, experiments were performed in presumptive mitral/tufted (M/T) cells.

We used our previous morphological criteria (Trombley and Westbrook, 1990), which correspond well with our prior immunocytochemical/cell marker criteria (i.e., VGLUT1, GAD) (Blakemore et al., 2018), to identify presumptive M/T cells and interneurons in OB cultures. M/T cells were identified based on soma size (20 to 40 μ m) and shape (pyramidal, multipolar) and were differentiated from the larger population of small-diameter (5–10 μ m) interneurons based on soma size. As there are multiple types of tufted cells, the term “M/T cells” is used in the present study to refer to mitral cells and the largest (internal) tufted cells (>20 μ m) (Nagayama et al., 2014).

Electrophysiology

Previously described methods were used to obtain whole-cell patch-clamp recordings in cultured OB neurons (Trombley et al., 1999; Blakemore et al., 2006; Blakemore et al., 2018). Briefly, whole-cell voltage-clamp recordings were obtained from M/T cells at room temperature after cells had been in culture for 2 to 14 days. The 35-mm culture dish was used as the recording chamber, and this dish was perfused at 0.5-2.0 ml/min with a bath solution containing (in mM): 162.5 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose. The pH was adjusted to 7.3 with NaOH. The final osmolarity was 325 mosmol/l. Patch electrodes were pulled from borosilicate glass to a final electrode resistance of 4-6 MΩ. These electrodes were filled with a solution containing (in mM): 145 CsCl or KMeSO₄, 1 MgCl₂, 10 HEPES, 4 Mg²⁺-ATP, 0.5 Mg²⁺-GTP, and 1.1 EGTA (pH 7.2; 310 mosmol/l).

Drugs were diluted in the bath solution and then applied using a gravity-fed flow-pipe perfusion system, in which flow pipes were assembled from 600 μm diameter square glass barrels. An electronic manipulator (Warner Instruments; Hamden, CT, USA) was used to position the flow pipes close to the cell, and drug flow was controlled with pinch clamps. The speed of solution changes allowed peak drug responses to occur within 100 ms. A barrel containing bath solution was used to continuously perfuse the neurons except during drug application. The applied drugs were AMPA (50 μM), ZnCl₂ (30 μM, 100 μM, and 1 mM; referred to here as “zinc”), CTZ (100 μM), and tetrodotoxin (TTX; 1 μM)(Sigma-Aldrich, St. Louis, MO, USA). CTZ was dissolved in dimethyl sulfoxide (DMSO) at 10 mM before being diluted with extracellular solution.

Procedures

To investigate membrane currents evoked by AMPA, whole-cell recordings were obtained from neuronal cell bodies with an Axoclamp 2B amplifier (Axon Instruments; Sunnyvale, CA) in discontinuous (switch frequency of 10-15 kHz) or continuous voltage-clamp mode. Membrane currents were filtered at 1-3 kHz, digitized at 5-10 kHz, and analyzed using AxoGraph software (John Clements).

Zinc's effects at AMPARs were evaluated by applying ZnCl₂ (30 μM, 100 μM, or 1 mM) during currents evoked by 50 μM AMPA. This involved either sequential application of drugs (i.e., application of AMPA alone followed by coapplication of AMPA + zinc) or coapplication of zinc in the middle (steady-state portion) of a current evoked by AMPA. For both types of application of zinc, the degree of current potentiation or inhibition by zinc was measured and expressed as a percentage of the control (AMPA-alone) current. For sequential application of zinc, effects of zinc on both the steady-state and peak components of the current were determined using the following formula: ([steady-state or peak] amplitude of AMPA + Zn current / [steady-state or peak] amplitude of AMPA-alone current) X 100. For application of zinc in the middle (steady-state portion) of an AMPA-evoked current, the degree of current potentiation or inhibition by zinc was estimated, as previously described (Blakemore and Trombley, 2004), using the following formula: (amplitude of AMPA + Zn portion of current / steady-state amplitude of AMPA-alone portion of current) X 100. For both types of application of zinc, when determining whether there was an effect of zinc on

the current, data from all cells (i.e., those with potentiation, inhibition, or no effect) were included in the statistical analysis.

Cyclothiazide's effects on potentiation or inhibition of AMPA-evoked currents by zinc were evaluated by coapplying CTZ (100 μ M) and ZnCl_2 (30 μ M, 100 μ M, or 1 mM) during currents evoked by 50 μ M AMPA over a trace span of approximately 15-20 seconds. To quantify the effects of CTZ on zinc-mediated potentiation or inhibition, the following formula was used: (amplitude of CTZ + AMPA + Zn portion of current / amplitude of CTZ + AMPA portion of current) X 100. For these experiments, due to the desensitizing nature of the current, the amplitude of the CTZ + AMPA + Zn portion of current was determined based on the average of 2 values (measured 1 second after the onset of application of CTZ + AMPA + zinc and 1 second before the end of application of CTZ + AMPA + zinc). The amplitude of the CTZ + AMPA portion of current was determined based on the average of 2 values (measured 1 second before the onset of application of CTZ + AMPA + zinc and 1 second after the end of application of CTZ + AMPA + zinc). To determine the effects of CTZ on zinc-mediated potentiation or inhibition, the post (with)-CTZ degree of current potentiation or inhibition by zinc was compared with the pre-(without) CTZ degree of current potentiation or inhibition by zinc (both expressed as a percentage of the control current).

For kinetic analyses, the degree of AMPAR desensitization was determined by calculating the ratio of the steady-state current amplitude (S) to the peak current amplitude (P)(S:P ratio) of the current evoked by 50 μ M AMPA, as previously described (Blakemore and Trombley, 2003).

Data Analysis

GraphPad Prism (version 8.0.1; GraphPad Software Inc. La Jolla, CA, USA) was used for all statistical analyses. The Kolmogorov-Smirnov (KS) normality test and Shapiro-Wilk (SW) normality test were used to test for normal distributions of data. The F test was used to assess for homogeneity of variance. All data were normally distributed and had equal variances. To determine whether a given concentration of zinc significantly potentiated or inhibited the AMPA-evoked current, the paired t-test was used to compare the mean (steady-state or peak) current before application of zinc (AMPA alone) with the mean current after application of zinc (AMPA + zinc), measured in pA, in the same cell. For paired normalized data (% of control current), the one-sample t-test was used to determine if there was a significant difference between the mean degree of zinc-mediated potentiation or inhibition of the steady-state current before (pre-CTZ) and after (post-CTZ) application of CTZ in the same cell. The unpaired t-test was used to determine whether the degree of zinc-mediated potentiation or inhibition of the AMPA-evoked current differed based on the portion of the current measured (steady-state vs. peak) or type of zinc application (coapplication in the middle of the current vs. sequential application). To protect against Type 1 errors, we used the Holm-Sidak test (alpha level=0.05) to correct for multiple comparisons and determined a threshold for significance of $P = 0.0051$. For the sake of simplicity, a threshold for significance of $P = 0.005$ is used in presenting results of statistical analyses. Values of data analyzed with t-tests are expressed as the mean $\pm$ SEM. Differences between means are

reported as $t(df)=x.xx$, $P = 0.0x$. Pearson correlation analysis (two-tailed) was used to evaluate the kinetic data (relationships between the degree of zinc-mediated potentiation or inhibition and the S:P ratio), and the associated correlation coefficient (r) and P value are presented for each analysis. As subject matching was effective ($P < 0.05$), one-way repeated measures ANOVA with a Geisser-Greenhouse correction was used to compare effects of 30 μ M, 100 μ M, and 1 mM zinc on AMPAR-mediated currents in a subset of cells. The Holm-Sidak post-hoc multiple comparisons test was used after ANOVA to determine differences between individual groups and P values adjusted for multiple comparisons. P values < 0.05 were considered significant for this analysis.

RESULTS

Zinc has largely biphasic effects at AMPARs on OB neurons: Effects of zinc without CTZ application

We have previously reported that zinc has heterogeneous effects at AMPARs on OB neurons, including potentiation at low zinc concentrations (e.g., 100 μ M) and inhibition at high zinc concentrations (e.g., 1 mM) (Blakemore and Trombley, 2004). Similar results were observed in the present study, in which experiments were exclusively performed in M/T cells.

We first examined the effects of 100 μ M zinc on AMPAR-mediated currents by sequentially applying AMPA then AMPA+zinc. Application of 50 μ M AMPA evoked a current in 8 cells with a steady-state amplitude of 42.8 ± 16.3 pA and a peak current amplitude of 149.8 ± 55.7 pA (Fig. 1A1). Subsequent application of 100 μ M zinc with 50 μ M AMPA increased the steady-state portion of the current evoked by AMPA in 7 cells (to $144.2 \pm 9.9\%$ of control) and had no effect in one cell. Overall, zinc significantly increased the steady-state current from 42.8 ± 16.3 pA to 53.2 ± 18.4 pA in these 8 cells (to $139.9 \pm 9.6\%$ of control) ($N=8$; paired t-test: $t(7)=4.026$, $P = 0.005$) (Fig. 1A2, Fig. 1B). Application of 100 μ M zinc with 50 μ M AMPA increased the peak portion of the current evoked by AMPA in 6 cells (to $129.1 \pm 11\%$ of control), decreased the peak current in one cell (to 91.4% of control), and had no effect in one cell. Overall, zinc increased the peak current from 108.3 ± 26.7 pA to 123.6 ± 28.3 pA in these 8 cells (to $121.3 \pm 9.6\%$ of control) ($N=8$; paired t-test: $t(7)=3.276$, $P > 0.005$) (Fig. 1A2, Fig. 1B). Although this potentiation of the peak current was significant in the uncorrected analysis ($P = 0.05$), it was no longer significant with the Holm-Sidak correction for multiple comparisons ($P > 0.005$). Whereas the degree of zinc-mediated potentiation of the steady-state current ($139.9 \pm 9.6\%$ of control; $N=8$) appeared to be greater than that of the peak current ($121.1 \pm 9.6\%$ of control; $N=8$), the difference was not statistically significant ($N=16$; unpaired t-test: $t(14)=1.385$, $P > 0.05$) (Fig. 1B).

We next determined the effects of 100 μ M zinc by coapplying it in the middle (steady-state portion) of a current evoked by AMPA. Coapplication of 100 μ M zinc with 50 μ M AMPA increased the steady-state amplitude of the current evoked by AMPA in 14 cells (to $139.8 \pm 7.2\%$ of control), inhibited the current in one cell (to 84.9% of control), and had no effect in 4 cells. Overall, zinc significantly increased the steady-state current to $128.5 \pm 6.9\%$ of control in these 19 cells ($N=19$; paired t-test: $t(18)=3.576$, $P = 0.005$) (Fig. 1C). AMPARs on the 4 cells with no effect of zinc were considered to be insensitive to zinc for the

purposes of further analysis. The degree of current potentiation of the middle (steady-state) portion of the current ($128.5 \pm 6.9\%$ of control; $N=19$) was similar to that seen with the above sequential application of AMPA and AMPA+zinc ($139.9 \pm 9.6\%$ of control; $N=8$) ($N=27$; unpaired t-test: $t(25)=0.9255$, $P > 0.05$)(Fig. 1D).

We next determined the effects of a higher concentration of zinc (1 mM) on AMPAR-mediated currents by sequentially applying AMPA then AMPA+zinc. Application of $50\ \mu\text{M}$ AMPA evoked a current in 10 cells with a steady-state amplitude of $18.1 \pm 6.5\ \text{pA}$ and a peak amplitude of $65.2 \pm 17.4\ \text{pA}$ (Fig. 2A1). Subsequent application of 1 mM zinc with $50\ \mu\text{M}$ AMPA decreased the steady-state portion of the current evoked by AMPA in 8 cells (to $47.5 \pm 12.6\%$ of control), increased the steady-state current in one cell (to 122.4% of control), and had no significant effect on the current in one cell. Overall, zinc significantly decreased the steady-state current from $20.1 \pm 6.4\ \text{pA}$ to $15.1 \pm 5.9\ \text{pA}$ (to $60.0 \pm 13.1\%$ of control) in these 10 cells ($N=10$; paired t-test: $t(9)=3.993$, $P < 0.005$)(Fig. 2A2, Fig. 2B). Application of 1 mM zinc with $50\ \mu\text{M}$ AMPA decreased the amplitude of the peak portion of the current evoked by AMPA in 8 cells (to $89.2 \pm 1.2\%$ of control) and increased the peak current in 2 cells (to $144.7 \pm 26.3\%$ of control). Overall, zinc did not have a significant effect on peak currents when all 10 cells were analyzed together, insignificantly changing the peak current from $65.2 \pm 17.4\ \text{pA}$ to $63.8 \pm 16.5\ \text{pA}$ (to $100 \pm 8.4\%$ of control)($N=10$; paired t-test: $t(9)=0.3048$, $P > 0.05$)(Fig. 2A2, Fig. 2B). In the uncorrected analysis, there was a significant difference between the degree of zinc-mediated inhibition of the steady-state current ($60.0 \pm 13.1\%$ of control; $N=10$) and the peak current ($100 \pm 8.4\%$ of control; $N=10$) ($N=20$; unpaired t-test; $t(18)=2.584$, $P = 0.05$)(Fig. 2B). However, this difference was no longer significant with the Holm-Sidak test correction for multiple comparisons ($P > 0.005$).

We next determined the effects of 1 mM zinc by coapplying it in the middle (steady-state portion) of a current evoked by AMPA. Coapplication of 1 mM zinc with $50\ \mu\text{M}$ AMPA decreased the steady-state amplitude of the current evoked by AMPA in 8 cells (to $48.7 \pm 4.4\%$ of control), potentiated the current in 1 cell (to 136.4% of control), and had no significant effect on the current in 4 cells. Overall, zinc significantly decreased the steady-state current to $78.2 \pm 8.5\%$ of control in these 13 cells ($N=13$; paired t-test: $t(12)=3.577$, $P = 0.005$)(Fig. 2C). AMPARs on the 4 cells with no effect of zinc were considered to be insensitive to zinc for the purposes of further analysis. The degree of current inhibition of the middle (steady-state) portion of the current ($78.2 \pm 8.5\%$ of control; $N=13$) was similar to that seen with the above sequential application of AMPA and AMPA+1 mM zinc ($60.0 \pm 13.1\%$ of control; $N=10$)($N=23$; unpaired t-test, $t(21)=1.210$, $P > 0.05$)(Fig. 2D).

Relationship between zinc effects and AMPA receptor desensitization kinetics

It has been suggested by several groups of investigators that zinc potentiates AMPARs by decreasing AMPAR desensitization (Smart et al., 1994; Bresink et al., 1996; Lin et al., 2001). Therefore, we evaluated whether the potentiating or inhibiting effects of zinc at AMPARs on M/T cells correlated with a quantitative measure of the degree of AMPAR desensitization (S:P ratio). The S:P ratio for cells with AMPARs modulated by zinc was determined as described in "Procedures."

For these experiments, we used the degree of zinc-mediated potentiation or inhibition of the middle (steady-state) portion of the AMPA-evoked current. As the degree of potentiation or inhibition of this portion of the current was similar in cells with different types of zinc application (sequential vs. middle of the current), we used data from both sets of cells. In the 21 cells with AMPAR-mediated currents potentiated by 100 μ M zinc, there was a significant negative correlation between the degree of current potentiation and the S:P ratio (N=21; Pearson's $r = -0.6435$, $P = 0.0016$) (Fig. 3A). However, in the 16 cells with AMPAR-mediated currents inhibited by 1 mM zinc, there was no significant correlation between the degree of inhibition and the S:P ratio (N=16; Pearson's $r = -0.05413$, $P = 0.8422$) (Fig. 3B).

CTZ blocks potentiating but not inhibitory effects of zinc at AMPARs: Effects of zinc with CTZ application

Cyclothiazide (CTZ) is a drug that inhibits AMPAR desensitization (Yamada and Tang, 1993; Yamada, 1998). Yamada and Tang (1993) reported that CTZ (0.3-100 μ M) reduced desensitization of whole-cell currents activated by quisqualate in a concentration-dependent manner (Yamada and Tang, 1993). CTZ (10 μ M) also increased spontaneous miniature EPSC amplitude and duration in cultured rat hippocampal neurons (Yamada and Tang, 1993). This suggests that AMPAR desensitization has *in vivo* effects on synaptic activity.

Bresink et al. (1996) first proposed that zinc may decrease/inhibit AMPAR desensitization and high-affinity [3H]AMPA binding in a manner similar to CTZ (Bresink et al., 1996). Several groups of investigators have since examined the effects of CTZ on zinc-mediated potentiation (Lin et al., 2001; Sun et al., 2010) and inhibition (Zhang et al. 2002; Sun et al., 2010) of AMPARs. In the present study, we used CTZ to determine whether zinc-mediated potentiation and/or inhibition of AMPAR-mediated currents in OB neurons reflect(s) AMPAR desensitization. For these experiments, we only included cells with AMPARs potentiated or inhibited by zinc in which zinc (100 μ M or 1 mM) was coapplied in the middle (steady-state) portion of the AMPA-evoked current.

In the present study, CTZ had significant effects on the steady-state component of currents evoked by 50 μ M AMPA alone. In the 14 cells with AMPARs potentiated by 100 μ M zinc, application of 100 μ M CTZ increased the steady-state component of the AMPA-evoked current to $1152.0 \pm 287.2\%$ of control. In the 8 cells with AMPARs inhibited by 1 mM zinc, application of 100 μ M CTZ significantly increased the steady-state component of the AMPA-evoked current to $857.8 \pm 162.1\%$ of control.

In the 14 cells with AMPAR-mediated currents initially potentiated by 100 μ M zinc (to $139.8 \pm 7.2\%$) (Fig. 4A), coapplication of 100 μ M CTZ with 50 μ M AMPA and 100 μ M zinc completely blocked the potentiating effect of zinc on the steady-state component of the AMPAR-mediated current in 13 cells (Fig. 4B) and reduced the degree of potentiation in 1 cell (from 131.1% of control to 107.0% of control) (N=14; one-sample t-test: $t(13) = 7.246$, $P = 0.005$). In the 4 cells with AMPARs initially insensitive to 100 μ M zinc (Fig. 4C), coapplication of 100 μ M CTZ with 50 μ M AMPA and 100 μ M zinc revealed mild underlying inhibition of the current in all cells (to $91.3 \pm 4.6\%$ of control) (N=4; one-sample t-test: $t(3) = 1.881$, $P > 0.05$) (Fig. 4D). Figures 4E and 4F show summary data (histograms) for 4A-B and 4C-D, respectively.

In the 8 cells with AMPAR-mediated currents initially inhibited by 1 mM zinc (Fig. 5A), coapplication of 100 μ M CTZ with 50 μ M AMPA and 1 mM zinc had no significant effect on the degree of inhibition of the steady-state component of the AMPAR-mediated current by zinc (Fig. 5B). The mean pre-(without) CTZ degree of zinc-mediated inhibition of AMPAR-mediated currents was $48.7 \pm 4.4\%$ of control, which did not significantly differ from the mean post-(with) CTZ degree of current inhibition of $64.4 \pm 6.4\%$ of control ($N=8$; one-sample t-test: $t(7)=1.644$, $P>0.05$)(Fig. 5C). In the 4 cells with AMPARs that initially were insensitive to 1 mM zinc, coapplication of 100 μ M CTZ and 1 mM zinc had no significant effect on the current.

Effects of 3 concentrations of zinc on AMPARs in the same cell

To evaluate co-existing potentiation and inhibition of AMPARs by zinc, we investigated the effects of 3 concentrations of zinc (30 μ M, 100 μ M, and 1 mM) on AMPA-evoked currents in the same cell before and after application of 100 μ M CTZ. Table 1 summarizes the effects of these 3 concentrations of zinc (potentiation, no effect, inhibition) in 6 M/T cells, expressed as a percentage of the control current. Before CTZ treatment, we observed a biphasic response (potentiation by 30 μ M and/or 100 μ M zinc and inhibition by 1 mM zinc of the current) in 2 cells, uniphasic potentiation in 2 cells, uniphasic inhibition in 1 cell, and no effect of any concentration of zinc in 1 cell.

One-way ANOVA for repeated measures showed a significant difference between the effects of 30 μ M zinc (mean: $130.4 \pm 11.7\%$ of control), 100 μ M zinc (mean: $119.8 \pm 9.7\%$ of control), and 1 mM zinc (mean: $86.6 \pm 10.3\%$ of control) on AMPAR-mediated currents in these cells before application of CTZ ($n=6$, $F[1.929, 9.643] = 10.25$; $P=0.0043$). Holm-Sidak post-hoc multiple comparisons test revealed a significant difference between the effects of 30 μ M zinc and 1 mM zinc (adjusted $P=0.0234$) and 100 μ M zinc and 1 mM zinc (adjusted $P=0.0299$), but not between 30 μ M zinc and 100 μ M zinc (adjusted $P=0.3743$).

In one of these 6 cells (Table 1), 30 μ M zinc potentiated (162.6% of control), 100 μ M zinc had no effect on (100% of control), and 1 mM zinc inhibited (88.1% of control) the current. This finding supports the hypothesis that the lack of an effect of 100 μ M zinc in some cells reflects a balance of zinc-mediated potentiation and inhibition. The finding in another cell that the degree of potentiation of the current decreased with increasing zinc concentration (159.8% vs. 145.7% vs. 108.3% of control, respectively) further suggests that co-existing inhibitory effects appear at higher concentrations of zinc that partially occlude zinc's potentiating effect.

Effects of CTZ on effects of 3 concentrations of zinc on AMPARs in the same cell

To further evaluate co-existing potentiation and inhibition of AMPARs by zinc, we investigated the effects of CTZ on the effects of 3 concentrations of zinc (30 μ M, 100 μ M, and 1 mM) on AMPA-evoked currents in the same cell. Table 1 summarizes the effects of zinc (potentiation, no effect, inhibition) in 6 M/T cells before and after application of CTZ, expressed as a percentage of the control current.

In 4 of these 6 cells, 30 μ M and/or 100 μ M zinc potentiated AMPAR-mediated currents and 1 mM zinc potentiated ($N=2$) or inhibited ($N=2$) currents before application of CTZ. In 2

cells (Table 1, Cells #1, 2) with initial potentiation of the current by all concentrations of zinc, coapplication of CTZ with zinc blocked the potentiation and/or revealed underlying inhibition. In a third cell (Cell #3) with currents initially potentiated by 30 μ M zinc, unaffected by 100 μ M zinc, and inhibited by 1 mM zinc, coapplication of CTZ reduced the degree of current potentiation by 30 μ M zinc, revealed underlying inhibition of the current by 100 μ M zinc, but had no effect on the degree of current inhibition by 1 mM zinc. AMPARs in a fourth cell (Cell #4) were potentiated by 30 μ M and 100 μ M zinc and inhibited by 1 mM zinc before CTZ treatment. CTZ application reduced the degree of potentiation of the current when coapplied with 30 μ M zinc, blocked the potentiation by 100 μ M zinc, but had no effect on the degree of inhibition by 1 mM zinc.

AMPArs in the remaining 2 cells were largely insensitive to zinc before application of CTZ. In a fifth cell (Cell #5), coapplication of CTZ with 30 μ M and 100 μ M zinc revealed underlying inhibition of the current but did not affect the degree of initial inhibition of the current by 1 mM zinc. Currents in the final cell (Cell #6) showed no response to any concentration of zinc before CTZ treatment. Coapplication of CTZ had no effect on the current when coapplied with 30 μ M zinc, but revealed underlying inhibition of the current when coapplied with 100 μ M zinc or 1 mM zinc.

Thus, coapplication of CTZ with zinc blocked the potentiating effects of 3 concentrations of zinc on AMPAR-mediated currents in most cells and revealed underlying inhibition in some cells. It also revealed a current response to zinc in some cells with zinc-insensitive AMPARs. For example, coapplication of CTZ with 100 μ M zinc changed the response from no effect to underlying inhibition of the current ($85.9 \pm 4.2\%$ of control) in 3 cells (Cells #3, 5, and 6). However, in 3 of 6 cells with currents initially inhibited by 1 mM zinc (Cells #3, 4, 5), CTZ had no significant effect. The Pre-CTZ ($68.3 \pm 11.9\%$ control) and Post-CTZ ($68.4 \pm 12.8\%$ control) degrees of current inhibition by 1 mM zinc did not significantly differ ($N=3$; one-sample t-test: $t(2)=1.025$, $P > 0.05$).

DISCUSSION

Summary of findings

Consistent with our previous findings (Blakemore and Trombley, 2004; Blakemore et al., 2013), we largely found biphasic effects of zinc at AMPARs on M/T cells (i.e., potentiation with 30 μ M and/or 100 μ M zinc; inhibition with 1 mM zinc), with a subset of AMPARs that were insensitive to zinc. Suggestive of a potential relationship between the potentiating effects of zinc and AMPAR desensitization, we found a significant (negative) correlation between the degree of potentiation of the AMPAR-evoked current by 100 μ M zinc and the AMPAR S:P ratio. That is, AMPARs that desensitize more completely (lower S:P ratio) are more sensitive to potentiation by zinc. However, no significant correlation was found between the degree of inhibition of the AMPAR-evoked current by 1 mM zinc and the S:P ratio. Coapplication of CTZ with AMPA and zinc completely blocked the potentiating effects of 100 μ M zinc on AMPAR-mediated currents in most cells but had no significant effect on the degree of current inhibition by 1 mM zinc. Together, these findings suggest that low concentrations of zinc potentiate AMPARs expressed by rat OB neurons by decreasing

receptor desensitization but that the inhibitory effects of higher concentrations of zinc are mediated by a separate mechanism.

Zinc-sensitive and insensitive AMPA receptors

We have previously reported that AMPARs on some OB neurons are insensitive to zinc (Blakemore and Trombley, 2004; Blakemore et al., 2013). We observed similar findings in the present study in M/T cells. The co-existence of dual populations of zinc-sensitive and zinc-insensitive AMPARs has also been observed by other investigators (Mayer et al., 1989; Lin et al., 2001) and may partially reflect diversity of AMPAR subunit and/or splice variant composition.

In *Xenopus* oocytes, currents mediated by homomeric GluA3 but not homomeric GluA1 receptors were enhanced by low zinc concentrations (4–7.5 μ M), implying that the GluA3 subunit is integral to zinc potentiation (Dreixler and Leonard, 1994). No zinc potentiation was observed in heteromeric receptors composed of GluA3 and GluA2 or GluA1 and GluA2 (Dreixler and Leonard, 1994), suggesting that the AMPAR zinc potentiation site involving the GluA3 subunit may be altered by the receptor's inclusion of the GluA2 subunit (Dreixler and Leonard, 1997). Thus, the absence or presence of certain subunits in the AMPAR may explain why we did not observe zinc-mediated modulation in a subset of cells.

Other factors that may contribute to the diverse response of AMPARs to zinc are variability in the extracellular (calcium) environment (Dreixler and Leonard, 1997) and alternative splicing of the AMPAR gene (Shen and Yang, 1999). The latter generates flip and flop AMPAR splice variants, and flip variants appear to be more sensitive to zinc than flop variants (Shen and Yang, 1999), including among AMPARs within the OB (Blakemore and Trombley, 2003; Blakemore and Trombley, 2004). Diversity of subunits and/or splice variants of AMPARs expressed on OB neurons may help explain the results of our kinetic analysis (see "Implications of our kinetic analysis"). Lin et al. (2001) hypothesized that variations in the zinc sensitivity of AMPARs seen in their study of hippocampal CA3 neurons may reflect different subunit and/or splice variant compositions of synaptic versus extrasynaptic AMPARs (Lin et al., 2001).

The simultaneous binding of zinc to distinct potentiating and inhibiting binding sites on different AMPAR subunits could also explain the existence of some apparently zinc-insensitive AMPARs. For example, the absence of an effect of zinc in some neurons may be due to the appearance of inhibition just sufficient to overcome co-existing potentiation (Blakemore and Trombley, 2004). Some support for this hypothesis comes from the M/T cells tested with 3 concentrations of zinc (30 μ M, 100 μ M, and 1 mM). In one cell, 30 μ M zinc potentiated, 100 μ M zinc had no significant effect on, and 1 mM zinc inhibited the AMPAR-mediated current. In another cell, the degree of current potentiation decreased with increasing zinc concentration, consistent with the appearance of co-existing inhibitory effects at higher concentrations of zinc and partial occlusion of zinc's potentiating effects. The lack of inclusion of either an inhibiting or potentiating binding site for zinc in AMPARs could also explain some of our results. In the 6 M/T cells tested with 3 concentrations of zinc, there was uniphasic potentiation of the AMPAR-mediated current in response to all concentrations of zinc in 2 cells and uniphasic inhibition in response to 1 mM zinc in 1 cell.

Further support for the existence of dual zinc binding sites on AMPARs comes from our findings with CTZ application (see “Mechanism(s) of zinc-mediated potentiation”).

Additional evidence to support the presence of discrete potentiating and inhibiting zinc binding sites comes from a 1997 study (Dreixler and Leonard, 1997). Under normal physiological conditions, activation of cloned GluA1 or GluA3 receptors allows Ca^{2+} ions to pass through the receptor channel pore complex. Dreixler and Leonard hypothesized that as zinc concentrations become sufficiently high, zinc's ability to block the channel pore might exceed its ability to cause allosteric potentiation of channel activity, resulting in the blockade of most of the ionic flow (i.e., current inhibition) (Dreixler and Leonard, 1997). Zinc binding at some site other than the channel pore may lead to a conformational change, resulting in greater current flow (i.e., current potentiation) (Dreixler and Leonard, 1997).

Mechanism(s) of zinc-mediated potentiation

It has been suggested that low concentrations of zinc potentiate AMPARs by decreasing AMPAR desensitization in a manner similar to CTZ (Smart et al., 1994; Bresink et al., 1996). Therefore, several groups of investigators have used CTZ as a means of exploring the mechanisms underlying zinc-mediated potentiation of AMPARs.

Lin et al. (2001) found that 100 μM CTZ reversibly reduced, without completely eliminating, potentiation of AMPAR-mediated currents by 100 μM zinc in hippocampal CA3 neurons (Lin et al., 2001). They identified 3 possible explanations for this effect: 1) competition of CTZ and zinc for an allosteric modulating site, 2) CTZ is maximally relieving AMPAR desensitization, hence, its competing mechanism of action prevents zinc's effect, and 3) CTZ may be combining with/chelating zinc, thus, decreasing its effects. Based on their data and other results (Bresink et al., 1996), Lin et al. (2001) concluded that the second mechanism appears to be the most likely explanation. Furthermore, they found that zinc inhibited desensitization but did not modify the recovery from desensitization and concluded that zinc may slow AMPARs' transition into a desensitized state, while exerting minimal effects on recovery from desensitization (Lin et al., 2001).

In a subsequent study, Sun et al. (2010) also used CTZ to investigate mechanisms of zinc's modulatory effects on calcium-permeable AMPARs (CP-AMPA) on carp retinal horizontal cells (Sun et al., 2010). They reported that the potentiating effect of 10 μM zinc on the peak amplitude of CP-AMPA-mediated currents was more marked when 20 μM CTZ was present. However, more consistent with previous findings, supplementary data from their study showed that higher concentrations of CTZ (>100 μM) abolished zinc's potentiating effect, potentially by altering the current desensitization process. Additional experiments showed that the potentiating effects of 10 μM zinc were not affected by the extracellular calcium concentration. Based on their collective results, Sun et al. (2010) concluded that potentiating effect of a low (10 μM) concentration of zinc involves interaction with the CTZ-binding site on CP-AMPA (Sun et al., 2010).

This conclusion by Sun et al. (2010) is in contrast to the conclusion by Lin et al. (2001) that competition of zinc and CTZ for a binding site was unlikely given the differing molecular structures of zinc and the thiazides. However, there is an alternative interpretation of Sun and

colleagues' findings that 10 μ M zinc enhanced AMPAR-mediated currents in the presence of 20 μ M CTZ but not >100 μ M CTZ (Sun et al., 2010). Concentration-response curves for CTZ-mediated enhancement of the steady-state current and the peak current activated by 1 mM quisqualate revealed EC50 values for CTZ of 14 μ M and 12 μ M, respectively (Yamada and Tang, 1993). Thus, the effects of 20 μ M CTZ on AMPAR desensitization in the study by Sun et al. (2010) may have been sub-maximal, allowing 10 μ M zinc to still potentiate AMPARs by a similar mechanism (i.e., decreasing receptor desensitization). This interpretation is also consistent with the findings by Shen & Yang (1999) who found that, in the presence of 10 μ M CTZ, 30 μ M zinc further potentiated the peak current evoked by application of 3 mM glutamate (Shen and Yang, 1999).

In the present study, we found that coapplication of 100 μ M CTZ with 100 μ M zinc completely blocked the potentiating effect of zinc on currents evoked by 50 μ M AMPA in 13 of 14 M/T cells. In 6 M/T cells tested with 3 concentrations of zinc (30 μ M, 100 μ M, and 1 mM), coapplication of CTZ completely blocked the potentiating effects of the 3 zinc concentrations on AMPA-evoked currents in most cells and revealed underlying inhibition of the current in some cells. These findings provide further support for the notion that zinc potentiates AMPAR-mediated currents by decreasing AMPAR desensitization. We hypothesize that this apparent "reversal" of zinc's potentiating effect (potentiation to inhibition) is because CTZ and zinc both potentiate AMPARs by the same mechanism (i.e., relief of receptor desensitization). As 100 μ M CTZ maximally relieved AMPAR desensitization, zinc could no longer potentiate the receptor by the same mechanism. That is, CTZ "preempted" zinc's potentiating effects and unmasked coexisting zinc-mediated inhibition. Following coapplication of CTZ and zinc (30 μ M, 100 μ M, or 1 mM), we also found inhibition of the current in a subset of cells with AMPARs that were initially insensitive to zinc. This suggests that the lack of an original effect of zinc was due to similar levels of co-existing potentiation and inhibition and that CTZ blocked the potentiation and revealed remaining inhibition. These findings provide further support for the notion that zinc can simultaneously bind to discrete potentiating and inhibiting binding sites on AMPAR subunits.

Structural data also support a link between AMPAR desensitization and zinc. Residues involved in zinc binding or desensitization map to the GluA2 S1/S2 domains, and intersubunit interactions have been proposed to alter the conformation of the ligand binding core (Armstrong and Gouaux, 2000).

Mechanism(s) of zinc-mediated inhibition

Several groups of investigators have evaluated the effects of CTZ on zinc-mediated inhibition of AMPARs by higher concentrations of zinc. Shen & Yang (1999) reported that 300 μ M zinc suppressed the peak component of the current evoked by 3 mM glutamate in the presence of 10 μ M CTZ in isolated carp retinal horizontal cells (Shen and Yang, 1999).

Zhang et al. (2002) subsequently used patch-clamp recording to examine zinc-mediated modulation of glutamate receptors on cultured retinal horizontal cells (Zhang et al., 2002). They found that the zinc (30 μ M and 300 μ M) suppression of the whole-cell peak current response to 200 μ M glutamate persisted with coapplication of 10 μ M CTZ. As

administration of 30 μ M GYKI 52466 (AMPA antagonist) completely abolished glutamate responses of outside-out patches, they concluded that the receptor target for zinc's effects on horizontal cells is AMPARs. They also found that zinc (300 μ M) did not alter the rate of AMPAR desensitization. Based on their collective results, they concluded that zinc does not inhibit AMPAR responses via enhancement of receptor desensitization and that zinc is unlikely to bind at the CTZ-binding site.

Consistent with previous results, Sun et al. (2010) reported that the inhibition of the peak CP-AMPA-mediated current on carp retinal horizontal cells by a high concentration of zinc (1 mM) was not affected by the coapplication of 20 μ M CTZ (Sun et al., 2010). They concluded that this suggests that a separate mechanism is associated with zinc's inhibitory effect, which is unrelated to the CTZ binding site.

In the present study, we found that degree of inhibition of AMPAR-mediated currents by 1 mM zinc was not affected by coapplication of 100 μ M CTZ. We also found no correlation between the degree of zinc-mediated inhibition of AMPARs by 1 mM zinc and the degree of AMPAR desensitization, as measured by the S:P ratio. These results suggest that higher concentrations of zinc likely do not inhibit AMPAR-mediated responses by altering AMPAR desensitization.

Our findings also advance results of previous studies in several ways. First, we evaluated the effects of a higher concentration of CTZ (100 μ M versus 10 μ M and 20 μ M), which is more likely to maximize the effects of CTZ on AMPARs (Yamada and Tang, 1993).

Determination of concentration-response curves for CTZ-mediated enhancement of quisqualate-activated (AMPA-mediated) steady-state and peak currents revealed EC50 values for CTZ of 14 μ M and 12 μ M, respectively (Yamada and Tang, 1993). In addition, we evaluated the effects of CTZ and zinc on the steady-state versus the peak component of the AMPAR-evoked current, which would be more likely to show a change in AMPAR desensitization in response to 1 mM zinc if such an effect was present (see "Strengths and limitations of our recording technique"). Yamada & Tang (1993) reported that, compared to the control response, 100 μ M CTZ caused a 400-fold increase in the steady-state current compared with only a 4-fold increase in the peak current (Yamada and Tang, 1993).

A variety of studies have shed light on potential mechanisms of zinc-mediated inhibition of AMPARs. Bresink et al. (1996) concluded that the antagonist effects of high zinc concentrations may mask the effects of low zinc concentrations (Bresink et al., 1996). They proposed that zinc's antagonist effects may reflect a decrease in agonist affinity, based on the concentration-dependent slowing of the onset rate of AMPA-induced currents.

It also has been proposed that the inhibition of AMPAR-mediated currents in *Xenopus* oocytes by high concentrations of zinc is due to channel blockade (Rassendren et al., 1990). These results are in agreement with the hypothesis that as zinc concentrations become sufficiently high, zinc's ability to block the channel pore might outweigh its ability to cause allosteric potentiation of channel activity, resulting in the blockade of most ionic flow (Dreixler and Leonard, 1997).

Sun et al. (2010) found that the inhibitory effect of 1 mM zinc on CP-AMPA receptors was eliminated or greatly decreased in Ca^{2+} -free (0 mM) Ringer's solution, but increased in high- Ca^{2+} (20 mM) Ringer's solution (Sun et al., 2010). They concluded that the extracellular calcium concentration influences the effects of a high inhibitory (1 mM) but not a low potentiating (10 μM) concentration of zinc. These findings contrast with findings in skate retinal horizontal cells (Kreitzer et al., 2009). Kreitzer et al. (2009) reported that inhibition of AMPAR-mediated responses to application of 100 μM glutamate by high concentrations of zinc (300 μM and 1 mM) was enhanced at lower extracellular concentrations of Ca^{2+} (0.2 mM Ringer's solution) (Kreitzer et al., 2009). They concluded that zinc- and calcium-mediated inhibition of glutamate receptor currents may reflect effects of these ions at a common extracellular site.

In a recent study, inhibition of EPSCs mediated by GluA1-3-expressing AMPARs at zinc-containing hippocampal synapses by endogenous zinc was attributed to direct binding and modulation of AMPARs by synaptically released zinc (Kalappa et al., 2015). This conclusion was partially based on structural data demonstrating that the ligand-binding domain (LBD) of the AMPAR subunit GluA2 contains a number of zinc binding sites (Armstrong and Gouaux, 2000). Based on additional structural data, Kalappa et al. (2015) proposed that the LBD is a most likely site for zinc-mediated allosteric modulation of AMPARs (Kalappa et al., 2015).

Strengths and limitations of our recording technique

There are strengths and limitations to our recording methods compared with previous techniques that largely explored the effects of zinc and CTZ on peak components of AMPAR-mediated currents. As AMPARs are characterized by rapid desensitization (Zorumski and Thio, 1992), whole-cell patch techniques may not allow one to change solutions rapidly enough to determine the peak of the AMPAR-mediated currents. While this may be a limitation for peak current analysis, a strength of our using whole-cell recording methods in cultured OB neurons is they enabled us to determine the effects of CTZ and zinc on the steady-state portion of the AMPA-evoked current. Because the steady-state portion of the current better represents the desensitization equilibrium, it allowed greater analysis of zinc's effects on the desensitization component of AMPAR kinetics. Another advantage of our method of whole-cell recording is that each electrophysiology trace is 15-20 seconds in duration, thus, shows both the peak and desensitizing portion of the current. Each trace also shows the time before drug application (baseline), the time spanning drug application (zinc effect), and the time after drug application (recovery), thus, serves as its own control. Finally, our method of whole-cell recording from cultured neurons allowed the rapid exchange of solutions (control, drug) that enveloped the entire neuron, which is not possible to achieve in brain slice preparations.

Another potential limitation of our recording from cultured neurons is variability in effects of zinc attributable to different neuronal populations (see "Neuronal Identity"). As we only recorded from M/T cells in the present experiments, this controls for our previously observed differences in the effects of zinc at AMPARs on M/T cells versus interneurons (Blakemore and Trombley, 2004). However, subpopulations of mitral and tufted cells exist in

the OB (Nagayama et al., 2014), so we cannot rule out the possibility that cell-specificity explains some of the variability in the effects of zinc. It is noteworthy that most of our statistical analyses were carried out in paired sets of data from the same cell (e.g., paired t-test, one-sample t-test), thus, were designed to control for cell-to-cell variability (each cell serves as its own control).

AMPA receptor desensitization

A variety of models of AMPAR kinetics and desensitization have been proposed, including models in which agonists bind with greater affinity to the desensitized receptor state (Trussell and Fischbach, 1989; Patneau and Mayer, 1991; Colquhoun et al., 1992; Raman and Trussell, 1992; Robert et al., 2001). High AMPA concentrations evoke currents that desensitize rapidly (tens of milliseconds) and almost completely (>90%) depending on receptor subunit composition (Kiskin et al., 1986; Trussell et al., 1988; Mosbacher et al., 1994; Partin et al., 1994; Koike et al., 2000; Blakemore and Trombley, 2003). Also, data from the effects on ligand binding by allosteric modulators demonstrate that AMPAR desensitization is accompanied by a 5- to 10-fold increase in AMPA affinity (Kessler et al., 1996). Crystallographic data reveal histidine residues in S1S2 of the GluR2 binding core as likely zinc binding sites that may bridge neighboring subunits and modulate receptor activity (Armstrong and Gouaux, 2000). Collectively, these data suggest the ability of synaptically released zinc to alter AMPAR desensitization and influence glutamate binding and synaptic efficacy.

Implications of our kinetic analysis

Inclusion of the flop isoform in an AMPAR causes it to desensitize more rapidly (faster desensitization time constants) and more completely (lower S:P ratio) than inclusion of the flip isoform (Mosbacher et al., 1994; Dingledine et al., 1999). In the present study, we found a significant negative correlation between the S:P ratio and the degree of current potentiation by a low concentration (100 μ M) of zinc. This suggests that cells with AMPARs with a low S:P ratio (flop-like kinetics) are more strongly affected by a low zinc concentration than AMPARs with a high S:P ratio (flip-like kinetics). This is consistent with the hypothesis that zinc's potentiating effects are due to inhibition of desensitization, since cells with AMPARs with a very high S:P ratio are already in a state of minimal receptor desensitization, thus, zinc cannot exert much of a potentiating effect by further decreasing AMPAR desensitization. However, cells with AMPARs with a very low S:P ratio are maximally desensitized, thus, zinc can potentiate the AMPARs by decreasing desensitization.

We have previously reported that AMPARs on OB interneurons desensitize more rapidly and completely than those on M/T cells, suggesting that AMPARs on interneurons versus M/T cells are composed of a greater proportion of flop versus flip subunits (Blakemore and Trombley, 2003). Consistent with the results of the present kinetic analysis, we also previously reported that the magnitude of the effect of a low (100 μ M) concentration of zinc on AMPAR-mediated currents was greater in interneurons (with flop-like kinetics) than M/T cells (with flip-like kinetics) (Blakemore and Trombley, 2004). In the present study, 100 μ M zinc significantly potentiated the steady-state component of AMPAR-mediated currents in the majority of M/T cells. Collectively, our previous electrophysiology (Blakemore and

Trombley, 2004) and molecular (Horning et al., 2004) results from AMPARs combined with the present findings suggest that, while amounts of flip and flop in AMPARs expressed by OB neurons vary with cell type, AMPARs on M/T cells are likely to heterogeneously express AMPAR subunits with both a high S:P ratio (flip-like kinetics) and low S:P ratio (flop-like kinetics). Thus, M/T cells, which are primary targets of zincergic innervation in the OB (see “Timing of zincergic innervation and AMPA receptor subunit expression in the OB”), are good subjects for these experiments.

Timing of zincergic innervation and AMPA receptor subunit expression in the OB

Relatively little is known about the timing and distribution of zinc-containing axon terminals (zincergic innervation) in the developing brain, including the OB. We recently used Zinpyr-1 (ZP1) (Burdette et al., 2001), a zinc-sensitive, fluorescent probe, to investigate the location and release of vesicular zinc in living OB slices obtained from 16- to 21-day-old rats (Blakemore et al., 2013). Consistent with previous findings in fixed tissues of older rats (Jo et al., 2000), we found high-intensity ZP1 fluorescence, reflecting vesicular zinc, in both the glomerular layer (vesicular zinc from olfactory sensory neuron terminals) and the granule cell layer (vesicular zinc from centrifugal fibers). Our unpublished data show ZP1 fluorescence in OB slices from even younger animals (e.g., postnatal days 3-5).

Our findings are consistent with Brown et al. (2003) who reported a mature pattern of zinc staining by postnatal day (PD) 12 in the mouse somatosensory cortex, with the presence of histochemically reactive zinc as early as PD3 (Brown et al., 2003). As further supported by our previous reports of effects of zinc on amino acid receptors (NMDA, GABA, glycine, AMPA) on OB neurons from P0-P5 rats (Trombley and Shepherd, 1996; Blakemore and Trombley, 2004; Trombley et al., 2011; Blakemore et al., 2013), vesicular zinc is available to modulate AMPARs in animals of the age (P0-P4) used in the present experiments.

Subunit composition of many neurotransmitter receptors is developmentally regulated. Data from other brain regions (e.g., hippocampus) suggest that expression of some AMPAR subunits (e.g., GluA2) (Pellegrini-Giampietro et al., 1992) and the flip and flop splice variants (Standley et al., 1995) change over time. However, little is known about any developmental changes in AMPAR subunit expression in the OB. Of significance to the present experiments, we have previously shown expression of mRNA for all AMPAR (GluA1-4) subunits and both AMPAR splice variants in the olfactory bulbs of 5-day-old rats (Horning et al., 2004). The relative amounts of mRNAs for GluA1-4 differed, i.e., GluA2 > GluA1 > GluA4 > GluA3. Also, while similar amounts of both flip and flop splice variants were found in GluA2 and GluA4 transcripts, GluA1 and GluA3 transcripts consisted mostly of flip. This heterogeneous expression of AMPAR subunits and splice variants in young (PD5) rats supports the interpretation of our kinetic data. However, it is possible that changes in zincergic innervation and/or AMPAR subunit expression between P0 and P4 may have contributed to variability in our findings.

Implications to OB function

Our findings may have significant implications in regard to synaptic transmission in the brain as a whole as well as the OB. In other areas of the brain, the brief exposure to

glutamate that occurs with synaptic release causes rapid desensitization of AMPARs, and AMPAR desensitization plays a role in determining the amplitude and timing of synaptic events. As zinc may potentiate AMPARs by inhibiting receptor desensitization, a potential role of the zinc co-released with glutamate from zinc-enriched neurons is to modify glutamate's desensitizing effects. In these ways, zinc may alter temporal aspects of synaptic efficacy in many brain regions, including the OB as we have recently demonstrated (Blakemore et al., 2013).

In various immunohistochemical studies involving fixed tissue, high concentrations of zinc have been found in the glomerular layer (GL) and granule cell layer (GCL) of the OB (Friedman and Price, 1984; Jo et al., 2000; Sekler et al., 2002). As previously mentioned, we recently used the zinc-sensitive, fluorescent probe Zinpyr-1 (ZP1) (Burdette et al., 2001) to investigate the location of vesicular zinc in living OB slices (Blakemore et al., 2013). Our collective results with the ZP1 probe combined with zinc chelators revealed that most vesicular zinc is located in the GL and GCL (Blakemore et al., 2013).

The fact that zinc and glutamate are co-expressed in some zinc-enriched neurons (Beaulieu et al., 1992; Slomianka, 1992; Dyck et al., 1993; Frederickson and Moncrieff, 1994; Perez-Clausell, 1996; Ichinohe and Rockland, 2005), combined with findings that depolarizing stimuli cause the release of zinc from synaptic vesicles (Assaf and Chung, 1984; Howell et al., 1984), suggests that zinc is released during synaptic transmission. We also recently investigated the release of vesicular (synaptic) zinc in the OB using a combination of zinc imaging and electrophysiology (Blakemore et al., 2013). OB slices from young adult rats were incubated with ZP1 and electrical stimulation the olfactory nerve (ON) layer was used to induce zinc release. In this manner, we showed for the first time that terminals of olfactory sensory neurons (OSNs) within individual glomeruli respond to patterned (sniff frequency) OB stimulation with the release of vesicular zinc (Blakemore et al., 2013).

The amount of zinc that is released from presynaptic terminals into the synaptic cleft has been a matter of controversy. Measurements of concentrations of synaptically released zinc appear to be influenced by a variety of factors (e.g., animal age, temperature, use of CaEDTA, choice of fluorescent probe) (Frederickson et al., 2006). While early studies suggested that neuronal depolarization resulted in extracellular zinc concentrations of 10 to 300 μM (Frederickson et al., 1983; Assaf and Chung, 1984; Howell et al., 1984; Xie and Smart, 1991), it has been suggested that such concentrations are unlikely under normal physiological conditions (McAllister and Dyck, 2017). More recent evidence suggests that, following exocytosis, peak synaptic cleft concentrations of zinc range from 10 nM to >100 μM (Vogt et al., 2000; Ueno et al., 2002; Paoletti et al., 2009; Vergnano et al., 2014; Goldberg et al., 2016; Blakemore and Trombley, 2017). Thus, zinc concentrations (30 μM and 100 μM) that have been shown to potentiate OB AMPARs (Blakemore and Trombley, 2004) via potential AMPAR desensitization are physiologically relevant.

In our 2013 study, we also showed that zinc (100 μM) potentiated AMPAR-mediated excitatory postsynaptic potentials (EPSPs) and/or excitatory postsynaptic currents (EPSCs) in both M/T cells and interneurons (Blakemore et al., 2013), suggestive of zinc effects at synaptic AMPARs. Zinc-mediated potentiation, via inhibition of desensitization, of synaptic

AMPA receptors has implications to OB function, thus, odor-information processing. For instance, zinc-mediated potentiation via inhibition of desensitization of AMPARs could enhance the limited role of AMPARs in reciprocal inhibition between M/T cells and interneurons (Isaacson and Strowbridge, 1998; Schoppa et al., 1998) by increasing and/or prolonging the relatively small and transient current mediated by AMPARs.

Synchronization of neural activity is an important aspect of neuronal networks in the CNS. In the OB, AMPAR activation plays a role in the synchronization of mitral and tufted cell activity within a glomerulus in response to patterns of glutamate release from olfactory sensory neurons (Carlson et al., 2000; Schoppa and Westbrook, 2001; Schoppa and Westbrook, 2002; Christie and Westbrook, 2006; Schoppa, 2006). This synchronization generates odor-evoked oscillations with temporal and spatial characteristics that contribute to olfactory information encoding within the OB. Our 2013 study findings suggest that zinc-mediated potentiation of synaptic events mediated by AMPARs enhances synaptic summation (Blakemore et al., 2013) and summation plays a role in synchronization. Thus, via altering glomerular network activity, synaptically released zinc may influence spatial and temporal patterns of network behavior involved with sensory coding by the OB.

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ABBREVIATIONS

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA_s	AMPA receptors
CNS	central nervous system
CP-AMPA	calcium-permeable AMPAR
CTZ	cyclothiazide
DMSO	dimethyl sulfoxide
EC₅₀	half maximal effective concentration
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase

GCL	granule cell layer
GL	glomerular layer
GYKI 52466	1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride
KS	Kolmogorov-Smirnov
LBD	ligand-binding domain
MEM	Minimal Essential Medium
M/T cells	mitral/tufted cells
NMDA	N-methyl-D aspartate
OB	olfactory bulb
ON layer	olfactory nerve layer
OSN	olfactory sensory neuron
SEM	standard error of mean
S:P ratio	steady-state: peak ratio
SS	steady-state
SW	Shapiro-Wilk
TTX	tetrodotoxin
VGLUT1	vesicular glutamate transporter 1
ZnCl₂	zinc chloride
ZP1	Zinpyr-1

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HIGHLIGHTS

- Zinc has biphasic concentration-dependent effects at most AMPA receptors (AMPA receptors) expressed by olfactory bulb (OB) neurons.
- Cyclothiazide, a drug that blocks AMPAR desensitization, blocked zinc's potentiating but not inhibitory effects at AMPARs.
- The degrees of AMPAR-mediated current potentiation by 100 μ M zinc and AMPAR desensitization were negatively correlated.
- There was no correlation between the degrees of AMPAR-mediated current inhibition by 1 mM zinc and AMPAR desensitization.
- Low (100 μ M) zinc concentrations potentiate AMPARs expressed by rat OB neurons by decreasing receptor desensitization.

Figure 1.

A low concentration of zinc (100 μ M) potentiates the steady-state component of AMPA receptor-mediated currents. **A1)** Application of 50 μ M AMPA evoked a current in all (n=8) cells examined. **A2)** Compared to the control current (AMPA alone), coapplication of 100 μ M zinc with 50 μ M AMPA significantly increased the steady-state (SS) component of the current evoked by AMPA in most cells. **B)** Histogram showing the effects of 100 μ M zinc on different components of the current with sequential application of AMPA (A1) and AMPA +zinc (A2), expressed as a percentage of the control current. The degree of potentiation of the SS current (N=8; *P = 0.005 compared to control) and peak current (N=8; **P > 0.005 compared to control) did not significantly differ (N=16; #P > 0.05). **C)** Coapplication of 100 μ M zinc during the SS portion (middle) of the current evoked by 50 μ M AMPA significantly increased the SS amplitude of the current in most cells examined. **D)** Histogram showing the effects of 100 μ M zinc on the SS current with different types of zinc application, expressed as a percentage of the control current. The degree of current potentiation with sequential application of AMPA and AMPA+zinc (N=8; * P = 0.005 compared to control) versus application of zinc during the SS portion (middle) of the current (N=19; **P = 0.005 compared to control) was similar (N=27; #P > 0.05).

Figure 2.

A high concentration of zinc (1 mM) inhibits the steady-state component of AMPA receptor-mediated currents. **A1)** Application of 50 μ M AMPA evoked a current in all (n=10) cells examined. **A2)** Compared to the control current (AMPA alone), coapplication of 1 mM zinc with 50 μ M AMPA significantly decreased the steady-state (SS) component of the current evoked by AMPA in most cells. **B)** Histogram showing the effects of 1 mM zinc on different components of the current with sequential application of AMPA (A1) and AMPA+zinc (A2), expressed as a percentage of the control current. The degree of inhibition of the SS current (N=10; * $P = 0.005$ compared to control) and peak current (N=10; ** $P > 0.05$ compared to control) significantly differed in the uncorrected analysis (N=20; $P = 0.05$) but not after Holm-Sidak correction for multiple comparisons (N=20; # $P > 0.005$). **C)** Coapplication of 1 mM zinc during the SS portion (middle) of the current evoked by 50 μ M AMPA significantly decreased the SS amplitude of the current evoked by AMPA in most cells examined. **D)** Histogram showing the effects of 1 mM zinc on the SS current with different types of zinc application, expressed as a percentage of the control current. The degree of current inhibition seen with sequential application of AMPA and AMPA+zinc (N=10; * $P = 0.005$ compared to control) versus application of zinc during the SS portion (middle) of the current (N=13; ** $P = 0.005$ compared to control) was similar (N=23; # $P > 0.05$).

Figure 3.

Relationship between the effects of zinc (100 μ M, 1mM) on AMPA receptors (AMPA_Rs) and the degree of AMPAR desensitization (measured as the steady-state to peak [S:P] ratio of the AMPA-evoked current). **A)** In cells with AMPAR-mediated currents potentiated by 100 μ M zinc, there was a significant negative correlation between the degree of current potentiation (expressed as a % of the control current) and the S:P ratio (N=21; Pearson's $r = -0.6435$; $P=0.0016$). **B)** However, there was no significant correlation between the degree of inhibition of AMPAR-mediated currents by 1 mM zinc (expressed as a % of the control current) and the S:P ratio (N=16; Pearson's $r = -0.05413$, $P=0.8422$).

Figure 4.

Coapplication of cyclothiazide (CTZ) blocks the potentiating effects of 100 μ M zinc on AMPA receptor (AMPA)-mediated currents. **A)** Prior to (without) CTZ treatment, coapplication of 100 μ M zinc during the steady-state (SS) portion (middle) of the current evoked by 50 μ M AMPA significantly increased the SS amplitude of the current in 14 cells ($P < 0.005$). **B)** Coapplication of 100 μ M CTZ with 50 μ M AMPA and 100 μ M zinc completely blocked the potentiating effect of zinc on the current in 13 of 14 of these cells. **C)** Prior to (without) CTZ treatment, coapplication of 100 μ M zinc during the SS portion (middle) of the current evoked by 50 μ M AMPA had no effect in a subset of cells (N=4). **D)** In these cells with zinc-insensitive AMPARs, coapplication of 100 μ M CTZ with 50 μ M AMPA and 100 μ M zinc revealed mild underlying inhibition of the current in all 4 cells. **E)** Histogram showing the effects of 100 μ M zinc on the SS component of AMPAR-mediated currents in the 14 cells potentiated by zinc relative to CTZ treatment, expressed as a percentage of the control current. There was a significant difference between the mean pre- (without) CTZ (139.8 $\pm$ 7.2% of control) and post- (with)-CTZ (100.5 $\pm$ 0.5% of control) degrees of current potentiation by zinc (N=14; # $P < 0.005$). **F)** Histogram showing effects of

100 μ M zinc on the SS component of AMPAR-mediated currents in the 4 cells with AMPARs initially insensitive to zinc relative to CTZ treatment, expressed as a percentage of the control current. Whereas mild inhibition (to 91.3 ± 4.6 % of control) of the current was observed post-CTZ treatment, the current amplitude did not significantly differ from the pre-CTZ current amplitude ($N=4$; $\#P > 0.05$).

Figure 5.

Coapplication of cyclothiazide (CTZ) does not significantly affect inhibition of AMPA receptor (AMPA)-mediated currents by 1 mM zinc. **A)** Prior to (without) CTZ treatment, coapplication of 1 mM zinc during the steady-state (SS) portion (middle) of the current evoked by 50 μ M AMPA significantly decreased the SS amplitude of the current in 8 cells ($P = 0.005$). **B)** Inhibition of the AMPAR-mediated current by 1 mM zinc persisted in the presence of 100 μ M CTZ in all cells. **C)** Histogram showing the effects of 1 mM zinc on the SS component of AMPAR-mediated currents relative to time of CTZ treatment, expressed as a percentage of the control current. The mean pre-(without) CTZ and post (with)-CTZ degrees of current inhibition by zinc did not significantly differ ($N=8$; $\#P > 0.05$).

Table 1.

Effects of 3 concentrations of zinc on AMPA receptors before and after application of cyclothiazide

Cell #	Pre-CTZ 30 μ M zinc			Pre-CTZ 100 μ M zinc			Pre-CTZ 1 mM zinc			Post-CTZ 30 μ M zinc			Post-CTZ 100 μ M zinc			Post-CTZ 1 mM zinc		
	+	0	-	+	0	-	+	0	-	+	0	-	+	0	-	+	0	-
1	143			151			109					97			92			54
2	160			146			108				100			100				90
3	163				100				88	104					91			89
4	117			122					68	105				100				70
5		100			100				46			80			77			45
6		100			100			100			100				89			59

Numbers in table represent % of control current. + = potentiation; 0 = no effect; - = inhibition; CTZ = cyclothiazide