

Zinc Potentiates Neuronal Nicotinic Receptors by Increasing Burst Duration

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Hsiao B, Mihalak KB, Magleby KL, Luetje CW. Zinc potentiates neuronal nicotinic receptors by increasing burst duration. *J Neurophysiol* 99: 999–1007, 2008. First published December 9, 2007; doi:10.1152/jn.01040.2007. Micromolar zinc potentiates neuronal nicotinic acetylcholine receptors (nAChRs) in a subtype-dependent manner. Zinc potentiates receptor function even at saturating agonist concentrations, without altering the receptor desensitization rate. Potentiation could occur through an increase in the number of available receptors, an increase in single-channel current amplitude, or an increase in single-channel open probability. To distinguish among these possibilities, we examined rat neuronal nAChRs expressed in *Xenopus* oocytes. Blockade of a large fraction of ACh activated $\alpha 4\beta 4$ or $\alpha 4\beta 2$ receptors by the open channel blocker hexamethonium failed to change the extent of potentiation by zinc, suggesting that zinc does not change the number of available receptors. The single-channel amplitudes of ACh (1 μ M) activated $\alpha 4\beta 4$ receptors in outside-out patches were similar in the absence and the presence of 100 μ M zinc (3.0 ± 0.1 and 2.9 ± 0.1 pA, respectively). To determine the effect of zinc on single-channel open probability, we examined $\alpha 4\beta 4$ receptors in cell-attached patches. The open probability at 100 nM ACh (0.011 ± 0.002) was increased 4.5-fold by 100 μ M zinc (0.050 ± 0.008), accounting for most of the potentiation observed at the whole cell level. The increase in open probability was due to an increase in burst duration, which increased from 207 ± 38 ms in the absence of zinc to 830 ± 189 ms in the presence of zinc. Our results suggest that potentiation of neuronal nAChRs by zinc is due to a stabilization of the bursting states of the receptor.

INTRODUCTION

Ionic zinc is found in neurons throughout the brain, with the highest concentrations in the cerebral cortex and limbic areas (Frederickson et al. 2000). Zn^{2+} is packaged in synaptic vesicles and released during neuronal activity (Assaf and Chung 1984; Howell et al. 1984). Zinc serves as an endogenous modulator of nervous system function (Li et al. 2003; Smart et al. 2004). For example, synaptically released Zn^{2+} serves as a modulator of long-term potentiation in the hippocampus and the amygdala (Izumi et al. 2006; Kodirov et al. 2006; Ueno et al. 2002; Vogt et al. 2000). The effects of Zn^{2+} are thought to occur through the modulation of a variety of ligand-gated ion channels, including glutamate and γ -aminobutyric acid receptors (GABARs), as well as glycine and adenosine triphosphate (ATP) receptors (Huang 1997; Smart et al. 2004).

Neuronal nicotinic acetylcholine receptors (nAChRs) are also modulated by Zn^{2+} (Garcia-Colunga 2001; Hsiao et al. 2001; Palma et al. 1998). Micromolar concentrations of Zn^{2+}

inhibit homomeric $\alpha 7$ receptors (Palma et al. 1998) and heteromeric $\alpha 3\beta 2$ receptors (Hsiao et al. 2001). In contrast, the effect of Zn^{2+} on many heteromeric neuronal nAChRs is biphasic. These receptors are potentiated by micromolar Zn^{2+} and inhibited by millimolar Zn^{2+} (Hsiao et al. 2001). The extent of potentiation varies depending on subunit composition, with $\alpha 4$ -containing receptors displaying the most dramatic potentiation. At low ACh concentrations, the $\alpha 4\beta 2$ and $\alpha 4\beta 4$ receptors are potentiated by Zn^{2+} , with EC_{50} values of 16 and 22 μ M (respectively) and with maximum potentiation of approximately 2.5- and 5-fold (respectively) reached at 100 μ M Zn^{2+} . Inhibition of both receptors occurs at $[Zn^{2+}] > 100$ μ M (Hsiao et al. 2001). Estimates of extracellular synaptic $[Zn^{2+}]$ during neuronal activity vary widely, from 7 to 8 μ M to perhaps as much as 100–300 μ M (Assaf and Chung 1984; Li et al. 2001; Ueno et al. 2002; Vogt et al. 2000; Xie et al. 1994; but see Kay 2003). Zn^{2+} modulation of neuronal nAChRs occurs throughout this concentration range (Hsiao et al. 2001).

There are a variety of possible mechanisms through which Zn^{2+} might potentiate the function of neuronal nAChRs. Our earlier demonstration that Zn^{2+} potentiates neuronal nAChRs even at saturating ACh concentrations (Hsiao et al. 2001) suggests that potentiation cannot be explained solely by a lateral shift in the macroscopic dose–response relationship. Our recent work has identified the sites at which Zn^{2+} binds and potentiates the receptors as the subunit–subunit interfaces that alternate with interfaces that bind ACh (Hsiao et al. 2006). In the context of the recent “C-loop closure” model of receptor activation (Hansen et al. 2005), Zn^{2+} might act by stabilizing an open or bursting conformation of the receptor. Here we examine the effect of Zn^{2+} on the parameters that contribute to the total current (I) through a population of receptors (as described by the equation $I = niP_o$), where n is the number of active receptors, i is the single-channel current amplitude, and P_o is the single-channel open probability. We rule out an effect of Zn^{2+} on n and i , finding that an increase in P_o , due to an increase in burst duration, accounts for the potentiation of neuronal nAChRs by Zn^{2+} .

METHODS

Materials

Xenopus laevis frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *X. laevis* were approved by the University of Miami Animal Research Committee and meet guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin,

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TX). Collagenase B was from Boehringer Mannheim (Indianapolis, IN). All other reagents were from Sigma (St. Louis, MO). Zinc-containing solutions were prepared prior to each experiment from a 1 M stock solution of zinc acetate. We previously showed that zinc acetate solutions yield results that are identical to those obtained with zinc chloride solutions (Hsiao et al. 2001).

Neuronal nAChR expression in *X. laevis* oocytes

m⁷G(5')ppp(5')G capped cRNA transcripts encoding nAChR subunits were prepared by in vitro transcription from linearized template DNA encoding the $\alpha 4$, $\beta 2$, and $\beta 4$ subunits. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester and oocytes were surgically removed. Follicle cells were removed by treatment with Collagenase B for 2 h at room temperature. Stage V oocytes were injected with 1–30 ng of each cRNA in 15–50 nl of water and incubated at 18°C in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM CaNO₃, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 100 μ g/ml gentamicin, and 15 mM HEPES; pH 7.6) for 1–7 days.

Whole cell recording

Current responses were measured under two-electrode voltage clamp. Oocytes were perfused at room temperature (20–25°C) with perfusion solution (115 mM NaCl, 1.8 mM CaCl₂, 2.5 mM KCl, 0.1 μ M atropine, and 10 mM HEPES; pH 7.2) at a rate of about 4 ml/min. Acetylcholine, hexamethonium, and zinc were diluted in the perfusion solution and applied under control of solenoid valves. Micropipettes were filled with 3 M KCl and had resistances of 0.3–2.0 M Ω . Oocytes were held at –70 mV (Fig. 1) or –85 mV (Fig. 2). Experiments were performed using a TEV-200 voltage-clamp unit (Dagan, Minneapolis, MN). Current responses were filtered (four-pole, Bessel low-pass) at 20 Hz (–3 dB) and sampled at 100 Hz. Data were acquired and analyzed on a Macintosh Power PC 7100 computer using AxoData 1.2.2 and AxoGraph 4.6 software (Molecular Devices, Union City, CA) or on a Pentium III PC running pClamp 8 software (Molecular Devices).

Patch clamp

Current flowing through single $\alpha 4\beta 4$ nAChRs was recorded from patches of oocyte surface membrane in both the outside-out and cell-attached configurations. $\alpha 4\beta 4$ nAChRs were used in these experiments because this subunit combination shows robust expression in oocytes and displays much less desensitization than $\beta 2$ -containing receptors. Vitelline membranes were manually removed from the oocytes prior to patch formation. Recording pipettes were pulled from borosilicate glass capillary tubing (Warner Instruments) to a resistance of 7–15 M Ω . During outside-out patch recordings, the recording chamber solution consisted of 150 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, and 10 mM HEPES (pH adjusted to 7.2 with NaOH). The recording pipette solution consisted of 80 mM CsCl, 60 mM CsF, 10 mM EGTA, 1 mM CaCl₂, and 10 mM HEPES (pH adjusted to 7.2 with KOH). Recording chamber solution containing 1 μ M ACh or 1 μ M ACh with 100 μ M Zn²⁺ was applied to the patches using a Warner Instruments SF-77B "Perfusion Fast-Step" stepper with a three-barreled, square glass tube. Based on preliminary experiments involving steps between solutions of differing concentrations of KCl, solution changes were achieved in 20–30 ms. In cell-attached patch experiments, the recording pipette solution was identical to the recording chamber solution (see earlier text) with the addition of either 100 nM ACh alone or 100 nM ACh with 100 μ M Zn²⁺. Patches were voltage clamped using an Axopatch 200A integrating patch clamp (Molecular Devices) and held at –100 mV. A +60-mV holding command voltage was applied to hold the patch at approximately –120 mV (assuming a resting potential of –60 mV). Current re-

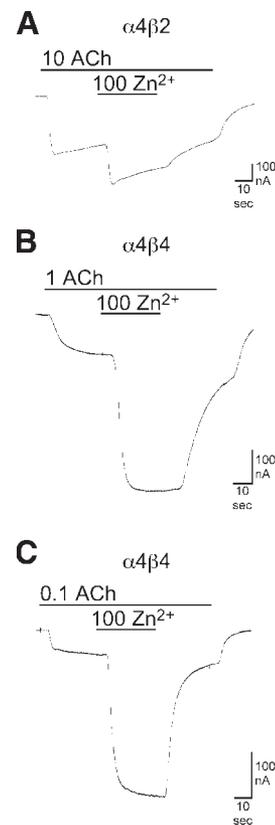


FIG. 1. Zinc potentiates neuronal nicotinic receptors. *A*: current response of an $\alpha 4\beta 2$ -expressing oocyte to 10 μ M acetylcholine (ACh) before, during, and after coapplication of 100 μ M Zn²⁺. *B*: current response of an $\alpha 4\beta 4$ -expressing oocyte to 1 μ M ACh before, during, and after coapplication of 100 μ M Zn²⁺. *C*: current response of an $\alpha 4\beta 4$ -expressing oocyte to 100 nM ACh before, during, and after coapplication of 100 μ M Zn²⁺. *B* and *C* show responses from different oocytes. The wide variation in expression level from oocyte to oocyte results in these responses to 2 different ACh concentrations having similar amplitudes.

sponses were filtered (eight-pole, Bessel low-pass) at 4 kHz (–3 dB) and sampled at 15.4 kHz using a Digidata 1322A (Molecular Devices) and a PC running Clampex 8.2 (Molecular Devices).

Data analysis

Prism software (GraphPad, San Diego, CA) was used to assess statistical significance using a two-tailed unpaired *t*-test or one-way ANOVA with a Dunnett's post test, as appropriate. The magnitude of Zn²⁺ potentiation in Figs. 1 and 2 was measured as previously described (Hsiao et al. 2001, 2006). Briefly, when no desensitization was apparent (all $\alpha 4\beta 4$ currents), control current in response to ACh was determined from a 1-s average beginning 29 s after initiation of agonist application and compared with a 1-s average of baseline current immediately prior to ACh application. Current levels during Zn²⁺ coapplication were determined from a 1-s average beginning 29 s after initiation of Zn²⁺ application and compared with the control current. When desensitization was apparent (all $\alpha 4\beta 2$ currents), the initial 30-s ACh response in the absence of Zn²⁺ was fit to a single-exponential decay function. This fit was projected over the next 30 s during which both ACh and Zn²⁺ were coapplied. The degree of modulation was measured by taking a 1-s average 29 s after initiation of Zn²⁺ application and comparing it to a 1-s average of the projected response to ACh alone during the same time period. Single-channel data were analyzed using Clampfit 8.2 software (Molecular Devices), custom software developed by K. L. Magleby, and the QUB software suite, v. 1.1.0.2538, developed by Qin, Auerbach, and Sachs at

the State University of New York at Buffalo (Qin et al. 1996). Data were preconditioned for analysis using Clampfit by low-pass fourth-order Bessel filtering at 1.5 kHz, adjusting for baseline variation and eliminating artifactual events.

Single-channel amplitude was measured in outside-out patches. Single-channel activity was observed following inactivation of the multiple-channel response. The average amplitude of all openings at the single-channel level was measured from each patch. Outside-out patches were not used to measure open probability because channel activity ceased in the outside-out configuration within 1 min of continual agonist application. Even after extended periods of wash in control solution and the addition of ATP in the pipette solution,

channel activity was never restored. This phenomenon, termed "run-down," has been reported by others (Ballivet 1988; Buisson 1996). For this reason, cell-attached patches were used to obtain data to evaluate open probability.

Open probability of single channels in cell-attached patches was assessed using both 50% threshold analysis with custom software and segmental K-means idealization (SKM) in the QUB software suite. Only patches lasting for >10 min were analyzed. Binomial analysis has shown that when P_o is low, it is not possible to estimate the maximum number of channels in a patch from overlap of channel opening (Horn 1991). For this reason, we will represent our measurement of open probability as nP_o , which should be regarded as an upper estimate of P_o . Patches containing stretch-activated channels were excluded from analysis (Silberberg and Magleby 1997).

Stable data, as evaluated with stability plots (Weiss and Magleby 1990), were used for the burst analysis, based on six patches with ACh alone and five patches with ACh and Zn^{2+} . Burst analysis was conducted using custom software by first defining the critical time (t_{crit}), the length of time between bursts as described by Magleby and Pallotta (1983). The t_{crit} was determined by fitting the distributions of closed-interval durations with the sum of several exponential components. The intervals in the exponentials with the longest time constants were then used to define the gaps between bursts. Because there was typically a difference of about three orders of magnitude in the mean durations of the closed intervals generating gaps between bursts from the much briefer duration closed intervals generating gaps within bursts, there was no ambiguity in assigning exponential components to gaps between bursts or within bursts. The t_{crit} was defined such that the number of closed intervals between bursts misclassified as closed intervals within bursts was equal to the number of closed intervals within bursts misclassified as closed intervals between bursts. Thus errors resulting from misclassification of intervals between and within bursts would tend to cancel out. Analysis of the intraburst subconductance architecture was accomplished by using the t_{crit} to isolate individual bursts in QUB, assigning each of the four states (closed, two subconductance levels, and fully open; see Fig. 4) to a corresponding state in a model, and idealizing the data using the SKM method in QUB.

As subsequently shown in RESULTS, Zn^{2+} increased the ACh-induced peak currents recorded from oocytes at the whole cell level by about four- to fivefold, while having much less of an effect on the peak macroscopic currents recorded from outside patches. To determine whether this difference might be associated with the rapid rundown/desensitization observed with outside-out patches, simula-

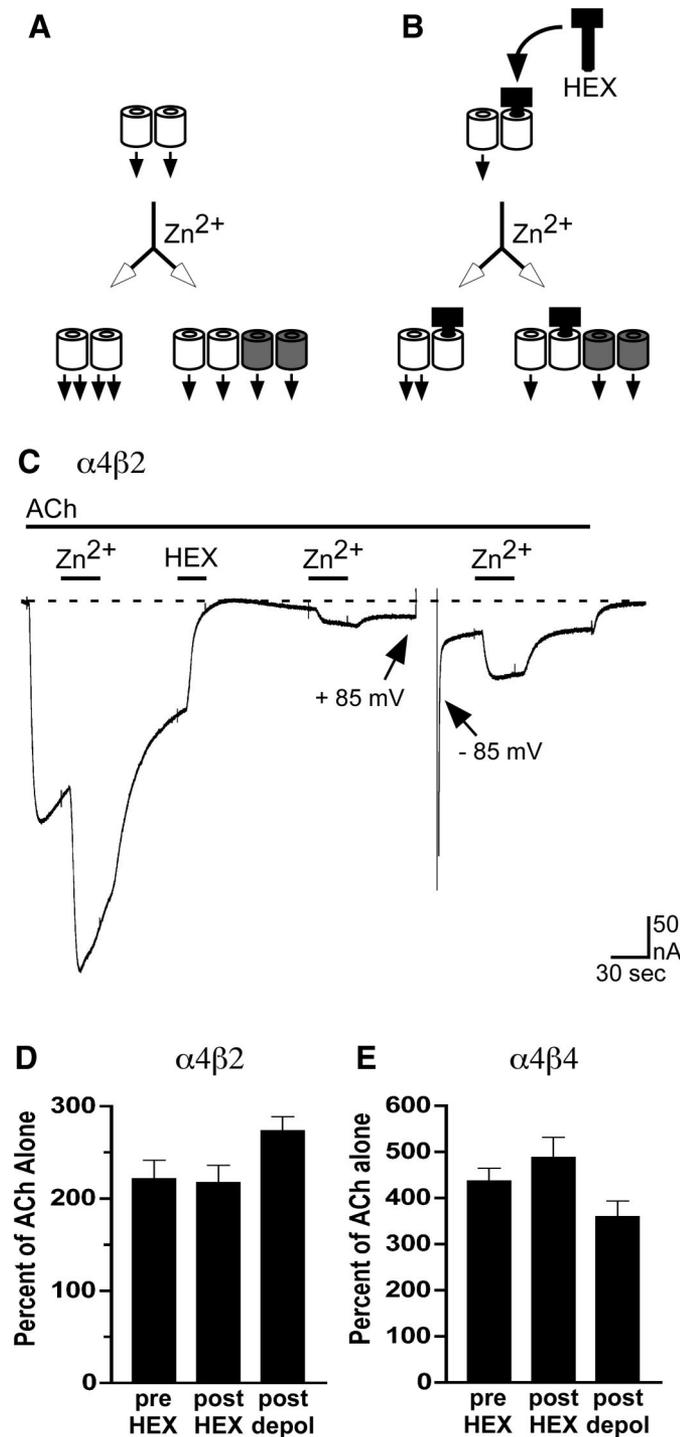


FIG. 2. Zinc does not increase n , the number of available receptors. *A*: potentiation of nicotinic acetylcholine receptors (nAChRs) by Zn^{2+} may be due to an increase in the activity of individual receptors (*bottom left*) or an increase in the number of receptors due to activation of a previously inactive population (*bottom right*). *B*: after open channel blockade of a portion of the active receptors by hexamethonium (HEX), the extent of Zn^{2+} potentiation should be similar if the properties of individual receptors change (*bottom left*) or should be greater if a previously inactive population of receptors becomes active (*bottom right*). *C*: potentiation of the ACh (10 μ M) response of an $\alpha4\beta2$ -expressing oocyte by 100 μ M Zn^{2+} is shown before and after application of HEX (30 μ M) and following reversal of blockade by depolarization to +85 mV. The dashed line indicates the baseline current level. *D*: open channel blockade does not alter the extent of potentiation of $\alpha4\beta2$ receptors. The response of $\alpha4\beta2$ -expressing oocytes to coapplication of 10 μ M ACh and 100 μ M Zn^{2+} is plotted as a percentage of the response to ACh alone (means \pm SE, $n = 3$). There were no significant differences in potentiation by Zn^{2+} before and after application of 30 μ M HEX or following reversal of HEX blockade ($P = 0.128$, one-way ANOVA). *E*: open channel blockade does not alter the extent of potentiation of $\alpha4\beta4$ receptors. The response of $\alpha4\beta4$ -expressing oocytes to coapplication of 1 μ M ACh and 100 μ M Zn^{2+} is plotted as a percentage of the response to ACh alone (means \pm SE, $n = 5$). There were no significant differences in potentiation by Zn^{2+} before and after application of 10 μ M HEX or following reversal of HEX blockade ($P = 0.073$, one-way ANOVA).

tions with a model for desensitization were carried out. Since little is known about desensitization of neuronal ACh receptors, a simple model without cyclic recovery was assumed: D-C-O-D, where D, C, and O are desensitized, closed, and open states, respectively. Similar results were obtained with a more complex model: D-C-C-O-D. Rate constants were selected to obtain a response like that in Fig. 3 for ACh and then the rate constants were changed to make the open probability fourfold greater and the response recalculated. This was not meant to be a study of desensitization, but to answer the question of whether desensitization could differentially alter the peak responses, depending on the open probability.

RESULTS

Zn^{2+} potentiates neuronal nicotinic receptors

The $\alpha 4\beta 2$ and $\alpha 4\beta 4$ neuronal nAChRs are potentiated by Zn^{2+} (Fig. 1). We previously found that maximal potentiation of these receptors occurred at $100 \mu M Zn^{2+}$ and that potentiation was most pronounced at low ACh concentrations (Hsiao et al. 2001). For this reason, we used $100 \mu M Zn^{2+}$ and low ACh concentrations ($\leq EC_{10}$) throughout the present study. Coapplication of $100 \mu M Zn^{2+}$ potentiates the response of $\alpha 4\beta 2$ receptors to $10 \mu M$ ACh by $215 \pm 26\%$ ($n = 4$), whereas the response of $\alpha 4\beta 4$ receptors to $1 \mu M$ ACh is potentiated by $435 \pm 30\%$ ($n = 7$). These values are similar to those in our previous work (Hsiao et al. 2001, 2006).

Zn^{2+} does not increase the number of available receptors

One possible mechanism for Zn^{2+} potentiation of the macroscopic current response is through an increase in n , the number of available receptors. In this mechanism, Zn^{2+} might reveal a population of neuronal nAChRs that were previously inactive despite the presence of agonist. We used the antagonist hexamethonium (HEX) to test this possibility. Experiments with both parasympathetic ganglion neurons and $\alpha 4\beta 2$ -

expressing *Xenopus* oocytes have shown that in the presence of agonist and under hyperpolarizing conditions, HEX can become trapped within the ion pore of the receptor (Ascher et al. 1979; Bertrand et al. 1990; Gurney and Rang 1984). Depolarization in the presence of agonist is required to eject HEX from the pore and allow normal function of the channel to resume (Bertrand et al. 1990; Gurney and Rang 1984). We took advantage of these properties of HEX to determine whether a fixed population of receptors is passing additional current in the presence of Zn^{2+} (Fig. 2A, bottom left) or whether the number of available receptors is increased in the presence of Zn^{2+} (Fig. 2A, bottom right). If a portion of the active receptors is blocked by HEX, the extent of potentiation of the remaining unblocked receptors should allow us to distinguish between these two possibilities. If, after partial HEX blockade, the remaining unblocked receptors pass additional current (Fig. 2B, bottom left), then the extent of potentiation should be similar to that seen prior to HEX blockade. If there is an increase in the number of available receptors (Fig. 2B, bottom right), then the extent of potentiation should be greater than that seen prior to HEX blockade.

A representative trace is provided in Fig. 2C to illustrate the HEX experiment. Before application of HEX, the response of $\alpha 4\beta 2$ receptors to $10 \mu M$ ACh is potentiated on coapplication of $100 \mu M Zn^{2+}$ by approximately twofold (left portion of trace). Following a 20-s application of $30 \mu M$ HEX, a substantial fraction of the ACh response is blocked ($\sim 85\%$, when desensitization is taken into account). The initial blockade of the receptor activity by HEX is a combination of both competitive block and open channel block (discussed earlier). After a brief wash period (~ 1 min) to remove the HEX, only the open channel blockade remains. Potentiation of the remaining ACh response by the second application of $100 \mu M Zn^{2+}$ was also approximately twofold (middle portion of trace). Results with multiple $\alpha 4\beta 2$ -expressing oocytes are provided in Fig. 2D. Potentiation of $\alpha 4\beta 4$ receptors was also unaffected by HEX blockade. Potentiation before and after HEX ($10 \mu M$) application had blocked approximately half of the active receptors was 437 ± 28 and $488 \pm 44\%$, respectively (Fig. 2E). For both $\alpha 4\beta 2$ and $\alpha 4\beta 4$, potentiation after relief of HEX block (by depolarization in the presence of ACh) did not differ from the extent of potentiation prior to HEX application. For both receptors, the similar extent of potentiation before and after HEX blockade indicates that Zn^{2+} does not reveal a new population of receptors. Instead, Zn^{2+} must act by altering the properties of a fixed population of receptors.

Zn^{2+} does not increase the single-channel current amplitude

A second possible mechanism for Zn^{2+} potentiation is through an increase in the current through individual channels. If a change in single-channel current amplitude were to account for the potentiation of $\alpha 4\beta 4$ receptors by Zn^{2+} , then single-channel current amplitude would have to increase four- to fivefold in the presence of Zn^{2+} . To examine this possibility, channel activity of $\alpha 4\beta 4$ receptors in response to $1 \mu M$ ACh was recorded in patches in the outside-out configuration in the absence and the presence of $100 \mu M Zn^{2+}$ (Fig. 3). The current recordings in Fig. 3 were selected to be of similar peak amplitude for ease of comparison. After an initial multichannel response, the current decayed to a level where individual

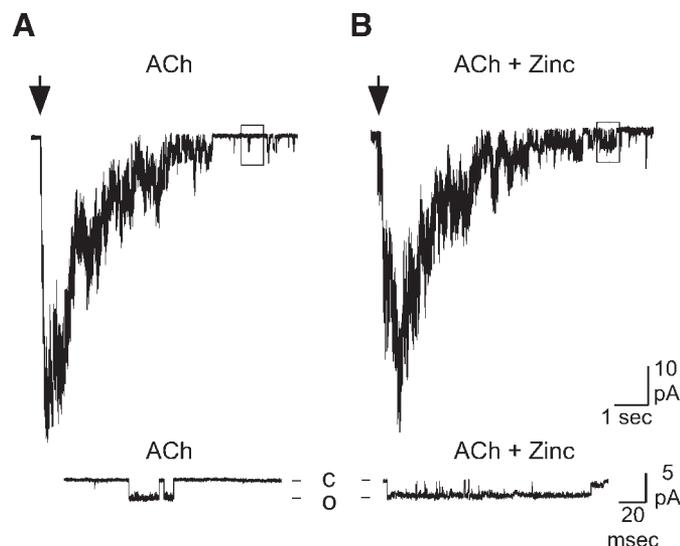


FIG. 3. Zinc does not increase i , the single-channel current amplitude. Recordings were obtained from outside-out patches (holding potential: -100 mV) containing multiple $\alpha 4\beta 4$ receptors in response to application (arrows) of $1 \mu M$ ACh alone (A), or $1 \mu M$ ACh and $100 \mu M Zn^{2+}$ (B). The multichannel response rapidly inactivated during maintained application of agonist until single-channel events could be discerned. At the bottom of each panel, the indicated sections of each recording are expanded to show the amplitude of a single channel in the absence (A) and presence (B) of Zn^{2+} .

channels could be observed. Channel amplitude was assessed from these single-channel events (here we are measuring the largest and most prevalent channel amplitude; two subconductance levels are discussed in the following text). The mean current amplitude for 33 open intervals from three separate patches in the presence of ACh alone and 34 open intervals from three patches in the presence of ACh and Zn^{2+} were measured. When activated by $1 \mu M$ ACh, the single-channel amplitude was 3.0 ± 0.1 pA. When activated by $1 \mu M$ ACh in the presence of $100 \mu M Zn^{2+}$, the single-channel amplitude was 2.9 ± 0.1 pA. Thus the single-channel current amplitude of these receptors is not altered by the presence of Zn^{2+} ($P = 0.24$, two-tailed, unpaired *t*-test).

The current recordings in Fig. 3 were selected to be of similar peak amplitude for ease of comparison. Results from four patches exposed to ACh yielded a peak amplitude of 44 ± 12 pA, whereas four patches exposed to ACh + Zn^{2+} yielded a peak amplitude of 88 ± 29 pA (means \pm SE). These values were not significantly different ($P = 0.21$, two-tailed, unpaired *t*-test). The large variability presumably reflects differing numbers of channels in the patches. The question that arises is why are not the peak amplitudes in the presence of zinc fourfold greater than the peak amplitudes in the absence of zinc? In contrast to Fig. 1, these responses are obtained in excised outside-out patches that display rapid rundown/desensitization. To explore whether rapid rundown/desensitization might be involved in determining peak amplitudes, simulations of gating with simple models for rundown/desensitization (see METHODS) indicated that peak macro current amplitudes resulting from a step application of ACh in the presence of Zn^{2+} could be increased only 30–100% over that in ACh alone, even though mean open time before desensitization was increased 400%. These simulations (not shown) suggest that the pronounced rundown/desensitization associated with outside-out patches could lead to less of a difference between peak currents in ACh and ACh plus Zn^{2+} in the presence of rundown/desensitization than in its absence. In any case, these experiments in the outside-out patch configuration, which allows precise control of voltage, are sufficient to show that single-channel amplitude is unchanged by zinc. Because of the rapid rundown/desensitization, single-channel data for the remainder of our study were collected in the cell-attached configuration.

Zn^{2+} increases the single-channel open probability

A third potential mechanism for Zn^{2+} potentiation is an increase in the open probability of individual receptors. To examine this possibility, single-channel openings were recorded in the cell-attached patch configuration on $\alpha 4\beta 4$ -expressing oocytes. In initial experiments we applied $1 \mu M$ ACh, although this concentration resulted in a loss of channel activity in about 1 min, presumably due to desensitization. To obtain suitably long recordings, we tried lower ACh concentrations and found that use of 100 nM ACh would yield channel activity in individual patches that could be recorded for the duration of the patch (10–20 min). At the whole cell level, potentiation of the response to this low concentration of ACh by $100 \mu M Zn^{2+}$ was $548 \pm 39\%$ ($n = 7$, Fig. 1C).

In the cell-attached patch configuration the receptors are in constant contact with the agonist in the patch pipette, so that exposure of receptors in an individual patch to solutions with

and without agonist was not done. Thus it was important to establish that the channel activity we observe is due to receptor activation. First, channel activity was not observed when ACh was not included in the pipette solution. Second, the channel activity occurred in bursts lasting for hundreds of milliseconds, interspersed with longer periods of inactivity (Fig. 4A). This bursting activity is typical of $\beta 4$ -subunit-containing receptors (Papke and Heinemann 1991). Our use of a very low concentration of ACh (100 nM) suggests that each burst represents a single receptor activation—the channel activity during a single episode of agonist binding (Gibb and Colquhoun 1991). Last, two distinct subconductance levels (at ~ 34 and 69% of the amplitude of the fully open state) could be observed within the bursts (Fig. 4C). Bursting activity and the presence of two distinct subconductance levels were also observed in patches recorded in the outside-out configuration where channel activity can be compared in the presence and the absence of ACh (Fig. 3).

Analysis of the cell-attached patch recordings revealed that Zn^{2+} exerts a large effect on the nP_o of $\alpha 4\beta 4$ receptors (Fig. 5, Table 1). Using 50% threshold analysis (see METHODS), an nP_o of 0.0111 ± 0.0016 was observed in the presence of 100 nM ACh. Coapplication of $100 \mu M Zn^{2+}$ with 100 nM ACh increased nP_o to 0.0495 ± 0.0084 . Results were similar when using the SKM algorithm in QUB ($nP_o = 0.0118 \pm 0.0018$ in the absence of Zn^{2+} ; $nP_o = 0.0541 \pm 0.0083$ in the presence of Zn^{2+}). Thus nP_o was increased by approximately 4.5-fold in the presence of Zn^{2+} . This accounts for most of the 5.5-fold increase in current seen at the whole cell level when $100 \mu M Zn^{2+}$ is coapplied with 100 nM ACh. SKM also allowed analysis of the effect of Zn^{2+} on the individual subconductance levels. As we observed for the largest conductance level (see earlier text), the amplitudes of the two subconductance levels were unaffected by Zn^{2+} . Although Zn^{2+} did not significantly increase the open probability of the rare (accounting for $\sim 2\%$ of the time spent in bursts) lower subconductance level ($P = 0.089$, two-tailed unpaired *t*-test), the open probabilities of the more common upper subconductance state and the fully open conductance state were increased by 3.1- and 5.7-fold, respectively ($P < 0.0001$ and $P < 0.01$, respectively, two-tailed unpaired *t*-test).

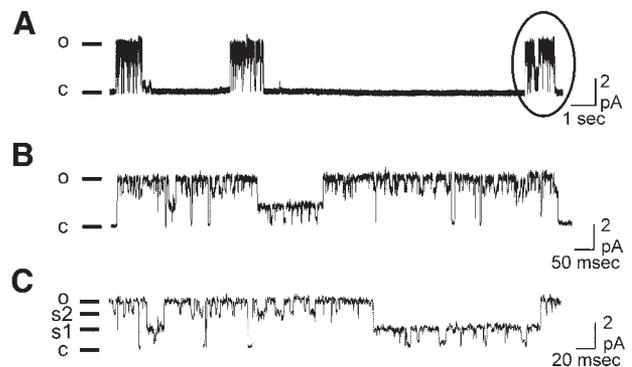


FIG. 4. Single-channel recording from a cell-attached patch of an $\alpha 4\beta 4$ -expressing oocyte. The recording pipette was filled with $0.1 \mu M$ ACh and $100 \mu M Zn^{2+}$. A: 3 successive bursts of activity are shown. B: an expanded view of the circled burst from A reveals multiple subconductance levels. C: further expansion of the third burst illustrates 2 distinct subconductance levels (s1 and s2) at 34 and 69% of the fully open state. The lower subconductance level (s1) was rare, accounting for about 2% of the time spent in bursts. This record was selected to illustrate this infrequent subconductance level.

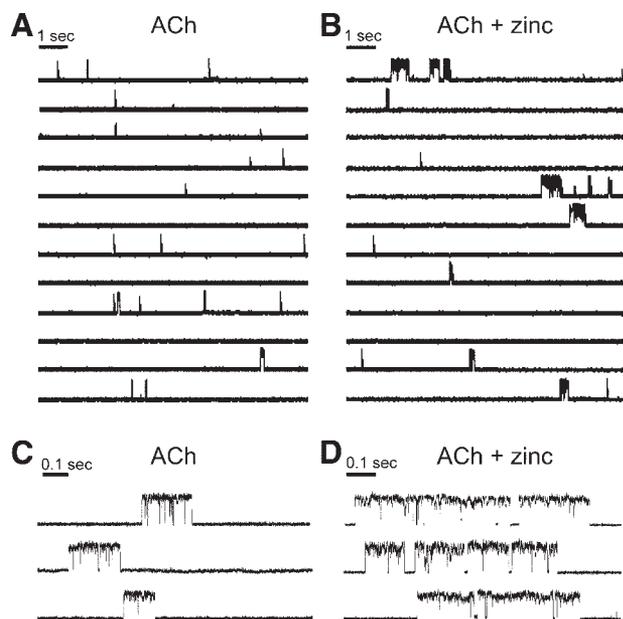


FIG. 5. *A*: a continuous trace from a single cell-attached patch on an $\alpha 4\beta 4$ -expressing oocyte in the presence of $0.1 \mu\text{M}$ ACh. Open events are in an upward direction and are about 3 pA when fully open. *B*: a continuous trace from a single cell-attached patch on an $\alpha 4\beta 4$ -expressing oocyte in the presence of both $0.1 \mu\text{M}$ ACh and $100 \mu\text{M}$ Zn^{2+} . *C*: representative bursts from cell-attached patches from $\alpha 4\beta 4$ -expressing oocytes are shown when only $0.1 \mu\text{M}$ ACh was in the recording pipette. *D*: representative bursts from patches when the recording pipette contained $0.1 \mu\text{M}$ ACh and $100 \mu\text{M}$ Zn^{2+} .

As expected from the stochastic nature of single-channel gating (Colquhoun and Hawkes 1982), a wide range of burst durations was observed in both the absence and the presence of Zn^{2+} , as shown by the representative traces in Fig. 5 where it can be seen that the presence of Zn^{2+} greatly increases the mean duration of the bursts. Analysis of the burst durations (Fig. 6, Table 1) reveals a fourfold increase in burst duration due to the coapplication of Zn^{2+} , which accounts for the increase in nP_o .

DISCUSSION

Zinc dramatically potentiates the function of $\alpha 4$ -containing neuronal nAChRs (Hsiao et al. 2001, 2006). We investigated

TABLE 1. *Single-channel and burst analysis of $\alpha 4\beta 4$ nAChRs*

Parameter	No Zn^{2+}	With Zn^{2+}	With Zn^{2+} /No Zn^{2+}
nP_o (half-threshold method)	0.0111 ± 0.0016	$0.0495 \pm 0.0084^{***}$	4.46
nP_o (SKM method)	0.0118 ± 0.0018	$0.0541 \pm 0.0083^{***}$	4.58
Critical time	$494 \pm 142 \text{ ms}$	$682 \pm 190 \text{ ms}$	1.38
Mean open time	$15 \pm 4 \text{ ms}$	$22 \pm 5 \text{ ms}$	1.47
Mean shut time	$1,466 \pm 513 \text{ ms}$	$667 \pm 210 \text{ ms}$	0.45
Mean number of bursts per patch	71 ± 19	81 ± 27	1.14
Number of intraburst openings	9 ± 1	$30 \pm 8^*$	3.33
Number of intraburst closings	8 ± 1	$29 \pm 8^*$	3.62
Duration of intraburst open intervals	$18 \pm 3 \text{ ms}$	$24 \pm 4 \text{ ms}$	1.33
Duration of intraburst shut intervals	$8 \pm 2 \text{ ms}$	$8 \pm 3 \text{ ms}$	1.00
Burst duration	$207 \pm 38 \text{ ms}$	$830 \pm 189 \text{ ms}^{**}$	4.01
Interburst gap duration	$15,710 \pm 5,300 \text{ ms}$	$14,370 \pm 4,720 \text{ ms}$	0.91
Single-channel amplitude	$3.0 \pm 0.1 \text{ pA}$	$2.9 \pm 0.1 \text{ pA}$	0.97

Values are means \pm SE, based on six patches without Zn^{2+} and five patches in the presence of Zn^{2+} , with an average of 560 open intervals per patch without Zn^{2+} and 1,860 per patch in the presence of Zn^{2+} . Values that are significantly different in the presence of Zn^{2+} are denoted with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

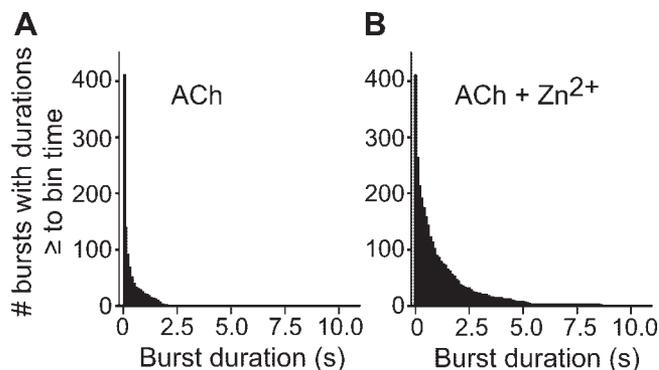


FIG. 6. Zinc increases burst duration. Cumulative histograms of burst durations are shown. Each bar represents the number of bursts of that duration or greater. *A*: burst durations in the presence of 100 nM ACh alone; 351 bursts from 6 patches are analyzed. The mean burst duration is $207 \pm 38 \text{ ms}$. The numbers of observations are multiplied by $415/351$ to allow direct comparison with *B*. *B*: burst durations in the presence of 100 nM ACh and $100 \mu\text{M}$ Zn^{2+} ; 415 bursts from 5 patches are analyzed. The mean burst duration is $830 \pm 189 \text{ ms}$.

the mechanism of Zn^{2+} potentiation by examining the effect of Zn^{2+} on the factors that contribute to the total amount of current passed by a population of these receptors. One possible mechanism is an increase in the total number of available receptors, either by activating a previously unresponsive population or by slowing the rate at which receptors enter a closed desensitized state. However, we found no evidence for the activation of a cryptic population of receptors by Zn^{2+} . We have also ruled out an effect of Zn^{2+} on the time course of desensitization onset in our previous work (Hsiao et al. 2006) and in the current study, where in Fig. 3 and additional experiments, the time constant for desensitization in the absence and the presence of Zn^{2+} was $\tau = 3.2 \pm 0.8$ and $3.0 \pm 0.7 \text{ s}$, respectively ($n = 4$ for each condition, $P = 0.836$, two-tailed unpaired t -test). Another possible mechanism for the potentiation by Zn^{2+} is an increase in the single-channel current amplitude. However, we found the amplitude of single-channel events to be unchanged in the presence and the absence of Zn^{2+} . Instead, we found Zn^{2+} potentiation is due to an increase in channel open probability.

Burst analysis of the recordings from cell-attached patches revealed that the Zn^{2+} -mediated increase in open probability was associated with a fourfold lengthening of the mean burst

duration, with little effect on the gap between bursts (Table 1). Thus once a channel enters a bursting mode, Zn^{2+} appears to stabilize it within the bursting states before it returns to the long-lasting closed states. If Zn^{2+} acted instead by facilitating the entrance of the channels into bursts by facilitating the binding of ACh, then the gap between bursts might be expected to decrease about fourfold. Our observation that the gap between bursts is unchanged by zinc most likely rules out the possibility that Zn^{2+} acts by facilitating the binding of ACh; i.e., Zn^{2+} does not act by inducing an allosteric conformational change that increases the access of ACh to its binding site or increases the probability that a collision of ACh with its binding site results in a successful binding. It should be noted, however, that a conclusion of a lack of effect of zinc on interburst gap duration requires that the average number of channels in patches examined with and without zinc be the same. We have no reason to suspect that the number of channels in each patch would be different, in part because some patches exposed to ACh or ACh plus zinc were obtained from the same oocyte. Thus the only way that zinc might increase the number of channels is by a mechanism of activating "silent" channels, a mechanism that we ruled out in Fig. 2. Therefore it is likely that zinc has little effect on the interburst gap duration and, consequently, is not facilitating the effective binding rate of ACh to its receptor as a mechanism of receptor potentiation. Our use of a very low agonist concentration (100 nM ACh) suggests that the termination of a burst represents a dissociation of agonist from the receptor, rather than the entry of the receptor into a desensitized state (Gibb and Colquhoun 1991). Further support for burst termination from agonist dissociation rather than desensitization with 100 nM ACh are the observations of no apparent desensitization with 100 nM or 1 μ M agonist application over many tens of seconds (Fig. 1, B and C). Desensitization becomes apparent only when the agonist concentration is increased to 10 μ M (data not shown), 100-fold greater than what we used for the bursting analysis. Taken together, these observations suggest that Zn^{2+} acts by stabilizing the agonist-bound set of open and closed states that generate bursts, mainly by decreasing the probability of leaving the bursting states. The dramatic increase in burst duration with Zn^{2+} in our experiments suggests that Zn^{2+} , at an appropriate concentration, might increase the durations of excitatory postsynaptic potentials generated by neuronal nicotinic receptors.

Our observations and other studies (Lewis et al. 1997; Sivilotti et al. 1997) suggest that the gating of neuronal ACh receptors is complex, with multiple conductance levels. We find that the $\alpha 4\beta 4$ channel enters at least two subconductance states during gating: one with a subconductance level of 69% of the fully open level that is a consistent feature of gating, and one with a subconductance level 34% of the fully open level that is entered only about 2% of the time. Zn^{2+} does not affect the amplitude of either of the subconductance states or the fully open state. However, Zn^{2+} does increase the open probability of both the upper subconductance state and the fully open state. Zn^{2+} does not appear to affect the open probability of the lower subconductance state.

Zn^{2+} also potentiates glycine receptors (Bloomenthal et al. 1994; Laube et al. 1995). Although the Zn^{2+} potentiation sites on glycine receptors and neuronal nAChRs are structurally distinct (Harvey et al. 1999; Hsiao et al. 2006; Laube et al.

2002), the mechanism of Zn^{2+} potentiation of these two receptor classes shows some similarity. Much like our findings with neuronal nAChRs, potentiating concentrations of Zn^{2+} increase the burst duration without altering the single-channel amplitude of glycine receptors (Laube et al. 2000). Zn^{2+} also modulates the function of GABA_A receptors, although this modulation is functionally distinct (GABA_A receptors are inhibited, not potentiated) and structurally distinct from neuronal nAChR modulation (Hosie et al. 2003; Hsiao et al. 2006; Smart et al. 2004).

The mechanism of Zn^{2+} potentiation of neuronal nAChRs shows some similarity to the mechanism of benzodiazepine potentiation of GABA receptors: both modulators increase the P_o . However, benzodiazepine potentiation of GABA receptors appears to be achieved by an increase in channel opening frequency, without an effect on burst duration (Rogers et al. 1994; Vicini et al. 1987). Importantly, the location of the Zn^{2+} potentiation sites of neuronal nAChRs, at the subunit-subunit interfaces that alternate with the interfaces that bind ACh (Hsiao et al. 2006), is analogous to the location of the high-affinity benzodiazepine-binding site of GABA receptors (Amin et al. 1997; Boileau et al. 1998; Kucken et al. 2000; Teissere and Czajkowski 2001). In particular, the $\alpha 4E59$ residue that we have localized to the zinc potentiation site of neuronal nAChRs (Hsiao et al. 2006) is in a location identical to that of the benzodiazepine-binding site residue $\gamma 2T81$ of GABA_A receptors (Kucken et al. 2003; Teissere and Czajkowski 2001).

The potentiating action of benzodiazepines is thought to occur through an allosteric mechanism in which the relative stability of the closed and open states is altered, shifting the equilibrium toward the open state (Campo-Soria et al. 2006; Downing et al. 2005). The increase in burst duration that we observe suggests that Zn^{2+} potentiates neuronal nAChRs by stabilizing the bursting states of the receptor. The recent examination of atomic structures of the soluble acetylcholine binding protein complexed with a variety of nicotinic ligands (Hansen et al. 2005) provides a structural framework for understanding this mechanism of Zn^{2+} potentiation. In the heteromeric neuronal nAChRs we are studying, agonist-binding sites are located at the α/β interface. When the agonist-binding sites are occupied by agonist, the C-loop of the α subunit accommodates the structure of the agonist by moving closer to the vertical axis of the receptor. This movement, termed "C-loop closure," may be part of a conformational change associated with channel opening (Hansen et al. 2005). We previously identified residues on the $\alpha 4$ subunit that mediate zinc potentiation and proposed that these sites are located at the $\beta-\alpha$ interfaces (Hsiao et al. 2006). It has also recently been proposed that these residues can participate in forming a zinc potentiation site at the $\alpha-\alpha$ interface in an $(\alpha 4)_3(\beta 2)_2$ receptor (Moroni et al. 2007). If the bursting episodes that we observe represent gating of agonist-bound receptors (see earlier text), then the increase in duration of these episodes in the presence of Zn^{2+} suggests that Zn^{2+} may be acting at the $\beta-\alpha$ interfaces (or possibly an $\alpha-\alpha$ interface) to stabilize C-loop closure, thus prolonging the burst and potentiating the macroscopic response. The structural similarity with the high-affinity benzodiazepine sites of GABA receptors together with the proposed C-loop closure model of subunit-subunit interface function make the Zn^{2+} potentia-

tion sites of neuronal nAChRs an attractive target for future drug development.

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REFERENCES

- Amin J, Brooks-Kayal A, Weiss DS. Two tyrosine residues on the alpha subunit are crucial for benzodiazepine binding and allosteric modulation of gamma-aminobutyric acid_A receptors. *Mol Pharmacol* 51: 833–841, 1997.
- Ascher P, Large WA, Rang HP. Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J Physiol* 295: 139–170, 1979.
- Assaf SY, Chung S-H. Release of endogenous Zn²⁺ from brain tissue during activity. *Nature* 308: 734–736, 1984.
- Ballivet M, Nef P, Couturier S, Rungger D, Bader CR, Bertrand D, Cooper E. Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes after cDNA injection. *Neuron* 1: 847–852, 1988.
- Bertrand D, Ballivet M, Rungger D. Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 87: 1993–1997, 1990.
- Bloomenthal AB, Goldwater E, Pritchett DB, Harrison NL. Biphasic modulation of the strychnine-sensitive glycine receptor by zinc. *Mol Pharmacol* 46: 1156–1159, 1994.
- Boileau AJ, Kucken AM, Evers AR, Czajkowski C. Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric γ -aminobutyric acid_A receptor subunits. *Mol Pharmacol* 53: 295–303, 1998.
- Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, Bertrand D. Human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor in HEK293 cells: a patch-clamp study. *J Neurosci* 16: 7880–7891, 1996.
- Campo-Soria C, Chang Y, Weiss DS. Mechanism of action of benzodiazepines on GABAA receptors. *Br J Pharmacol* 148: 984–990, 2006.
- Colquhoun D, Hawkes AG. On the stochastic properties of bursts of single ion channel openings and of clusters of bursts. *Philos Trans R Soc Lond B Biol Sci* 24: 1–59, 1982.
- Downing SS, Lee YT, Farb DH, Gibbs TT. Benzodiazepine modulation of partial agonist efficacy and spontaneously active GABA(A) receptors supports an allosteric model of modulation. *Br J Pharmacol* 145: 894–906, 2005.
- Frederickson CJ, Suh SW, Silva D, Frederickson CJ, Thompson RB. Importance of zinc in the central nervous system: the zinc-containing neuron. *J Nutr* 130: 1471S–1483S, 2000.
- Garcia-Colunga J, Gonzalez-Herrera M, Miledi R. Modulation of alpha2beta4 neuronal nicotinic acetylcholine receptors by zinc. *Neuroreport* 12: 147–150, 2001.
- Gibb AJ, Colquhoun D. Glutamate activation of a single NMDA receptor-channel produces a cluster of channel openings. *Proc Biol Sci* 243: 39–45, 1991.
- Gurney AM, Rang HP. The channel-blocking action of methonium compounds on rat submandibular ganglion cells. *Br J Pharmacol* 82: 623–642, 1984.
- Hansen SB, Sulzenbacher G, Huxford T, Marchot P, Taylor P, Bourne Y. Structures of Aplysia AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* 24: 3635–3646, 2005.
- Harvey RJ, Thomas P, James CH, Wilderspin A, Smart TG. Identification of an inhibitory Zn²⁺ binding site on the human glycine receptor $\alpha 1$ subunit. *J Physiol* 520: 53–64, 1999.
- Horn R. Estimating the number of channels in patch recordings. *Biophys J* 60: 433–439, 1991.
- Hosie AM, Dunne EL, Harvey RJ, Smart TG. Zinc-mediated inhibition of GABAA receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* 6: 362–369, 2003.
- Howell GA, Welch MG, Frederickson CJ. Stimulation-induced uptake and release of zinc in hippocampal slices. *Nature* 308: 736–738, 1984.
- Hsiao B, Dweck D, Luetje CW. Subunit-dependent modulation of neuronal nicotinic receptors by zinc. *J Neurosci* 21: 1848–1856, 2001.
- Hsiao B, Mihalak KB, Repicky SE, Everhart D, Mederos AH, Malhotra A, Luetje CW. Determinants of zinc potentiation on the alpha4 subunit of neuronal nicotinic receptors. *Mol Pharmacol* 69: 27–36, 2006.
- Huang EP. Metal ions and synaptic transmission: think zinc. *Proc Natl Acad Sci USA* 94: 13386–13387, 1997.
- Izumi Y, Auberson YP, Zorumski CF. Zinc modulates bidirectional hippocampal plasticity by effects on NMDA receptors. *J Neurosci* 26: 7181–7188, 2006.
- Kay A. Evidence for chelatable zinc in the extracellular space of the hippocampus, but little evidence for synaptic release of Zn. *J Neurosci* 23: 6847–6855, 2003.
- Kodirov SA, Takizawa S, Joseph J, Kandel ER, Shumyatsky GP, Bolshakov VY. Synaptically released zinc gates long-term potentiation in fear conditioning pathways. *Proc Natl Acad Sci USA* 103: 15218–15223, 2006.
- Kucken AM, Teissere JA, Seffinga-Clark J, Wagner DA, Czajkowski C. Structural requirements for imidazobenzodiazepine binding to GABA_A receptors. *Mol Pharmacol* 63: 289–296, 2003.
- Kucken AM, Wagner DA, Ward PR, Teissere JA, Boileau AJ, Czajkowski C. Identification of benzodiazepine binding site residues in the $\gamma 2$ subunit of the γ -aminobutyric acid_A receptor. *Mol Pharmacol* 57: 932–939, 2000.
- Laube B, Kuhse J, Betz H. Kinetic and mutational analysis of Zn²⁺ modulation of recombinant human inhibitory glycine receptors. *J Physiol* 522: 215–230, 2000.
- Laube B, Kuhse J, Rundstrom N, Kirsch J, Schmieden V, Betz H. Modulation by zinc ions of native rat and recombinant human inhibitory glycine receptors. *J Physiol* 483: 613–619, 1995.
- Laube B, Maksay G, Schemm R, Betz H. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses. *Trends Pharmacol Sci* 23: 519–527, 2002.
- Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D, Millar NS. The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J Physiol* 505: 299–306, 1997.
- Li Y, Hough CJ, Frederickson CJ, Sarvey JM. Induction of mossy fiber→CA3 long-term potentiation requires translocation of synaptically released Zn²⁺. *J Neurosci* 21: 8015–8025, 2001.
- Li YV, Hough CJ, Sarvey JM. Do we need zinc to think? *Sci STKE* 182: pe19, 2003.
- Magleby KL, Pallotta BS. Burst kinetics of single calcium-activated potassium channels in cultured rat muscle. *J Physiol* 344: 605–623, 1983.
- Moroni M, Carbone AL, Vijayan R, Biggin P, Bermudez-Diaz I. Identification of molecular determinants that mediate zinc effects on alternate stoichiometries of the human alpha4beta2 nicotinic acetylcholine receptors. *Soc Neurosci Abstr* 33: 677.7, 2007.
- Palma E, Maggi L, Miledi R, Eusebi F. Effects of Zn²⁺ on wild and mutant neuronal $\alpha 7$ nicotinic receptors. *Proc Natl Acad Sci USA* 95: 10246–10250, 1998.
- Papke RL, Heinemann SF. The role of the beta 4-subunit in determining the kinetic properties of rat neuronal nicotinic acetylcholine alpha 3-receptors. *J Physiol* 440: 95–112, 1991.
- Qin F, Auerbach A, Sachs F. Estimating single channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys J* 70: 264–280, 1996.
- Rogers CJ, Twyman RE, Macdonald RL. Benzodiazepine and beta-carboline regulation of single GABAA receptor channels of mouse spinal neurons in culture. *J Physiol* 475: 69–82, 1994.
- Silberberg SD, Magleby KL. Voltage-induced slow activation and de-activation of mechanosensitive channels in *Xenopus* oocytes. *J Physiol* 505: 551–569, 1997.
- Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, Colquhoun D. Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single channel behaviour. *J Physiol* 500: 123–138, 1997.
- Smart TG, Hosie AM, Miller PS. Zn²⁺ ions: modulators of excitatory and inhibitory synaptic activity. *Neuroscientist* 10: 432–442, 2004.

- Teissere JA, Czajkowski C.** A β -strand in the $\gamma 2$ subunit lines the benzodiazepine binding site of the GABA_A receptor: structural rearrangements detected during channel gating. *J Neurosci* 21: 4977–4986, 2001.
- Ueno S, Tsukamoto M, Hirano T, Kikuchi K, Yamada MK, Nishiyama N, Nagano T, Matsuki N, Ikegaya Y.** Mossy fiber Zn²⁺ spillover modulates heterosynaptic N-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. *J Cell Biol* 158: 215–220, 2002.
- Vogt K, Mellor J, Tong G, Nicoll R.** The actions of synaptically released zinc in hippocampal mossy fiber synapses. *Neuron* 26: 187–196, 2000.
- Weiss DS, Magleby KL.** Voltage dependence and stability of the gating kinetics of the fast chloride channel from rat skeletal muscle. *J Physiol* 426: 145–176, 1990.
- Xie X, Hider RC, Smart TG.** Modulation of GABA-mediated synaptic transmission by endogenous zinc in the immature rat hippocampus in vitro. *J Physiol* 478: 75–86, 1994.