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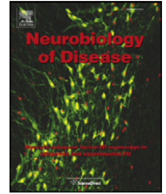

Tonic zinc inhibits spontaneous firing in dorsal cochlear nucleus principal neurons by enhancing glycinergic neurotransmission

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ABSTRACT

In many synapses of the CNS, mobile zinc is packaged into glutamatergic vesicles and co-released with glutamate during neurotransmission. Following synaptic release, the mobilized zinc modulates ligand- and voltage-gated channels and receptors, functioning as an inhibitory neuromodulator. However, the origin and role of tonic, as opposed to phasically released, zinc are less well understood. We investigated tonic zinc in the dorsal cochlear nucleus (DCN), a zinc-rich, auditory brainstem nucleus. Our results show that application of a high-affinity, extracellular zinc chelator (ZX1) enhances spontaneous firing in DCN principal neurons (fusiform cells), consistent with inhibition of this neuronal property by tonic zinc. The enhancing effect was prevented by prior application of strychnine, a glycine receptor antagonist, suggesting that ZX1 interferes with zinc-mediated modulation of spontaneous glycinergic inhibition. In particular, ZX1 decreased the amplitude and the frequency of glycinergic miniature inhibitory postsynaptic currents in fusiform cells, from which we conclude that tonic zinc enhances glycinergic inhibitory neurotransmission. The observed zinc-mediated inhibition in spontaneous firing is present in mice lacking the vesicular zinc transporter (ZnT3), indicating that non-vesicular zinc inhibits spontaneous firing. Noise-induced increase in the spontaneous firing of fusiform cells is crucial for the induction of tinnitus. In this context, tonic zinc provides a powerful break of spontaneous firing that may protect against pathological run-up of spontaneous activity in the DCN.

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Introduction

Since the discovery that zinc is loaded into glutamatergic vesicles and is exocytosed with glutamate during synaptic transmission, numerous studies have investigated the role of mobilized zinc in synapses. These studies are consistent with a model whereby zinc serves as an inhibitory neuromodulatory neurotransmitter, inhibiting NMDA receptors (NMDARs), reducing release probability in excitatory synapses, and potentiating glycinergic and GABAergic inhibitory neurotransmission (Xie and Smart, 1991; Hirzel et al., 2006; Nozaki et al., 2011; Pan et al., 2011; Perez-Rosello et al., 2013; Vergnano et al., 2014). Unlike many other neurotransmitter systems, where the actions of tonic levels of neurotransmitters are well studied, the role of tonic zinc remains less understood. Moreover, prior electrophysiological studies are limited and present conflicting results. For example, one study reports that, in mossy fiber synapses, ZnT3-dependent tonic zinc levels modulate NMDARs (Vogt et al., 2000), but more recent work at the same synapses, finds that ambient zinc levels are too low for NMDAR modulation (Vergnano et al., 2014). The role of ambient zinc therefore remains enigmatic.

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A major obstacle in determining the role of zinc (synaptic and tonic) has been the lack of a chelator with appropriate kinetic and thermodynamic properties for probing the temporal and spatial changes of mobile zinc at synapses (Radford and Lippard, 2013), as illustrated by the contrasting findings about the role of tonic zinc in previous work. Quite different zinc chelators were applied in these studies: a kinetically slow one (Vogt et al., 2000) and a faster, but low-affinity chelator (Vergnano et al., 2014). To assess the role and origin of tonic zinc we utilized the fast, high-affinity zinc chelator ZX1 (Pan et al., 2011; Radford and Lippard, 2013) as well as transgenic mice lacking ZnT3. We studied the role of tonic zinc on DCN fusiform cells. Fusiform cells generate spontaneous action potentials (Rhode et al., 1983; Hancock and Voigt, 2002; Leao et al., 2012) and, because they are embedded in a zinc-rich nucleus (Frederickson et al., 1988; Rubio and Juiz, 1998; Oertel and Young, 2004), they provide an ideal assay for testing the effects of tonic zinc on neuronal excitability.

Methods

Slice preparation

Experiments were conducted according to the methods approved by the Institutional Animal Care and Use Committee of the University of

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Pittsburgh. Coronal brainstem slices were prepared from ICR mice and ZnT3 knockout (ZnT3 KO) mice (P17–P25). ICR mice were purchased from Harlan and ZnT3 KO mice were purchased from The Jackson Laboratory. The preparation of coronal slices containing DCN has been described in detail previously (Tzounopoulos et al., 2004).

Electrophysiological recordings and analysis

Loose cell-attached voltage-clamp and whole cell voltage-clamp recordings were obtained from visually identified fusiform cells at a temperature of 31–33 °C. Fusiform cells were identified on the basis of morphological and electrophysiological criteria (Tzounopoulos et al., 2004). In preparing the external solution we removed contaminating zinc from our solutions with Chelex 100 resin (Biorad). After applying Chelex to the ACSF, high purity calcium and magnesium salts were added (99.995% purity, Sigma). All plastic and glassware were washed with 5% high purity nitric acid (Sigma). The external solution contained the following (in mM): 130 NaCl, 3 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 21 $NaHCO_3$, 3.5 HEPES, and 10 glucose, saturated with 95% O_2/5% CO_2. For loose cell-attached voltage-clamp recordings assessing spontaneous firing, pipettes (1.5–2.5 MΩ) were filled with modified external solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2$, 25 $NaHCO_3$, and 25 glucose. Seal resistance was maintained between 10 and 30 MΩ with command potential at 0 mV. To measure the time course of ZX1 (100 μM, purchased from Strem) and strychnine (500 nM) on spontaneous firing frequency, instantaneous frequency was measured, averaged every minute, and then normalized to baseline. For miniature IPSCs (mIPSCs) and spontaneous IPSCs (sIPSCs) experiments, fusiform cells were clamped at the reversal of excitatory responses (~0 mV) with pipettes containing a cesium-based internal solution (containing in mM: Cs (CH_3O_3S) 126, KCl 4, HEPES 10, Na_2ATP 4, Tris-GTP 0.3, Tris-phosphocreatine 10, CsEGTA 1, QX-314 1, sodium ascorbate 3, pH = 7.25, 295 mOsm). Miniature IPSCs were recorded in the presence of tetrodotoxin (TTX, sodium channel blocker, 0.5 μM), SR95531 ($GABA_A$ receptor antagonist, 20 μM), AP-5 (NMDA receptor antagonist, 50 μM), and DNQX (AMPA receptor antagonist, 50 μM). Spontaneous IPSCs were recorded in the presence of SR95531 (20 μM). Ten-second blocks of mIPSCs and sIPSCs were acquired at a sample rate of 50 kHz and low pass filtered at 10 kHz. Negative-voltage pulses (−5 mV, 50 ms) were delivered every 10 s to monitor input and access resistance. Voltage-clamp experiments were not included if the series and/or input resistance changed by more than 20% during the experiment. mIPSCs and sIPSCs were detected and analyzed using Mini-analysis software (Synaptosoft) with amplitude and area thresholds set at 3 times the noise level. All events were verified by visual inspection. Amplitude values were obtained by subtracting the average baseline from the amplitude at the local maximum during the event. Electrophysiological data were acquired and analyzed using pClamp, IGOR PRO (Wavemetrics), and GraphPad Prism (GraphPad Software). Statistical comparisons were made using analysis of variance, and paired and unpaired two-tailed Student's *t* tests. Statistical significance was based on p values <0.05. All means are reported ±SEM.

Results

Tonic zinc decreases spontaneous firing in fusiform cells by enhancing glycinergic neurotransmission

To test whether tonic zinc modulates spontaneous firing in fusiform cells, we examined the effect of ZX1, a high-affinity extracellular zinc chelator (Pan et al., 2011; Radford and Lippard, 2013), on the rate of spontaneous action potentials. In cell-attached recordings, 100 μM ZX1 increased the spontaneous firing rate in fusiform cells; this effect was reversed upon removal of ZX1 from the bath (Figs. 1A, B; ZX1: 148 ± 14% of control, n = 7, p = 0.01 when compared to control; wash out: 95 ± 10%, p = 0.5 when compared to control). These results indicate that tonic zinc inhibits spontaneous firing rates in fusiform cells.

Spontaneous firing in fusiform cells is generated by intrinsic neuronal properties (Leao et al., 2012); however, synaptic activity, mainly inhibitory, modulates spontaneous firing in fusiform cells (Roberts and Trussell, 2010). Fusiform cells receive robust glycinergic (and GABAergic inhibition) from cartwheel, stellate, and tuberculoventral cells (Oertel and Young, 2004; Mancilla and Manis, 2009; Roberts and Trussell, 2010; Kuo et al., 2012; Apostolides and Trussell, 2013, 2014a), and zinc potentiates glycinergic neurotransmission in other brain areas (Laube et al., 2000; Suwa et al., 2001). We therefore hypothesized that tonic zinc exerts its inhibitory effect on fusiform cell firing by potentiating spontaneous glycinergic inhibition. Consistent with this hypothesis, we found that 500 nM strychnine, a glycine receptor (GlyR) antagonist, increased spontaneous firing and blocked the effect of zinc chelation (Figs. 2A, B; strychnine: 179 ± 28% of control, n = 5, p = 0.03 when compared to control; ZX1: 112 ± 8% of strychnine, n = 5, p = 0.24 when compared to strychnine). The absence of an effect of ZX1 on spontaneous firing after strychnine application is not the result of a "ceiling effect", because the magnitude of ZX1 enhancement was not correlated with the initial spontaneous firing frequency (Suppl Fig. 1A, $r^2 = 0.004$, p = 0.88). Moreover, the effect of ZX1 in the presence of strychnine was smaller than in control conditions even when the initial firing frequency prior to ZX1 application was similar between control and strychnine (Suppl. Fig. 1B). Together, these results suggest that tonic zinc inhibits spontaneous firing rates in fusiform cells, at least in part by potentiating spontaneous glycinergic neurotransmission.

Tonic zinc potentiates GlyRs and increases Pr in glycinergic terminals

Tonic zinc may increase glycinergic inhibition onto fusiform cells presynaptically, either by increasing glycine release and/or by increasing the spontaneous firing rate of glycinergic interneurons that contact fusiform cells, or postsynaptically, by potentiating glycine receptors (GlyRs). To determine whether tonic zinc causes a potentiation in postsynaptic GlyRs we measured the effect of ZX1 on the amplitude of

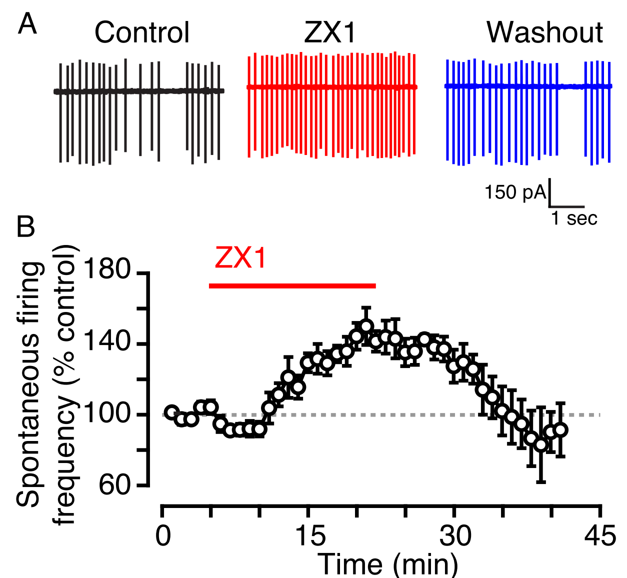

Fig. 1. Tonic zinc decreases fusiform cell spontaneous firing rate. A) Representative cell-attached recordings showing fusiform cell spontaneous firing in control (black), in the presence of ZX1 (red), and after ZX1 washout (blue). B) Time course of the effect of ZX1. ZX1 significantly increased fusiform cell spontaneous firing rate; the firing rate returned to baseline after ZX1 washout.

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