Published in final edited form as:

Neuroscience. 2020 January 21; 428: 252–268. doi:10.1016/j.neuroscience.2019.11.041.

Zinc modulates olfactory bulb kainate receptors

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Abstract

Kainate receptors (KARs) are glutamate receptors with ionotropic and metabotropic activity composed of the GluK1-GluK5 subunits. We previously reported that KARs modulate excitatory and inhibitory transmission in the olfactory bulb (OB). Zinc, which is highly concentrated in the OB, also appears to modulate OB synaptic transmission via actions at other ionotropic glutamate receptors (i.e., AMPA, NMDA). However, few reports of effects of zinc on recombinant and/or native KARs exist and none have involved the OB. In the present study, we investigated the effects of exogenously applied zinc on OB KARs expressed by mitral/tufted (M/T) cells. We found that 100 μ M zinc inhibits currents evoked by various combinations of KAR agonists (kainate or SYM 2081) and the AMPA receptor antagonist SYM 2206. The greatest degree of zinc-mediated inhibition was observed with coapplication of zinc with the GluK1- and GluK2- preferring agonist SYM 2081 plus SYM 2206. This finding is consistent with prior reports of zinc's inhibitory effects on some recombinant (homomeric GluK1 and GluK2 and heteromeric GluK2/GluK4 and GluK2/GluK5) KARs, although potentiation of other (GluK3, GluK2/3) KARs has also been described. It is also of potential importance given our previously reported molecular data suggesting that OB neurons express relatively high levels of GluK1 and GluK2. Our present findings suggest that a physiologically relevant concentration of zinc modulates KARs expressed by M/T cells. As M/T cells are targets of zinc-containing olfactory sensory neurons, synaptically released zinc may influence odor information-encoding synaptic circuits in the OB via actions at KARs.

Keywords

glutamate receptors; olfaction; kainate; SYM 2081; SYM 2206

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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.

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DECLARATION OF INTERESTS

None.

INTRODUCTION

Glutamate mediates the majority of excitatory transmission in the mammalian brain, including the olfactory bulb (OB), acting at ionotropic and metabotropic receptors. Ionotropic glutamate receptors consist of three subtypes: N-methyl-D-aspartate receptors (NMDARs), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors), and kainate receptors (KARs). KARs are tetramers composed of the GluK1-5 glutamate receptor subunits, which were initially referred to as GluR5, GluR6, GluR7, KA1, and KA2. In 2009, new nomenclature re-named GluR5-7 as GluK1-3 and KA1-2 as GluK4-5 (Collingridge et al., 2009), and this new nomenclature is used here (replacing older subunit nomenclature used in the original literature).

KARs are broadly distributed in the central nervous system (CNS), where their primary role is to modulate synaptic transmission (Contractor et al., 2011; Rodrigues and Lerma, 2012; Lerma and Marques, 2013; Sihra and Rodriguez-Moreno, 2013; Evans et al., 2019). In general, presynaptic KARs regulate transmitter release, while postsynaptic KARs mediate synaptic transmission and regulate neuronal excitability (Rodrigues and Lerma, 2012; Lerma and Marques, 2013; Sihra and Rodriguez-Moreno, 2013; Evans et al., 2019). KARs differ from the other ionotropic glutamate receptors, because while some modulation of synaptic transmission by KARs occurs via traditional ionotropic receptor activity, other modulation involves metabotropic (G protein-coupled)/non-canonical signaling (Rodriguez-Moreno and Sihra, 2007; Rodrigues and Lerma, 2012; Lerma and Marques, 2013; Negrete-Diaz et al., 2018).

Until relatively recently, little was known about the distribution and function of KARs in the OB. A 2003 study using AMPAR/KAR antagonists revealed that KAR-mediated currents contribute to mitral cell somatodendritic excitation (Lowe, 2003). A 2006 study employing patterned stimulation of the olfactory nerve suggests that KARs help mediate synaptic events evoked in granule cells with a dynamic stimulus (Schoppa, 2006a). We subsequently showed that KAR activation increases the frequency of excitatory spontaneous activity in OB interneurons, possibly by depolarizing mitral/tufted (M/T) cells closer to their threshold for spiking and enhancing glutamate release (Davila et al., 2007). Recently, we used combinations of KAR agonists and AMPAR antagonists to explore the distribution and circuit functions of KARs in the OB (Blakemore et al., 2018). Our findings suggest that a variety of OB neuron subtypes express functional KARs, which modulate excitatory and inhibitory transmission.

Zinc is a trace element that is highly concentrated in the OB (Donaldson et al., 1973; Gulya et al., 1991; Ono and Cherian, 1999), which also appears to modulate synaptic transmission (Bitanhirwe and Cunningham, 2009; Nakashima and Dyck, 2009; Paoletti et al., 2009; McAllister and Dyck, 2017; Blakemore and Trombley, 2017). Some brain zinc (15%) is contained in synaptic vesicles (Frederickson, 1989). Most of this vesicular zinc is co-localized with glutamate in presynaptic terminals of subsets of glutamatergic zinc-enriched neurons (Haug, 1967; Crawford and Connor, 1973; Frederickson et al., 1983; Perez-Clausell and Danscher, 1985; Frederickson, 1989; Beaulieu et al., 1992) from where it is thought to be released with neuronal activity (Assaf and Chung, 1984; Howell et al., 1984; Li et al.,

2001; Blakemore et al., 2013). While the amount of vesicular zinc released following neuronal depolarization is unclear, peak synaptic cleft zinc concentrations appear to range from 10 nM to >100 μ M (Howell et al., 1984; Assaf and Chung, 1984; Vogt et al., 2000; Li et al., 2001; Ueno et al., 2002; Frederickson et al., 2006; Paoletti et al., 2009; Vergnano et al., 2014; Goldberg et al., 2016; Blakemore and Trombley, 2017).

In regard to the distribution of vesicular zinc, results in fixed tissue suggest that zinc-enriched terminals in the (mouse) OB are from two main sources: (1) olfactory sensory neuron (OSN) terminals synapsing with dendrites of M/T cells and periglomerular (PG) cells and (2) centrifugal fibers synapsing with granule cells (Jo et al., 2000). We recently extended these findings by demonstrating high-intensity fluorescence with a fluorescent zinc probe Zinpyr-1 (ZP1) (Burdette et al., 2001), indicating the presence of vesicular zinc, in the glomerular layer (GL) and granule cell layer (GCL) of living rat OB slices (Blakemore et al., 2013). We also showed that patterned (sniff frequency) stimulation of the olfactory nerve causes OSN terminals to release vesicular zinc (Blakemore et al., 2013), providing further evidence that zinc is released in response to neuronal activity, and thus, is available to modulate synaptic transmission in the OB.

Modulation of amino acid receptors (e.g., AMPA, NMDA, GABA, glycine) and synaptic transmission by zinc is well described (Smart et al., 1994; Bitanhirwe and Cunningham, 2009; Nakashima and Dyck, 2009; Paoletti et al., 2009; McAllister and Dyck, 2017; Blakemore and Trombley, 2017), including that demonstrated by us in the OB (Trombley and Shepherd, 1996; Trombley et al., 1998; Blakemore and Trombley, 2004; Trombley et al., 2011; Blakemore et al., 2013; Blakemore and Trombley, 2019). However, relatively little is known about effects of zinc on isolated KARs. Initial studies showed that zinc inhibited some recombinant KARs (Hoo et al., 1994; Fukushima et al., 2003). Mott et al. (2008) extended these observations by reporting that exogenous and/or endogenous zinc inhibited native (hippocampal) KARs in addition to recombinant (expressed in *Xenopus* oocytes) KARs (Mott et al., 2008). Veran et al. (2012) subsequently showed that zinc inhibited some combinations of KAR subunits in recombinant (HEK293 cells) receptors but potentiated others (Veran et al., 2012). As the OB contains high zinc concentrations, expresses functionally diverse KARs, and has a highly defined function (odor-information processing), the OB represents an ideal structure to investigate the modulatory effects of zinc on KARs. In the present study, we built on our recent investigations of OB KARs (Blakemore et al., 2018) and zinc modulation of OB AMPARs (Blakemore and Trombley, 2019) by investigating modulation of OB KARs by physiologically relevant concentrations of zinc (30 and 100 μ M).

Data from recombinant receptors suggest that zinc modulates KARs in a subunit-dependent manner (Hoo et al., 1994; Fukushima et al., 2003; Mott et al., 2008; Veran et al., 2012), which has potential implications for zinc modulation of OB KARs. Studies employing in situ hybridization (ISH) (Gall et al., 1990; Davila et al., 2007), autoradiography (Nadi et al., 1980; Bailey et al., 2001), immunocytochemistry (ICC) (Petrulia et al., 1994; Montague and Greer, 1999; Davila et al., 2007), and semiquantitative reverse transcription polymerase chain reaction (RT-PCR) (Davila et al., 2007) suggest that the OB expresses all KARs subunits but in a heterogeneous manner. Collectively, these results suggest that M/T cells,

the bulb's principal neurons that form glutamatergic synapses with OSNs (Berkowicz et al., 1994; Ennis et al., 1996), express a diverse array of KAR subunits localized to their cell bodies and/or dendrites. Given this, combined with evidence that M/T cells express functional KARs (Blakemore et al., 2018) and OSNs release zinc (Blakemore et al., 2013), we focused on the effects of zinc on KARs expressed by M/T cells.

Early data showed that zinc inhibited recombinant GluK2 and GluK2/GluK5 KARs (Hoo et al., 1994; Fukushima et al., 2003). It was subsequently reported that, while zinc inhibited all tested subunit combinations of recombinant KARs, it inhibited GluK4- and GluK5-containing heteromeric KARs with a greater affinity than GluK1 and GluK2 homomeric KARs (Mott et al., 2008). This is potentially significant given GluK4 and GluK5 combine with GluK1–GluK3 subunits to form functional receptors (Lerma, 2006; Pinheiro and Mulle, 2006; Lerma and Marques, 2013; Carta et al., 2014) and it is unclear if native homomeric KARs exist (Carta et al., 2014). In another study, zinc inhibited recombinant GluK2 receptors but potentiated GluK3 and GluK2/GluK3 receptors (Veran et al., 2012). We, therefore, applied various combinations of KAR agonists and an AMPAR antagonist to evoke currents mediated by KARs composed of a variety of subunits and tested the effects of exogenously applied zinc.

We first used an agonist (kainate) with demonstrated effects at recombinant and native KARs composed of a wide array of KAR subunits (Huettner, 1990; Sommer et al., 1992; Lerma et al., 1993; Schiffer et al., 1997; Jones et al., 1997; Sahara et al., 1997; Wilding and Huettner, 1997; Lerma et al., 2001; Alt et al., 2004). The concentration of kainate (100 μ M) used was based on reported half maximal effective concentrations (EC50s) for kainate at these KARs. As in our previous study (Blakemore et al., 2018), 100 μ M kainate evoked currents in all cells examined. While kainate is many-fold more selective for KARs than AMPARs (Huettner, 1990; Lerma et al., 1993; Wilding and Huettner, 1996; Clarke et al., 1997; Lerma et al., 2001), even relatively low kainate concentrations have effects on AMPARs (Lerma et al., 2001). Therefore, to ensure we were testing the effects of zinc on currents mediated by KARs, we coapplied zinc and kainate with the AMPAR antagonist 4-(4-Aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxypythalazine (SYM 2206) at a concentration (100 μ M) that effectively blocks AMPARs (Pelletier et al., 1996; Li et al., 1999; Rodriguez-Moreno et al., 2000; Blakemore et al., 2018).

Our previous RT-PCR data showed relatively high levels of GluK1 and GluK2 in the OB, and our ISH data suggested that M/T cells express GluK1 and GluK5 (Davila et al., 2007). Therefore, we also tested the effects of zinc (30 μ M, 100 μ M) on currents evoked by application of 10 μ M (2S,4R) 4-methylglutamic acid (SYM 2081), a powerful agonist of homomeric GluK1 and GluK2 KARs (Donevan et al., 1998; Small et al., 1998). SYM 2081 is 500- to 2000-fold more selective for homomeric GluK1 and GluK2 KARs than AMPARs (Donevan et al., 1998; Small et al., 1998). However, to ensure we were isolating the effects of zinc on KARs, we coapplied 100 μ M zinc and SYM 2081 with SYM 2206 in another set of experiments.

We found that 100 μ M zinc inhibited KAR-mediated currents evoked in M/T cells, with significant degrees of inhibition for all KAR agonist/AMPA antagonist combinations tested. As KARs modulate both excitatory and inhibitory transmission in the OB (Blakemore et al., 2018) and M/T cells are targets of zinc-containing axon terminals (Jo et al., 2000; Blakemore et al., 2013), synaptically released zinc may influence odor information-encoding synaptic circuits in the OB via actions at KARs.

EXPERIMENTAL PROCEDURES

Animals

All experiments were carried out in accordance with the current edition (8th) of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Florida State University Institutional Animal Care and Use Committee approved all procedures. The total number of animals (Sprague–Dawley P0–P4 rat pups) used was 48 (6 rats per set of OB cultures).

Tissue Culture

Primary cultures of OB neurons were prepared using previously described methods (Trombley and Blakemore, 1999; Blakemore et al., 2018; Blakemore and Trombley, 2019). Briefly, the OBs were dissected from male and female Sprague-Dawley rats (Charles River, Wilmington, MA, USA), aged 0–4 postnatal days (P0 to P4). After removal of their meninges, OBs were cut into small pieces and incubated in a calcium–buffered papain solution (Worthington Biochemical, Lakewood, NJ, USA) for 1 hr at 37° C. The tissue was then dissociated into a single-cell suspension via gentle trituration with a glass pipette, and the cells were plated at varying densities (250,000–350,000 cells/dish) onto a confluent layer of OB astrocytes in 35-mm culture dishes. To prevent overgrowth of glial cells, cytosine- β -D-arabinofuranoside (10^{-5} M) was added to culture dishes on the day after plating. Neurons were fed and maintained in previously described neuronal growth media.

Neuronal Identity

All experiments were performed in presumptive M/T cells. Mitral and tufted cells are the bulb's two types of glutamatergic projection neurons, which receive synaptic input from OSN axons in glomeruli of the GL. Mitral and tufted cells make reciprocal synapses with dendrites of OB interneurons including PG cells in the GL and granule cells in the external plexiform layer (EPL). Multiple subtypes of projection neurons and interneurons exist (Nagayama et al., 2014).

M/T cells and interneurons in primary OB cultures were identified and distinguished from one another using our previously described morphological criteria (Trombley and Westbrook, 1990), which are consistent with our recently described immunocytochemical/cell marker criteria (i.e., VGLUT1, GAD) (Blakemore et al., 2018). M/T cells were identified and differentiated from the more abundant small-diameter (5–10 μ m) interneurons based on M/T cells' larger soma size (20 to 40 μ m) and shape (pyramidal, multipolar). As there are multiple types of tufted cells, the term “M/T cells” is used here to refer to mitral cells and the largest (internal) tufted cells (>20 μ m) (Nagayama et al., 2014).

Electrophysiology

Whole-cell patch-clamp recordings were obtained in cultured OB neurons using previously described methods (Trombley et al., 1999; Blakemore et al., 2006; Blakemore et al., 2018; Blakemore and Trombley, 2019). Briefly, whole-cell voltage-clamp recordings were obtained at room temperature from M/T cells in culture for 4 to 10 days. The 35-mm dish used to culture the neurons was also used as the recording chamber and 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 osmolality 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 KMeSO₄, 1 MgCl₂, 10 HEPES, 4 Mg²⁺-ATP, 0.5 Mg²⁺-GTP, and 1.1 EGTA (pH 7.2; 310 mosmol/l).

After being diluted in recording solution, drugs were applied to cells using a gravity-fed flow-pipe perfusion system. An electronic manipulator (Warner Instruments; Hamden, CT, USA) was used to position flow pipes made from small (600-μm diameter) square glass barrels in close proximity to the cell, and pinch clamps were used to control drug flow. The rapid speed of solution changes allowed peak drug responses to occur within 100 ms. Except during drug application, cells were continuously perfused with a barrel containing bath solution. The applied drugs were ZnCl₂ (30 μM, 100 μM; referred to here as “zinc”)(Sigma-Aldrich, St. Louis, MO, USA), kainate (100 μM)(Tocris, Minneapolis, MN, USA), SYM 2206 (100 μM)(Tocris), SYM 2081 (10 μM)(Tocris), and tetrodotoxin (TTX; 1 μM)(Sigma-Aldrich).

Procedures

To investigate membrane currents evoked by various combinations of KAR agonists and AMPAR antagonist, 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 KARs were evaluated by applying ZnCl₂ (30 μM, 100 μM) during currents evoked by KAR agonist/AMPA antagonist combinations (referred to here as “drugs”). This involved either coapplication of zinc in the middle (steady-state portion) of the evoked current or sequential application of drugs and zinc (i.e., application of drug(s) alone followed by separate coapplication of drug(s) + zinc). Although only zinc-mediated inhibition of currents was observed in the present study, zinc-mediated potentiation would be assessed using the same formulas (Blakemore and Trombley, 2004; Blakemore and Trombley, 2019). The degree of current inhibition or potentiation by zinc was measured and expressed as a percentage of the control (drug(s)-alone) current. For application of zinc in the middle (steady-state portion) of a drug-evoked current, the following formula was used to determine the degree of current inhibition or potentiation by zinc: (amplitude of drug(s) + Zinc portion of current / steady-state amplitude of drug(s)-alone portion of current) x 100. For sequential application of zinc, the following formula was used to determine the effect of

zinc on the steady-state or peak component of the current: ([steady-state or peak] amplitude of drug(s) + Zinc current / [steady-state or peak] amplitude of drug(s)-alone current) x 100.

Data Analysis

All statistical analyses were performed with Prism 8 for MacOS (version 8.2.1; GraphPad Software Inc., La Jolla, CA, USA). For data compared with t-tests, the Kolmogorov-Smirnov (KS), Shapiro-Wilk (SW), and D'Agostino-Pearson omnibus (K2) normality tests were used to determine whether residuals followed a normal distribution, and homogeneity of variance was assessed with the F test. All of these data were normally distributed and had equal variances. To determine whether zinc significantly inhibited the KAR-mediated current, the paired t-test was used to compare the mean (steady-state or peak) current before application of zinc (drug(s) alone) with the mean current following zinc application (drug(s) + zinc), measured in pA, in the same cell. The unpaired t-test was used to determine whether the degree of current inhibition by zinc differed based on the current portion measured (steady-state vs. peak) or type of zinc application (coapplication in the middle of the current vs. sequential application). Values of data analyzed with t-tests are expressed as the mean $\pm$ SEM, with differences between means reported as $t(df)=x.xx$, $P < 0.0x$. One-way ANOVA was used to compare (inhibitory) effects of 100 μ M zinc on KAR-mediated currents activated by the three KAR agonist/AMPA antagonist combinations. As the equal variance assumption for ordinary one-way ANOVA was not met, Brown-Forsythe and Welch ANOVA tests were used. Based on sample size, Dunnett's T3 post hoc test was used after ANOVA to compare means between groups and determine P values adjusted for multiple comparisons. P values < 0.05 were considered significant for this analysis.

RESULTS

Use of kainate as an agonist with SYM 2206

To investigate zinc's effects on KARs expressed by OB neurons, we first coapplied 100 μ M zinc with 100 μ M kainate and 100 μ M of the AMPA antagonist SYM 2206. This concentration of kainate was based on our previous finding that 100 μ M kainate evoked currents in all OB neurons examined (Blakemore et al., 2018) as well as previously reported application concentrations and EC50s. For example, EC50s for kainate at recombinant homomeric GluK1 and GluK2 receptors and heteromeric GluK1/2, GluK1/5, and GluK2/5 receptors range from 1.8 to 33.6 μ M (Sommer et al., 1992; Jones et al., 1997; Lerma et al., 2001; Alt et al., 2004). Kainate has less potent agonist effects at recombinant GluK3, GluK3/4, and GluK3/5 receptors (Schiffer et al., 1997). EC50s for kainate at native (dorsal root ganglion cells, trigeminal ganglion cells, hippocampal neurons) KARs range from 12–23 μ M (Huettnner, 1990; Lerma et al., 1993; Sahara et al., 1997; Wilding and Huettnner, 1997; Lerma et al., 2001).

EC50 values suggest that kainate has a 5- to 30-fold higher affinity for KARs than AMPARs (Huettnner, 1990; Lerma et al., 1993; Wilding and Huettnner, 1996; Clarke et al., 1997; Lerma et al., 2001). However, relatively low concentrations of kainate still can activate AMPARs (Lerma et al., 2001). Therefore, to ensure we were testing the effects of zinc on KARs, we coapplied zinc and kainate with 100 μ M SYM 2206. SYM 2206 is a relatively selective

antagonist of AMPARs (Pelletier et al., 1996; Li et al., 1999; Rodriguez-Moreno et al., 2000). In spinal cord, SYM 2206 (100 μ M) and AP5 (100 μ M) were used to block AMPARs and NMDARs, respectively, thus isolate the KAR-mediated EPSC component (Li et al., 1999). As this concentration (100 μ M) of SYM 2206 is thought to maximally inhibit AMPARs ($IC_{50} \sim 1$ -2 μ M) (Rodriguez-Moreno et al., 2000; Wilding and Huettner, 2001), we used the same concentration here. Consistent with the effective blockade of AMPARs, Figure 1A shows that coapplication of 100 μ M SYM 2206 blocked the large AMPAR-mediated portion of the current evoked by 100 μ M kainate and revealed the relatively small residual KAR-mediated component of the current (Fig. 1A).

We first investigated zinc's effects on KAR-mediated currents evoked by coapplication of kainate and SYM 2206. After pretreatment with 100 μ M SYM 2206, coapplication of 100 μ M kainate and 100 μ M SYM 2206 evoked currents in all cells examined ($N=9$), with a mean steady-state current amplitude of 30.7 ± 4.8 pA and a mean peak current amplitude of 34.0 ± 5.0 pA (Fig. 1B). Coapplication of 100 μ M zinc during the steady-state portion (middle) of the current evoked by 100 μ M kainate and 100 μ M SYM 2206 significantly decreased the steady-state amplitude of the current from 30.7 ± 4.8 pA to 11.5 ± 3.6 pA (to $36.0 \pm 9.7\%$ of control) ($N=9$; paired t-test: $t(8)=4.578$, $P < 0.01$) (Fig. 1C, D), with recovery of the current following a wash to $93.7 \pm 2.0\%$ of control ($N=9$; paired t-test: $t(8)=4.352$, $P < 0.01$) (Fig. 1D). Thus, zinc blocked $64.0 \pm 9.7\%$ of the KAR-mediated current, with a range of 12.9 to 100%.

Use of SYM 2081 as a KAR-subunit preferring agonist

As zinc-mediated inhibition of recombinant KARs varies with subunit composition, we also wanted to determine whether effects of zinc are influenced by OB KAR subunit composition. Our prior RT-PCR data suggest that GluK1 and GluK2 are highly expressed in the OB, and our previous ISH data suggest that M/T cells and interneurons mostly express GluK1, GluK2, and GluK5 (Davila et al., 2007). This is consistent with evidence that, while GluK2 and GluK1 are mainly expressed by principal cells and interneurons, respectively, in the hippocampus and cortex, GluK5 is more widely distributed throughout the brain (Lerma and Marques, 2013). Therefore, we tested the effects of zinc on currents in OB neurons (M/T cells) evoked by application of SYM 2081, a powerful GluK1 and GluK2 preferring agonist (Donevan et al., 1998; Small et al., 1998).

Interestingly, Mott et al. (2008) found that 3 μ M SYM 2081 substantially reduced slow (KAR-mediated) spontaneous mEPSCs isolated by coapplication of the AMPAR antagonist GYKI 52466 (Mott et al., 2008). This is consistent with the finding that 1 and 5 μ M SYM 2081 reduced or abolished residual KAR-mediated EPSCs isolated in spinal cord neurons with coapplication of AP5 and SYM 2206 (Li et al., 1999). In GluK2-expressing human embryonic kidney 293 (HEK293) cells, while higher SYM 2081 concentrations ($EC_{50} = 1.0 \pm 0.1$ μ M) evoked kainate-like, rapidly desensitizing inward currents, lower SYM 2081 concentrations reversibly blocked rapidly desensitizing kainate-evoked currents (Zhou et al., 1997). Thus, in addition to acting as an agonist, SYM 2081 has an antagonist-like effect at low concentrations that limits transmission at KARs by causing brief activation followed by

sustained desensitization (Zhou et al., 1997; Donevan et al., 1998; Li et al., 1999; Lerma et al., 2001).

In GluK2-expressing HEK293 cells, 1-100 μM SYM 2081 evoked inward desensitizing currents with an EC_{50} of $1.05 \pm 0.11 \mu\text{M}$ (Jones et al., 1997). However, amplitudes of peak currents evoked by application of 4 μM and 16 μM SYM 2081 to dorsal root ganglion (DRG) cells were only $20 \pm 1\%$ and $45 \pm 6\%$, respectively, of those evoked by 300 μM kainate (Jones et al., 1997). In our previous study of OB KARs, 10 μM SYM 2081 evoked currents in all cells examined ($N=41$), whereas a lower concentration of SYM 2081 (3 μM) failed to elicit a current in 5 of 19 cells (Blakemore et al., 2018). Here, our goal was to choose a concentration of SYM 2018 that would effectively function as an agonist at KARs with limited effects at AMPARs. Therefore, we tested the effects of zinc (30 and 100 μM) on currents evoked in OB neurons by 10 μM SYM 2081.

Application of 10 μM SYM 2081 evoked currents in all cells examined ($N=10$), with a mean steady-state current amplitude of $12.4 \pm 1.7 \text{ pA}$ and a mean peak current amplitude of $18.6 \pm 2.2 \text{ pA}$ (Fig. 2A). Coapplication of 100 μM zinc during the steady-state portion (middle) of the current evoked by 10 μM SYM 2081 significantly decreased the steady-state amplitude of the current from $12.4 \pm 1.7 \text{ pA}$ to $1.7 \pm 0.6 \text{ pA}$ (to $13.4 \pm 4.2\%$ of control)($N=10$; paired t-test: $t(9)=6.773$, $P < 0.01$) (Fig. 2B, C), with recovery of the current following a wash to $92.8 \pm 1.7\%$ of control ($N=10$; paired t-test: $t(9)=6.726$; $P < 0.01$)(Fig. 2C). Thus, zinc blocked $86.6 \pm 4.2\%$ of the KAR-mediated current, with a range of 67.3 to 100%. A lower concentration of zinc (30 μM) had qualitatively similar but quantitatively different effects. Coapplication of 30 μM zinc decreased the steady-state portion of the current evoked by 10 μM SYM 2081 to $39.9 \pm 5.8\%$ of control ($N=5$; paired t-test: $t(4)=2.945$, $P < 0.05$), representing a $60.1 \pm 5.8\%$ (range: 45.5–77.8%) block of the current (Fig. 2D). The degree of current inhibition with application of 100 μM zinc ($13.4 \pm 4.2\%$ of control; $N=10$) was significantly greater than that with 30 μM zinc ($39.9 \pm 5.8\%$ of control; $N=5$)($N=15$; unpaired t-test: $t(13)=3.676$; $P < 0.01$)(Fig. 2E).

In these 10 cells, we also examined the effects of 100 μM zinc on KAR-mediated currents evoked by sequential application of 10 μM SYM 2081 followed by 10 μM SYM 2081 plus 100 μM zinc. This allowed us to determine zinc's effects on peak currents in a subset of cells as well as compare zinc-mediated modulation of the steady-state portion of the current induced by two application methods. Application of 10 μM SYM 2081 evoked a current in all 10 cells, with a steady-state amplitude of $13.3 \pm 1.4 \text{ pA}$ and a peak current amplitude of $19.1 \pm 1.7 \text{ pA}$ (Fig. 3A). Subsequent coapplication of 100 μM zinc with 10 μM SYM 2081 significantly decreased the steady-state and peak portions of the current in all 10 cells to $2.3 \pm 0.8 \text{ pA}$ (to $17.5 \pm 4.9\%$ of control)($N=10$; paired t-test: $t(9)=8.700$, $P < 0.01$) and $4.4 \pm 1.1 \text{ pA}$ (to $20.7 \pm 4.8\%$ of control)($N=10$; paired t-test: $t(9)=12.53$, $P < 0.01$), respectively (Fig. 3B, C). The degrees of zinc-mediated inhibition of the steady-state ($17.5 \pm 4.9\%$ of control) and peak ($20.7 \pm 4.7\%$ of control) portions of the current did not significantly differ ($N=20$; unpaired t-test: $t(18)=0.4698$; $P = 0.6442$)(Fig. 3C). The degrees of zinc-mediated inhibition of the steady-state portion of the current with sequential application of SYM 2081 followed by SYM 2081 plus 100 μM zinc ($17.5 \pm 4.9\%$ of control; $N=10$) compared with coapplication of 100 μM zinc during the middle of the current evoked by 10 μM SYM 2081 ($13.4 \pm 4.2\%$

of control; N=10) were also similar (N=20; unpaired t-test: $t(18)=0.6319$; $P = 0.5354$)(Fig. 3D).

Use of SYM 2081 with SYM 2206

SYM 2081 has been shown to be three orders of magnitude more selective for KARs than AMPARs in binding and functional assays (Gu et al., 1995; Lerma et al., 2001). In *Xenopus* oocytes, EC₅₀s for SYM 2081 at homomeric GluK1 and GluK2 KARs were 0.12 ± 0.02 and 0.23 ± 0.01 μ M, respectively, compared with 132 ± 44 μ M and 453 ± 57 μ M at GluA1 and GluA3 AMPARs, respectively (Donevan et al., 1998). In rat neocortical neurons, high SYM 2081 concentrations (30-3000 μ M) induced nondesensitizing currents with waveforms resembling those induced by application of kainate (Zhou et al., 1997). However, a lower SYM 2081 concentration (3 μ M) evoked kainate-like, rapidly desensitizing, currents (Zhou et al., 1997). SYM 2081 was almost 5-fold less potent than kainate (EC₅₀ of 325 ± 23 vs. 70 ± 6 μ M, respectively) in activating AMPARs in these neurons (Zhou et al., 1997). Thus, the SYM 2081 concentration used here (10 μ M) was unlikely to have had significant effects at AMPARs. To ensure that zinc was not having effects on a portion of the current mediated by AMPARs, we coapplied 10 μ M SYM 2081 with 100 μ M SYM 2206 in another set of experiments. This concentration of SYM 2206 is thought to maximally inhibit AMPARs (IC₅₀ ~1-2 μ M) (Rodriguez-Moreno et al., 2000; Wilding and Huettner, 2001). As 100 μ M SYM 2206 has also been reported to inhibit KARs by 15–30% (Pelletier et al., 1996; Li et al., 1999; Lerma et al., 2001; Wilding and Huettner, 2001), coapplication of SYM 2206 with SYM 2081 may have reduced the size of KAR-mediated currents relative to those evoked by SYM 2081 alone.

After pretreatment with 100 μ M SYM 2206, coapplication of 10 μ M SYM 2081 and 100 μ M SYM 2206 evoked currents in all cells examined (N=8), with a mean steady-state current amplitude of 7.3 ± 1.4 pA and a mean peak current amplitude of 11.8 ± 1.5 pA (Fig. 4A). Coapplication of 100 μ M zinc during the steady-state portion (middle) of the current evoked by 10 μ M SYM 2081 and 100 μ M SYM 2206 significantly decreased the steady-state amplitude of the current from 7.3 ± 1.4 pA to 0.32 ± 0.14 pA (to $5.6 \pm 1.9\%$ of control)(N=8; paired t-test: $t(7)=4.923$, $P < 0.01$) (Fig. 4B, C), with recovery of the current following a wash to $80.8 \pm 2.9\%$ of control (N=8; paired t-test: $t(7)=4.521$; $P < 0.01$)(Fig. 4C). Thus, zinc blocked $94.4 \pm 1.9\%$ of the KAR-mediated current, with a range of 84.8-100%.

Comparison of degrees of zinc-mediated inhibition between groups

We used one-way ANOVA to evaluate differences in the effects of 100 μ M zinc on the steady-state currents evoked by the various KAR agonist/AMPA antagonist combinations tested. As the equal variance assumption was not met, we used the Brown-Forsythe and Welch ANOVA tests followed by the Dunnett's T3 multiple comparison post hoc test to compare mean degrees of zinc-mediated current inhibition (expressed as mean $\pm$ SEM % of the control current) for three groups: (A) SYM 2081 plus SYM 2206 ($5.6 \pm 1.9\%$ of control), (B) SYM 2081 alone ($13.4 \pm 4.2\%$ of control), and (C) kainate plus SYM 2206 ($36.0 \pm 9.7\%$ of control). Both the Brown-Forsythe and Welch ANOVA tests revealed a significant difference between groups (Brown-Forsythe ANOVA: $n=27$, $F [2.0, 11.74] = 6.197$; $P=0.0146$; Welch ANOVA: $n=27$, $F [2.0, 13.4] = 5.455$; $P=0.0185$). Dunnett's T3

multiple comparisons test showed that the mean degree of current inhibition in the SYM 2081 plus SYM 2206 group (group A) was significantly greater than that in the kainate plus SYM 2206 group (group C) (adjusted P value = 0.0379)(Fig. 5). There were no significant differences in mean values between groups A and B (adjusted P value = 0.2912) and groups B and C (adjusted P value = 0.1521).

DISCUSSION

Summary of findings

We found that 100 μ M zinc significantly inhibited KAR-mediated currents evoked by all tested combinations of KAR agonists and the AMPAR antagonist in M/T cells. The greatest degree of zinc-mediated inhibition ($5.6 \pm 1.9\%$ of control) was observed with coapplication of 100 μ M zinc with 10 μ M SYM 2081 plus 100 μ M SYM 2206, which was significantly greater than the degree of inhibition ($36.0 \pm 9.7\%$ of control) observed with coapplication of 100 μ M zinc with 100 μ M kainate plus 100 μ M SYM 2206. This finding may reflect differences in the effects of zinc on OB KARs containing the GluK1 and GluK2 subunits (activated with coapplication of SYM 2081 and SYM 2206) versus OB KARs containing a broader distribution of KAR subunits (activated with coapplication of kainate and SYM 2206), as further discussed in the next section (see “Zinc has subunit-specific effects at KARs”). The greater degree of zinc-mediated inhibition of currents evoked by SYM 2081 plus SYM 2206 is also of potential importance given our prior molecular data suggesting that OB neurons express relatively high levels of GluK1 and GluK2. Together, our findings suggest that a physiologically relevant concentration of zinc modulates KARs expressed by M/T cells in the OB. As KARs participate in OB synaptic transmission (Blakemore et al., 2018) and mitral cells are targets of zinc-containing axon terminals (Jo et al., 2000; Blakemore et al., 2013), synaptically released zinc may influence odor-encoding synaptic circuits via actions at KARs.

Zinc has subunit-specific effects at KARs

Our findings that 100 μ M zinc inhibited KAR-mediated currents evoked by KAR agonist/AMPA antagonist combinations to varying degrees are consistent with previous findings in recombinant KARs (Hoo et al., 1994; Fukushima et al., 2003; Mott et al., 2008; Veran et al., 2012) and native (hippocampal) KARs (Mott et al., 2008).

The initial investigations of effects of zinc at recombinant KARs showed that micromolar concentrations of zinc inhibited recombinant GluK2 and GluK2/GluK5 receptors (Hoo et al., 1994; Fukushima et al., 2003). Fukushima et al. (2003) examined the effects of 0 – 300 μ M zinc on currents induced by 100 μ M willardiine in HEK 293 cells co-expressing GluK2/GluK5 KARs (Fukushima et al., 2003). As RNA editing of the GluK2 subunit, which converts the glutamine (Q) residue to arginine (R), regulates ion permeability (Sommer et al., 1991; Burnashev et al., 1995), they investigated effects of zinc at both GluK2(Q)/GluK5 and GluK2(R)/GluK5 KARs (Fukushima et al., 2003). Zinc similarly inhibited both types of heteromeric receptors, with IC₅₀ values of 6.8 ± 2.5 μ M for GluK2(Q)/GluK5 and 6.4 ± 2.5 μ M for GluK2(R)/GluK5 KARs.

Mott et al. (2008) tested the effects of zinc on steady-state currents evoked in recombinant KARs expressed in *Xenopus* oocytes (Mott et al., 2008). Agonists varied with KAR subunit combination and included AMPA (30–300 μ M), 5-iodowillardiine, domoate (3–10 μ M), and kainate (30 μ M). Zinc (nM–mM range) inhibited currents mediated by all tested KAR subunit combinations, which was generally complete at the highest zinc concentrations used. An exception was incomplete inhibition of GluK2R/GluK4 receptors, which only reached a maximum of $67 \pm 4\%$ at 1 mM zinc.

Zinc inhibited both GluK1 and GluK2 homomeric KARs (Mott et al., 2008). Consistent with previous findings in heteromeric GluK2-containing KARs (Fukushima et al., 2003), RNA editing of the GluK2 subunit (i.e., GluK2R) did not significantly affect zinc inhibition (Mott et al., 2008). The IC_{50} for zinc inhibition of GluK2R homomeric receptors was $67 \pm 8 \mu$ M. While it is unclear if native KARs can exist as homomers (Carta et al., 2014), this IC_{50} is consistent with our finding that 100 μ M zinc inhibited currents activated by application of a GluK1- and GluK2-preferring agonist (SYM 2081) to M/T cells.

Interestingly, Mott et al. (2008) found that zinc inhibited GluK4- and GluK5-containing heteromeric KARs with a much greater affinity than GluK1 and GluK2 homomeric KARs (Mott et al., 2008). The IC_{50} for zinc inhibition of GluK2R/GluK4 receptors ($1.5 \pm 0.6 \mu$ M) was more than 40-fold less than that for GluK2R homomeric receptors ($67 \pm 8 \mu$ M) and was similar to the low micromolar IC_{50} s previously reported for GluK2/GluK5 receptors (Fukushima et al., 2003). Given the incomplete zinc inhibition of GluK2R/GluK4 receptors, the maximal inhibition of currents mediated by these receptors was less than that for GluK2R/GluK5 receptors (Mott et al., 2008). As GluK4 and GluK5 combine with the GluK1–GluK3 subunits to form functional KARs with varying agonist affinities (Lerma, 2006; Pinheiro and Mulle, 2006; Perrais et al., 2010; Lerma and Marques, 2013; Carta et al., 2014), the different degrees of zinc-mediated inhibition of native OB KARs we observed with various combinations of KAR agonists and AMPAR antagonists may partially reflect variability in GluK4 and GluK5 content.

The IC_{50} s for zinc inhibition of GluK4- and GluK5-containing heteromeric KARs and NR1a/NR2B NMDARs ($2.1 \pm 0.7 \mu$ M) were comparable (Mott et al., 2008). However, GluA3 and GluA2/GluA3 AMPARs had higher IC_{50} s for zinc inhibition ($> 100 \mu$ M) than any of the tested KAR subunit compositions. This is consistent with our previous reports that, while 100 μ M zinc inhibits AMPARs expressed by a subset of OB neurons, this concentration of zinc largely has potentiating effects at OB AMPARs while higher concentrations of zinc (e.g., 1 mM) inhibit AMPARs (Blakemore and Trombley, 2004; Blakemore and Trombley, 2019).

Veran et al. (2012) applied zinc and glutamate to HEK293 cells transfected with cDNA for various KAR subunits (Veran et al., 2012). In this manner, they investigated the effects of zinc on currents mediated by recombinant GluK2, GluK3, and GluK2/3 receptors. Consistent with findings in *Xenopus* oocytes (Mott et al., 2008), zinc inhibited GluK2-mediated currents evoked by 10 mM glutamate at all tested concentrations of zinc (1–1000 μ M), with an IC_{50} of $102 \pm 11 \mu$ M (Veran et al., 2012). Zinc (100 μ M) also inhibited GluK2-mediated currents ($48\% \pm 10\%$ of control) evoked by a concentration of glutamate

(500 μ M) below the EC₅₀ for GluK2. These findings are consistent with the inhibition of currents mediated by native OB KARs we observed with 100 μ M zinc, including those activated by a GluK1- and GluK2-preferring agonist.

However, Veran et al. (2012) also found that zinc markedly potentiated currents evoked by 10 mM glutamate in HEK293 cells mediated by GluK3 receptors ($193\% \pm 38\%$ of control by 100 μ M zinc), with an EC₅₀ of 46 ± 17 μ M (Veran et al., 2012). This zinc-mediated modulation of GluK3 was dose-dependent and biphasic: increasing zinc concentrations up to 100 μ M potentiated currents, whereas higher concentrations (up to 1000 μ M) progressively inhibited currents. All tested zinc concentrations (1-1000 μ M) potentiated currents mediated by GluK2/GluK3 heteromeric receptors, with an EC₅₀ of 477 ± 16 μ M. In contrast to homomeric GluK3 receptors, they did not observe inhibition of GluK2/3 receptors at zinc concentrations as high as 1 mM.

These findings in recombinant receptors may help explain some of our findings in native OB KARs. We found that 100 μ M zinc inhibited steady-state KAR-mediated currents induced by all three KAR agonist/AMPA antagonist combinations. However, the ANOVA post hoc analysis revealed a significant difference between the inhibitory effects of 100 μ M zinc on steady-state currents evoked by kainate plus SYM 2206 ($36.0 \pm 9.7\%$ of control) compared with SYM 2081 plus SYM 2206 ($5.6 \pm 1.9\%$ of control). Thus, the degree of zinc-mediated inhibition of currents mediated by GluK1- and GluK2-containing KARs (activated by SYM 2081 plus SYM 2206) was greater than the degree of inhibition of currents mediated by KARs composed of a potentially broader array of KAR subunits (activated by kainate plus SYM 2206).

The concentration of kainate (100 μ M) used in the present experiments may have activated receptors composed of a wide range of KAR subunits. Our prior RT-PCR experiments showed the expression of mRNA for all KAR subunits in the OB, although in varying amounts, i.e., GluK1 $\approx$ GluK2 $\approx$ GluK5 $>$ GluK4 $\gg$ GluK3 (Davila et al., 2007). Kainate has been shown to function as an agonist at recombinant homomeric GluK1 and GluK2 receptors and heteromeric GluK1/2, GluK1/5, and GluK2/5 receptors, with EC₅₀s ranging from 1.8 to 33.6 μ M (Sommer et al., 1992; Jones et al., 1997; Lerma et al., 2001; Alt et al., 2004). Kainate has less potent agonist effects at recombinant GluK3, GluK3/4, and GluK3/5 receptors (Schiffer et al., 1997). Thus, the lesser degree of inhibition by 100 μ M zinc we observed for currents evoked by kainate plus SYM 2206 may reflect mixed effects of zinc at KARs, i.e., inhibition at GluK1- and some GluK2-containing receptors and potentiation at GluK2/GluK3 and some other GluK3-containing receptors. Zinc has been shown to potentiate heteromeric GluK2/GluK3 receptors and homomeric GluK3 receptors, with an EC₅₀ for zinc at GluK3 of 46 ± 17 μ M (Veran et al., 2012). The higher degree of inhibition by 100 μ M zinc we observed for currents activated by SYM 2081 plus SYM 2206, in turn, is consistent with the purely inhibitory effects of zinc at homomeric GluK1 and GluK2 receptors, with reported IC₅₀s for zinc at GluK2 receptors of 67 ± 8 μ M to 102 ± 11 μ M (Mott et al., 2008; Veran et al., 2012).

Zinc's mechanism(s) of action at KARs

To determine zinc's mechanism of action, Mott et al. (2008) examined the effects of 100 μ M zinc on the concentration–response curve for domoate at recombinant GluK2R receptors (Mott et al., 2008). Zinc did not alter the EC50 for domoate at this receptor, suggesting that zinc does not alter KAR agonist affinity. Analysis of effects of zinc on current-voltage curves also suggested that zinc inhibition is only weakly voltage-dependent. Fukushima et al. (2003) reported that zinc inhibition of GluK2/GluK5 receptors was not voltage-dependent (Fukushima et al., 2003).

To determine whether zinc alters KAR desensitization, Mott et al. (2008) perfused oocytes with concanavalin A, an agent that blocks KAR desensitization (Partin et al., 1993). They found that this had no effect on zinc-mediated inhibition at GluK2R or GluK2R/GluK5 receptors, suggesting that zinc does not inhibit KARs by enhancing receptor desensitization (Mott et al., 2008). Consistent with this finding, Veran et al. (2012) found that 100 μ M zinc had no effect on GluK2 desensitization kinetics (Veran et al., 2012). However, kinetic analysis and data from GluK3 mutants showed that zinc's potentiation of currents mediated by GluK3 receptors is mostly attributable to reduced receptor desensitization. Similarly, we recently used cyclothiazide, a drug that blocks AMPAR desensitization (Yamada and Tang, 1993; Yamada, 1998), to show that 100 μ M zinc potentiates currents mediated by AMPARs on M/T cells by decreasing receptor desensitization (Blakemore and Trombley, 2019).

An interaction between zinc modulatory effects and pH has been reported for zinc binding sites at other (e.g., NMDA) receptors (Choi and Lipton, 1999; Low et al., 2000). This could be due to the potential for protons and zinc to interact with each other's respective binding sites (Low et al., 2000) or other allosteric mechanisms (Veran et al., 2012). As protons have been shown to tonically inhibit KARs (Mott et al., 2003), Mott et al. (2008) measured the zinc sensitivity of recombinant (GluK2R, GluK2R/GluK4, GluK2R/GluK5) and native postsynaptic (hippocampal) KARs at different pH levels to determine whether there was an interaction between proton- and zinc-mediated inhibition of KARs (Mott et al., 2008). They found that KARs were less sensitive to zinc-mediated inhibition at acidic pH. Protons shifted the zinc inhibition curve to the right, increasing the IC50 for zinc, and suggesting that protons suppress zinc inhibition of KARs. Thus, a decrease in pH, as may occur with neuronal activity (see “Implications to OB function”), in the presence of zinc potentiates KARs by relieving zinc inhibition.

Veran et al. (2012) also studied the interaction between pH and zinc effects (Veran et al., 2012). They found that pH had a potent effect on KAR (GluK3) function: In the absence of zinc, the amplitude of a glutamate-evoked current was much smaller at pH 8.3 and slightly greater at pH 6.8 compared with that at pH 7.4. Coapplication of zinc potentiated the current at pH 8.3 but inhibited the current at pH 6.8. They concluded that this loss of zinc-mediated potentiation of GluK3-mediated currents at pH 6.8 may be due to protonation of an amino acid, probably a histidine, at low pH. They did not investigate the effects of pH on zinc-mediated inhibition of GluK2-mediated currents.

Strengths and limitations of our methods

Our methods have various strengths and limitations compared with those used in prior studies. First, there are some inherent advantages to studying KARs in expression systems. Characteristics that make the HEK293 cell line popular for studying isolated receptor channels include that it is fast and easy to reproduce and maintain, transfection and protein production occur with high efficiency, and the moderate cell size with few processes facilitates voltage-clamp recording (Thomas and Smart, 2005). The relatively large size of *Xenopus* oocytes facilitates both the injection of complementary RNA or DNA and electrophysiological recording, and the efficiency at which *Xenopus* oocytes translate messages leads to large-amplitude currents (Buckingham et al., 2006). In the present study, we examined the effects of zinc on KARs expressed by M/T cells. In addition to providing data about a native population of KARs, an advantage of using M/T cells is that they are large (compared with OB interneurons) and generate relatively large-amplitude KAR-mediated currents (Blakemore et al., 2018).

The major advantage of the use of expression systems is the ability to create recombinant receptors of known subunit composition, which can be used to investigate ligand binding affinity, efficacy, and rates of association and desensitization (Buckingham et al., 2006). In the present study, we only recorded from native KARs expressed by M/T cells, thus, the receptor subunit compositions were less clear. However, our previous ICC, RT-PCR, and ISH data (Davila et al., 2007) provide some insights into the subunit composition of KARs expressed by OB neurons. To our knowledge, our study is the first to examine effects of zinc on currents evoked by exogenous application of various KAR agonist/AMPA antagonist combinations in a native population of KARs. We chose to study the effects of zinc on KARs expressed by M/T cells, as M/T cells are targets of zincergic OSNs (Jo et al., 2000; Blakemore et al., 2013).

One advantage of our method of whole-cell recording from cultured neurons is that it allows the efficient and relatively rapid exchange of solutions (control, drug, zinc) that envelope the whole neuron, which cannot be achieved in brain slice preparations. The speed of solution changes allows peak drug responses to occur within 100 ms. Given the smaller diffusion space compared with that in slice recording, one can be more confident of the effects of a given concentration of an agonist applied to a neuron in culture. KAR-mediated currents induced by application of KAR agonists such as kainate and SYM 2081 strongly and rapidly desensitize (Patneau and Mayer, 1991; Lerma et al., 1993; Jones et al., 1997; Zhou et al., 1997; Lerma et al., 2001). Thus whole-cell patch techniques, particularly those applied to neurons in brain slices, would not allow rapid enough exchange of solutions to permit measurement of peak currents evoked by exogenous application of these agents. An advantage of our recording from cultured OB neurons with a solution exchange time orders of magnitude faster than that in slice is that it maximized our opportunity to measure at least a portion of the peak component of the current evoked by various agonist/antagonist combinations. Given our better ability to measure steady-state currents and our desire to compare our results with previous findings in recombinant KARs (Mott et al., 2008), we largely examined the effects of zinc on the steady-state component of KAR-mediated currents. However, we did show in one set of experiments, with sequential application of

SYM 2081 followed by SYM 2081 plus zinc, that effects of 100 μ M zinc on steady-state and peak KAR-mediated currents were similar.

Whereas prior studies suggest that zinc does not affect GluK2 receptor desensitization (Mott et al., 2008; Veran et al., 2012), zinc appears to potentiate GluK3-mediated currents by reducing receptor desensitization (Veran et al., 2012). Another potential advantage of our recording method in which we coapplied zinc during the middle (steady-state) portion of a KAR-mediated current is that it allowed us to observe effects of zinc on the portion of the current potentially most relevant to desensitization. For AMPARs, the steady-state current is considered the to be most significant component of the current in this regard, as the peak current does not represent an equilibrium condition between the open and desensitized states (Yamada and Tang, 1993). This method of recording also generates a single 15- to 20-second electrophysiology trace, which shows the times prior to zinc application (baseline), during zinc application (zinc effect), and following zinc application (wash/recovery). Thus, each trace serves as its own control.

A potential limitation of our study is that the precise amount of vesicular zinc released into the synaptic cleft is unknown, so concentrations of zinc used (30 μ M, 100 μ M) were based on estimates of synaptic zinc release. Initial data suggested that extracellular zinc concentrations following neuronal depolarization range from 10 to 300 μ M (Frederickson et al., 1983; Assaf and Chung, 1984; Howell et al., 1984; Xie and Smart, 1991). More recent evidence suggests that peak synaptic cleft zinc concentrations after exocytosis range from 10 nM to >100 μ M (Vogt et al., 2000; Li et al., 2001; Ueno et al., 2002; Frederickson et al., 2006; Paoletti et al., 2009; Vergnano et al., 2014; Goldberg et al., 2016; Blakemore and Trombley, 2017). As peak synaptic concentrations of glutamate exceed 1 mM (Clements et al., 1992; Diamond and Jahr, 1997; Pal, 2018), micromolar concentrations of co-released synaptic zinc are certainly possible. Frederickson et al. (2006) investigated methods for studying zinc release that can influence the quantity, imaging, and calculated concentration of released zinc, including animal age, temperature, use of CaEDTA as a zinc chelator, and choice of zinc probe (Frederickson et al., 2006). They concluded that “common errors can cause up to a 3000-fold underestimation of the concentration of released zinc.” Thus, concentrations of released zinc are often under-estimated rather than over-estimated. The concentrations of zinc used in the present study are also similar to those used in our previous studies showing effects of zinc at other glutamate receptors (NMDA, AMPA) in the OB (Trombley and Shepherd, 1996; Trombley et al., 1998; Blakemore and Trombley, 2004; Blakemore et al., 2013; Blakemore and Trombley, 2019). We expect that lower concentrations of applied zinc (e.g., 10-30 μ M) would have had qualitatively similar effects on KAR-mediated currents.

Another potential limitation of our study relates to the physiological relevance of the KAR agonist/AMPA antagonist combinations used. The neurotransmitter mediating excitatory transmission at OB KARs is glutamate. To see the effects of zinc on the endogenous ligand (glutamate), it would have been necessary to use NMDAR blockers (in addition to AMPAR blockers), thus, further contributing to an environment of polypharmacy we wanted to avoid. As described earlier in the manuscript (Introduction and Results), the selective drugs used to evoke KAR-mediated currents in these experiments were consistent with those used in

multiple previous studies of recombinant and native KARs, including our own study of native KARs in the OB (Blakemore et al., 2018). Similarly, the concentrations of drugs applied were based on concentrations of drugs used and/or EC50s reported in multiple previous studies of recombinant and/or native KARs, including our own (Blakemore et al., 2018).

A final limitation of our study is that cultured neurons from which we recorded were from various subpopulations grouped together (see “Neuronal Identity”). As subtypes of mitral and tufted cells have been identified (Nagayama et al., 2014), it is possible that recording from different subpopulations of M/T cells accounts for some of the observed variability in zinc’s effects. However, most of our statistical analyses involved paired sets of data from the same cell (e.g., paired t-test), so the design of our analyses controlled for some cell-to-cell variability (each cell serves as its own control).

Timing of KAR expression including the OB

Many receptors are composed of subunits that are developmentally regulated. Immunocytochemical and molecular data from other brain regions (medial nucleus of the trapezoid body; substantia gelatinosa of the trigeminal subnucleus caudalis) and the spinal cord in rats and mice suggest that most KAR subunits (GluK1, GluK2, GluK3, GluK5) are expressed at an early age (e.g., E16-17, P0-P7) and that expression of some subunits changes over time (Lohrke and Friauf, 2002; Park et al., 2010; Cui et al., 2012). Thus, consistent with our functional (electrophysiology) data, there is evidence of KAR subunit expression in animals of the age we used for cultures (P0-P4).

Functional data from cultured cells support these findings and provide some information about expression of KAR subunits in vitro. In hippocampal cells cultured from embryonic rats (E17), most cells responded to kainate after 6–8 days in vitro (DIV), whereas cells were insensitive to the GluK1-selective agonist ATPA (Bleakman et al., 1999). In a subsequent study, application of kainate (with SYM 2206) or ATPA evoked currents in cultured hippocampal neurons prepared from embryonic (E17) or postnatal (1-5 day old) rats (Wilding and Huettner, 2001). Similarly, our previous (Blakemore et al., 2018) and current electrophysiology results showing that application of both kainate and SYM 2081 (with or without SYM 2206) evoked currents in cultured OB neurons prepared from P1-P5/P0-P4 rats suggest that OB KARs are present and functional in cultures prepared from animals of this age. However, information about developmental changes in OB KAR subunit expression, especially in vitro, is limited.

To investigate whether developmental changes in the expression of some KAR subunits could have influenced our findings, we looked at zinc sensitivity and number of DIV. For the experiments in which we applied 100 μ M zinc, the number of DIV for cells were 5 days (kainate + SYM 2206), 10 days (SYM 2081; applied in the middle of the current or sequentially), and 4 days (SYM 2081 + SYM 2206). The only significant difference in the effects of zinc between these groups was between kainate plus SYM 2206 (5 DIV) and SYM 2081 plus SYM 2206 (4 DIV), cells of comparable number of DIV. There was no significant difference in the effects of zinc between the kainate plus SYM 2206 (5 DIV) and SYM 2081 (10 DIV) groups or between the SYM 2081 plus SYM 2206 (4 DIV) and SYM 2081 (10

DIV) groups, so potential changes in the expression of KAR subunits between 4–5 DIV and 10 DIV did not result in differences in zinc sensitivity. We also observed a significant difference between the degrees of inhibition by 100 μ M zinc versus 30 μ M zinc of currents evoked by SYM 2081. However, for these experiments, the number of DIV (10) for both groups was the same, so changes in KAR expression over time do not explain this difference. Thus, whereas some KAR subunit expression may vary with developmental stage, this does not appear to explain our findings.

Implications to OB function

The neurotransmitter used at the majority of excitatory synapses in the mammalian brain, including the OB, is glutamate. In the OB, ionotropic glutamate receptors, including NMDARs, AMPARs, and/or KARs, contribute to processes such as glomerular synchronization (Carlson et al., 2000; Schoppa and Westbrook, 2001; Schoppa and Westbrook, 2002; Christie and Westbrook, 2006; Schoppa, 2006b) and reciprocal inhibition (Schoppa et al., 1998; Isaacson and Strowbridge, 1998; Schoppa, 2006a; Blakemore et al., 2018). Glutamate released by OSNs excites OB neurons which synchronize the activity of M/T cells projecting to the same glomerulus (Carlson et al., 2000; Schoppa and Westbrook, 2001; Schoppa and Westbrook, 2002; Christie and Westbrook, 2006; Schoppa, 2006b); this creates spatial (Rubin and Katz, 1999; Belluscio and Katz, 2001; Wachowiak and Cohen, 2001; Chen and Shepherd, 2002) and temporal (Schoppa and Westbrook, 2002; Christie and Westbrook, 2006; Schoppa, 2006b) patterns of odor-evoked oscillations among OB neurons that encode olfactory information. GABAergic inhibitory circuits modify this network behavior (Schoppa et al., 1998; Isaacson and Strowbridge, 1998; Christie et al., 2001; Nusser et al., 2001; Urban and Sakmann, 2002; Schoppa, 2006b), and excitatory transmission, mediated partially by AMPA/kainate receptors (Schoppa, 2006a; Blakemore et al., 2018), influences inhibitory circuit activity. Thus, glutamate plays an integral role in the processing of odor information.

Peak synaptic cleft concentrations of glutamate exceed 1 mM (Clements et al., 1992; Diamond and Jahr, 1997; Pal, 2018). In expression systems and in neurons from other brain regions, relatively low concentrations of glutamate cause rapid and strong desensitization of both AMPARs and KARs (Traynelis et al., 2010). For example, submaximal glutamate concentrations have been shown to substantially desensitize homomeric recombinant GluK1-3 receptors (Sommer et al., 1992; Heckmann et al., 1996; Schiffer et al., 1997; Paternain et al., 1998; Fisher and Fisher, 2014). Similarly, ~ 3 μ M glutamate causes half maximal desensitization of native KARs expressed by DRG cells (Jones et al., 1997) and hippocampal neurons (Paternain et al., 1998). As desensitization is a biophysical property that influences the timing and amplitude of synaptic responses, this desensitizing action of glutamate has important circuit implications. Synaptically released zinc is another factor that may alter KAR desensitization. We recently showed that 100 μ M zinc potentiates OB AMPARs by decreasing receptor desensitization (Blakemore and Trombley, 2019). Although zinc does not appear to inhibit GluK2-containing KARs by altering receptor desensitization (Mott et al., 2008; Veran et al., 2012), zinc's potentiation of currents mediated by GluK3 receptors was mostly attributable to reduced receptor desensitization (Veran et al., 2012). We found a lesser degree of zinc-mediated inhibition of currents evoked by kainate plus SYM

2206 compared with currents evoked by the GluK1- and GluK2-preferring agonist SYM 2081 (plus SYM 2206). This may reflect activation of KARs composed of a broader array of KAR subunits by kainate (compared with SYM 2081), and thus, mixed effects of zinc at these KARs, i.e., inhibition at GluK1- and some GluK2-containing receptors and potentiation at GluK2/GluK3 and some other GluK3-containing receptors. Thus, zinc may have potentiating effects at some GluK3-containing OB KARs by affecting receptor desensitization and, in this manner, synaptically released zinc could modulate KAR desensitization caused by glutamate in the OB.

In the present study, zinc inhibited currents evoked by all KAR agonist/AMPA antagonist combinations, suggesting zinc largely has inhibitory actions at OB KARs. There is precedence for inhibition of synaptic transmission mediated by KARs by zinc. In addition to determining zinc's actions at recombinant receptors, Mott et al. (2008) studied effects of zinc at native synaptic KARs in the hippocampus (Mott et al., 2008). They found that exogenously applied zinc (100 μ M) blocked slow KAR-mediated, but not fast AMPAR-mediated, spontaneous mEPSCs. Zinc (100 μ M) did not affect the frequency or amplitude of AMPAR-mediated mEPSCs, suggesting that glutamate release was not affected by presynaptic actions of zinc. Based on these results, they concluded that zinc selectively blocks postsynaptic KARs on CA3 pyramidal cells. They also used zinc chelators (BTC-5N and/or CaEDTA) and mice deficient in the ZnT3 zinc transporter (Kantheti et al., 1998; Vogt et al., 2000) to demonstrate that postsynaptic KARs at mossy fiber synapses are inhibited by synaptically released (endogenous) zinc. Collectively, their results suggest that physiologically relevant zinc concentrations modulate synaptic circuits involving KARs in the hippocampus (Mott et al., 2008). As KARs are located at some synapses where OSNs release zinc in the OB (Blakemore et al., 2018), endogenously released zinc could also modulate synaptic circuits mediated by KARs in the OB.

In our previous study, we explored whether KARs are expressed at synapses (Blakemore et al., 2018), including those where vesicular zinc is located. First, we stimulated the olfactory nerve layer (ONL) and evoked glutamate-mediated excitatory postsynaptic currents (EPSCs) in mitral cells and juxtglomerular (JG) cells (including PG cells and external tufted [ET] cells) in OB slices. We then used AP5 (to block NMDARs) and GYKI 52466 (to block AMPARs) to isolate KAR-mediated responses mediated by postsynaptic KARs expressed by these cells. We showed that KARs are located at OSN-mitral cell and OSN-JG cell synapses (Blakemore et al., 2018), where zinc is released (Blakemore et al., 2013). Additionally, we showed that exogenously applied kainate has presynaptic effects (potentiation by 50 nM kainate; inhibition by 500 nM kainate) on EPSCs evoked in mitral cells and JG cells by ONL stimulation. Thus, zinc released from OSNs could alter synaptic transmission in the OB by inhibiting postsynaptic and/or presynaptic KARs.

In our previous study, exogenous application of 1 μ M of the selective GluK1 agonist (RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid (ATPA) (Clarke et al., 1997; Hoo et al., 1999; Alt et al., 2004) decreased the mean amplitude of ONL-evoked glutamate-mediated EPSCs in mitral cells and JG cells, suggesting the presence of GluK1-containing presynaptic KARs (Blakemore et al., 2018). This is consistent with our previous ICC experiments in the OB showing that some GluK1 labeling colocalized with labeling for the

synapse-specific protein synapsin (Stone et al., 1994), especially in the glomerular and granule cell layers (Davila et al., 2007). This is potentially significant given the present findings that the greatest degree of zinc-mediated inhibition of KAR-mediated currents was for currents evoked by the GluK1 and GluK2-preferring agonist SYM 2081 (plus SYM 2206).

We also previously showed that KARs participate in dendrodendritic inhibition (DDI) between OB neurons (Blakemore et al., 2018). Kainate (300 nM, 5 μ M) or (ATPA)(1 μ M, 10 μ M) modulated (potentiated or inhibited) reciprocal inhibitory postsynaptic currents (IPSCs) evoked in mitral cells and ET cells using a protocol that induces reciprocal inhibition from granule cells and PG cells, respectively. The (mostly) inhibitory effects of kainate and ATPA on IPSCs could be due to activation of presynaptic KARs on mitral and tufted cells, reducing glutamate release, and/or the activation of postsynaptic KARs on PG cells and granule cells, reducing GABA release. Thus, glomerular zinc released from OSNs could modulate currents mediated by presynaptic and/or postsynaptic KARs localized to glomeruli, and thus, influence the role of KARs in DDI.

Another major conclusion of the study by Mott et al. (2008) was that pH significantly affects zinc-mediated inhibition of KARs (Mott et al., 2008). They found that a decrease in pH potentiated KARs by relieving zinc inhibition. Whereas protons suppressed zinc-mediated inhibition of all examined recombinant KARs, effects were strongest at GluK5-containing receptors, where zinc inhibition was tonically suppressed at a physiological pH. They further proposed that the alkaline and acidic shifts in the interstitial pH that occur with neuronal activity could influence this zinc modulation of KARs (Mott et al., 2008). Consistent with this notion, they found that even nanomolar concentrations of zinc significantly inhibited currents mediated by GluK2R/GluK5 receptors at an alkaline pH (pH 8.3). However, with zinc present, decreasing the pH from 7.5 to an acidic pH (6.7) potentiated currents mediated by these receptors. In this way, we propose that zinc could act as a high-pass filter of KAR-mediated activity in the OB. That is, weak odors could result in little in the way of neural activity, thus, permitting tonic zinc inhibition of KARs. Strong odors could lead to repetitive neuronal activity, acidifying the extracellular space and unmasking KAR-mediated neurotransmission that is tonically inhibited by synaptically released zinc.

With the present data, we have now shown that zinc modulates currents mediated by excitatory (NMDA, AMPA, kainate) and inhibitory (e.g., GABA_A, glycine) ionotropic amino acid receptors in the OB (Trombley and Shepherd, 1996; Trombley et al., 1998; Blakemore and Trombley, 2004; Trombley et al., 2011; Blakemore et al., 2013; Blakemore and Trombley, 2019). Collectively, these receptors contribute to a variety of synaptic circuits involved in odor-information processing. For example, both NMDARs and AMPARs contribute to the glutamate-mediated activation of OB neurons by OSNs (Berkowicz et al., 1994; Ennis et al., 1996; Aroniadou-Anderjaska et al., 1997; Chen and Shepherd, 1997; Ennis et al., 2001). Activation of the olfactory nerve results in prolonged excitation in M/T cell apical dendrites via actions at NMDARs and AMPA/kainate receptors, allowing incoming sensory information to be modulated and integrated (Aroniadou-Anderjaska et al., 1999). NMDA receptors mediate the majority of the late phase of synaptic excitation in these M/T cell dendrites, which may play an integral role in both synaptic integration on

these dendrites and synaptic plasticity (Aroniadou-Anderjaska et al., 1999). Thus, the co-release of zinc with glutamate from OSNs may influence synaptic integration and plasticity mediated by these receptors at the initial site of sensory processing in the olfactory system. Similarly, in the hippocampus, synaptically released zinc contributes to synaptic integration and plasticity by inhibiting postsynaptic GluN2A-NMDARs (Vergnano et al., 2014).

Synaptically released zinc also has been shown to fine-tune neurotransmission by modulating AMPAR- and NMDAR-mediated EPSCs and presynaptic release probability in various brain regions (Vogt et al., 2000; Pan et al., 2011; Perez-Rosello et al., 2013; Vergnano et al., 2014; Anderson et al., 2015; Kalappa et al., 2015; Kalappa and Tzounopoulos, 2017). Recent in vivo data from the auditory cortex suggest that synaptically released zinc modulates the gain of sound-evoked responses via both NMDAR-dependent and NMDAR-independent effects (Anderson et al., 2017) and also shapes cortical sound frequency tuning and discrimination (Kumar et al., 2019). As neuronal tuning to sensory stimuli is integral to the cortical sensory processing underlying behavior (Kumar et al., 2019), these findings are of significance and support the notion that synaptically released zinc could similarly modulate sensory-evoked responses in the OB and facilitate encoding of odors by actions at amino acid receptors.

Acknowledgments

FUNDING

This work was supported in part by the National Institute on Deafness and Other Communication Disorders (NIDCD) of the US National Institutes of Health [grant number: DC-04320].

ABBREVIATIONS

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
ANOVA	analysis of variance
AP5	2-amino-5-phosphonopentanoic acid
ATPA	(RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid
CNS	central nervous system
CTZ	cyclothiazide
DDI	dendrodendritic inhibition
DIV	days in vitro
DRG	dorsal root ganglion
EC50	half maximal effective concentration
EPSC	excitatory postsynaptic current

EPSP	excitatory postsynaptic potential
EPL	external plexiform layer
ET cells	external tufted cells
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GL	glomerular layer
GYKI 52466	1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride
IC50	half maximal inhibitory concentration
ICC	immunocytochemical
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
ISH	in situ hybridization
JG cells	juxtaglomerular cells
K2	D'Agostino-Pearson omnibus
KAR	kainate receptor
KS	Kolmogorov-Smirnov
MEM	Minimal Essential Medium
mEPSC	miniature EPSC
M/T cell	mitral/tufted cell
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
OB	olfactory bulb
ON	olfactory nerve
ONL	olfactory nerve layer
OSN	olfactory sensory neuron
PG cell	periglomerular cell
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean

SS	steady-state
SW	Shapiro-Wilk
SYM 2081	(2S,4R) 4-methylglutamic acid
SYM 2206	4-(4-Aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine
TTX	tetrodotoxin
VGLUT1	vesicular glutamate transporter 1
ZnCl₂	zinc chloride
ZPI	Zinpyr-1

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HIGHLIGHTS

- Prior results show that kainate receptors (KARs) modulate excitatory and inhibitory transmission in the olfactory bulb.
- A KAR agonist (kainate or SYM 2081) with or without an AMPA receptor antagonist (SYM 2206) evoked KAR-mediated currents.
- Zinc inhibited currents evoked by kainate plus SYM 2206, SYM 2081, or SYM 2081 plus SYM 2206 in the olfactory bulb.
- Coapplication of zinc with a GluK1- and GluK2- preferring agonist (SYM 2081) plus SYM 2206 caused the greatest inhibition.
- Zinc may influence odor-encoding synaptic currents by effects at KARs.

Figure 1.

Zinc (100 μ M) inhibits kainate receptor (KAR)-mediated currents evoked by coapplication of kainate and the AMPA receptor antagonist SYM 2206. (A) Application of 100 μ M SYM 2206 during a current evoked by 100 μ M kainate. SYM 2206 blocked the large AMPA receptor-mediated component of this current, revealing the relatively small KAR-mediated component. (B) Coapplication of 100 μ M kainate and 100 μ M SYM 2206, after pretreatment with SYM 2206, evoked a current in all cells (N=9) examined. (C) Coapplication of 100 μ M zinc during the steady-state portion (middle) of the current evoked by kainate and SYM 2206 significantly decreased the steady-state amplitude of the current in all cells. (D)

Histogram showing the effect of 100 μ M zinc on the steady-state component of the current evoked by 100 μ M kainate and 100 μ M SYM 2206, expressed as a percentage of the control current. Zinc significantly decreased the control (kainate + SYM 2206) current, with significant recovery of the current after a wash (N=9; *P < 0.01 compared to control; **P < 0.01 compared to zinc).

Figure 2.

Zinc (30 and 100 μM) inhibits kainate receptor (KAR)-mediated currents evoked by application of the GluK1- and GluK2-preferring agonist SYM 2081. **(A)** Application of 10 μM SYM 2081 evoked a current in all cells (N=10) examined. **(B)** Coapplication of 100 μM zinc during the steady-state portion (middle) of the current evoked by SYM 2081 significantly decreased the steady-state amplitude of the current in all cells. **(C)** Histogram showing the effect of 100 μM zinc on the steady-state component of the current evoked by 10 μM SYM 2081, expressed as a percentage of the control current. Zinc significantly

decreased the control (SYM 2081) current, with significant recovery of the current after a wash (N=10; *P < 0.01 compared to control; ** P< 0.01 compared to zinc). **(D)** Coapplication of 30 μ M zinc also significantly decreased the steady-state amplitude of the current evoked by 10 μ M SYM 2081 in all cells (N=5) examined. **(E)** Histogram showing the effects of 30 μ M and 100 μ M zinc on the steady-state component of the current evoked by 10 μ M SYM 2081, expressed as a percentage of the control current. The degree of current inhibition by 100 μ M zinc (N=10; ** P < 0.01 compared to control) was significantly greater than the degree of inhibition by 30 μ M zinc (N=5; *P < 0.05 compared to control)(N=15; ***P < 0.01).

Figure 3.

Zinc (100 μM) has similar effects on the steady-state (SS) and peak components of currents evoked by SYM 2081. Effects of 100 μM zinc on the SS and peak components of kainate receptor (KAR)-mediated currents were examined by sequential application of 10 μM SYM 2081 followed by 10 μM SYM 2081 plus 100 μM zinc. (A) Application of 10 μM SYM 2081 evoked a current in all (N=10) cells examined. (B) Subsequent coapplication of 100 μM zinc with 10 μM SYM 2081 significantly decreased both the SS and peak components of the current. (C) Histogram showing the effects of 100 μM zinc on different components of

the current evoked by sequential application of 10 μ M SYM 2081 (A) and 10 μ M SYM 2081 plus 100 μ M zinc (B), expressed as a percentage of the control current. Zinc significantly decreased both the SS and peak portions of the current (N=10; *P < 0.01 compared to control for both). The degrees of zinc-mediated inhibition of the SS and peak portions of the current were similar (N=20; #P = 0.6442). **(D)** Histogram showing the effects of 100 μ M zinc on the SS current evoked by 10 μ M SYM 2081 with different types of zinc application, expressed as a percentage of the control current. Both types of zinc application significantly decreased the current (N=10; *P < 0.01 compared to control for both). The degrees of zinc-mediated inhibition of the current with sequential application of SYM 2081 followed by SYM 2081 plus 100 μ M zinc versus coapplication of zinc during the middle of the current evoked by SYM 2081 were similar (N=20; #P = 0.5354).

Figure 4.

Zinc inhibits kainate receptor (KAR)-mediated currents evoked by SYM 2081 and the AMPA receptor antagonist SYM 2206. (A) Coapplication of 10 μ M SYM 2081 and 100 μ M SYM 2206, after pretreatment with SYM 2206, evoked currents in all (N=8) cells examined. (B) Coapplication of 100 μ M zinc during the steady-state portion (middle) of the current evoked by SYM 2081 and SYM 2206 significantly decreased the amplitude of the current in all cells. (C) Histogram showing the effect of 100 μ M zinc on the steady-state component of the current evoked by 10 μ M SYM 2081 and 100 μ M SYM 2206, expressed as a percentage

of the control current. Zinc significantly decreased the control (SYM 2081+ SYM 2206) current, with significant recovery of the current after a wash (N=8; *P < 0.01 compared to control; ** P< 0.01 compared to zinc).

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Figure 5.

Histogram summarizing the effects of 100 μ M zinc on kainate receptor (KAR)-mediated steady-state currents evoked by the three KAR agonist/AMPA receptor antagonist combinations tested: A) 10 μ M SYM 2081 plus 100 μ M SYM 2206 ($5.6 \pm 1.9\%$ of control), B) 10 μ M SYM 2081 ($13.4 \pm 4.2\%$ of control), and C) 100 μ M kainate plus 100 μ M SYM 2206 ($36.0 \pm 9.7\%$ of control). Both the Brown-Forsythe and Welch ANOVA tests revealed a significance difference in mean degrees of zinc-mediated inhibition of currents (expressed as mean $\pm$ SEM % of the control current) between groups (Brown-Forsythe ANOVA: $n=27$, $P=0.0146$; Welch ANOVA: $n=27$, $P=0.0185$). Post-hoc analysis with Dunnett's T3 multiple comparisons test showed a significant difference in the mean degree of current inhibition

between groups A and C (*adjusted P value = 0.0379), but not between groups A and B (#adjusted P value = 0.2912) or groups B and C (##adjusted P value = 0.1521).

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