

# Copper and zinc as modulators of neuronal excitability in a physiologically significant concentration range

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## Abstract

Evidence from several areas of neuroscience has led to the notion that copper and zinc could be modulators of neuronal excitability. In order to contribute to test this idea, we characterized the changes induced by these divalent metal ions on the extracellularly recorded action potential firing rates of undissociated olfactory epithelium neurons. Our main finding is that at low concentrations, 1–100 nM for Cu<sup>2+</sup> and 1–50 μM for Zn<sup>2+</sup>, they induced a concentration dependent increase in the neuronal firing rate. In contrast, at higher concentrations, 1–5 μM for Cu<sup>2+</sup> and 100–500 μM for Zn<sup>2+</sup>, they decreased the firing rate.

Based on these and previous results of our laboratory we propose that the biphasic effect of Cu<sup>2+</sup> and Zn<sup>2+</sup> exposure on neuronal firing may be explained by the interaction of these ions with high and low affinity sites in sodium channels whose occupancy leads to activation or inhibition of the sodium current, which is consistent with the proposed modulatory role of these metal ions on neuronal excitability.

**Keywords:** Copper; Zinc; Neuronal excitability; DTT; Redox-modulated-gating

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Copper and zinc are trace elements that play essential roles in several cell functions. They are present at high levels in the brain, particularly in regions such as the olfactory bulb, hypothalamus, and hippocampus (Donaldson et al., 1973; Ono and Cherian, 1999; Frederickson et al., 2000). These metal ions accumulate in synaptic vesicles, especially in some glutamatergic neurons (Kardos et al., 1989; Ebadi et al., 1995; Frederickson and Bush, 2001). There is evidence that they are co-released with the neurotransmitters during normal synaptic events (Assaf and Chung, 1984; Howell et al., 1984; Aniksztejn et al., 1987; Hartter and Barnea, 1988; Kardos et al., 1989; Sato et al., 1994) reaching free concentrations in the synaptic space of around 10 μM for copper and 60 μM for zinc, respectively, in normal conditions (Hopt et al., 2003).

During the last years evidence from several areas of neuroscience has led to propose that copper and/or zinc could be modulators of neuronal excitability. Several studies report the effects of these cations on neurotransmitter and voltage-gated currents studied in isolated cells in culture or in heterologous expression systems. Also there are evidences of a modulatory role of these ions on synaptic plasticity phenomena (Li et al., 2001; Ma and Zhao, 2001; Li et al., 2003; Quinta-Ferreira and Matias, 2004; Goldschmidt et al., 2005).

Most of the studies regarding the role of these ions on neuronal excitability have focused on neurotransmitter rather than on voltage-gated channels using concentrations around 10–100 μM. Narahashi et al. (1994) reported that Cu<sup>2+</sup> and Zn<sup>2+</sup> suppressed the GABA-induced currents in rat dorsal root ganglion neurons in primary culture. Also, Trombley and Shepherd (1996) found that Zn<sup>2+</sup> and Cu<sup>2+</sup> were effective antagonists of both NMDA and GABA-mediated currents in rat olfactory bulb neurons. In one study, (Sharonova et al., 1998) reported the reversible blockade of GABA(A)-activated channels in rat isolated cerebellar Purkinje cells by Cu<sup>2+</sup> in the low nanomolar range.

Acuña-Castillo et al. (2000) reported that zinc (10 μM) stimulated and copper (10–300 μM) inhibited ATP evoked

**Abbreviations:** CNS, central nervous system; DEPC, diethyl pyrocarbonate; DTT, dithiotreitol; GABA, gamma-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; NMDA, N-methyl-D-aspartate; SOD, super oxide dismutase; TTX, tetrodotoxin

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currents in the rat purinergic receptor P2X4 expressed in *Xenopus* oocytes.

Regarding voltage-gated currents, Gilly and Armstrong (1982) described a low potency effect of  $Zn^{2+}$  with sodium currents in squid giant axons. They found that 30 mM  $Zn^{2+}$  slowed the opening kinetics of sodium channels without altering the closing kinetics. More recently, Horning and Trombley (2001) reported the effects of micromolar  $Cu^{2+}$  and  $Zn^{2+}$  on voltage-gated currents in rat olfactory bulb neurons in primary culture. They found that 100  $\mu M$  zinc or 30  $\mu M$  copper reversibly inhibited TTX-sensitive sodium and delayed rectifier-type potassium currents but did not prevent the firing of evoked action potentials nor dramatically altered their kinetics.

A systematic study of the blocking effect of copper and zinc on voltage-activated  $Ca^{2+}$  currents from rat piriform cortical neurons has been reported by Magistretti et al. (2003) and Castellii et al. (2003). They basically found that  $Cu^{2+}$  ( $IC_{50} \sim 1 \mu M$ ) and  $Zn^{2+}$  ( $IC_{50} \sim 20 \mu M$ ) blocked the currents and also slowed their activation kinetics.

Gruss et al. (2004) examined the modulation by copper and zinc of two-pore-domain potassium channels. They reported activation of TREK-1 and inhibition of TASK-3 by copper with an  $IC_{50}$  of around 3  $\mu M$  for both and inhibition of both channels by zinc with  $IC_{50}$  of 700 and 13  $\mu M$ , respectively.

In a recent study (Delgado et al., 2006) we investigated the effect of both copper and zinc on voltage-gated conductances exploring a wider concentration range than in previous studies (from nM to  $\mu M$ ). We chose *C. caudiververa* olfactory neurons as a model system because these cells present only four voltage-gated currents which have been extensively characterized, there are no synaptic connections among them and, to our knowledge, they do not store either copper or zinc. These characteristics allow the study of the effects of externally added  $Cu^{2+}$  and  $Zn^{2+}$  on voltage-gated conductances without the influence of all the synaptic modulation present in CNS neurons.

These neurons present two voltage-gated outward  $K^+$  selective currents and two voltage-gated inward currents. The inward components are a TTX sensitive  $Na^+$  current modulated by external  $Ca^{2+}$  and an L-type  $Ca^{2+}$  current. The outward currents are a delayed rectifier  $K^+$  current and a  $Ca^{2+}$  activated K conductance (Delgado and Labarca, 1993; Madrid et al., 2003).

We found that copper and zinc presented a biphasic effect on sodium currents. At low concentrations (two-digit nM to one-digit  $\mu M$ ) they increased the amplitude and speeded up the activation and inactivation kinetics. At higher concentrations, (low  $\mu M$  for  $Cu^{2+}$  and two-digit  $\mu M$  for  $Zn^{2+}$ ) they inhibited the inward sodium currents. We found no effect of copper or zinc concentrations in the nanomolar range over the inward calcium current, nevertheless, in the micromolar range, they caused a dose-dependent inhibition of the  $Ca^{2+}$  currents and as a consequence, they also reduced the amplitude of the  $Ca^{2+}$ -dependent  $K^+$  outward currents. Our findings indicate that sodium currents are more sensitive to copper and zinc concentrations than calcium and potassium ones (Delgado et al., 2006).

Since neuronal excitability is determined in great extent by the sodium channel gating kinetics, our results predict that the biphasic effect of these metal ions on the sodium current should also cause a biphasic effect on the spontaneous firing frequency of these and other neurons. In the present study we tested this prediction and also discuss our results based on the models that have been proposed to explain the role of metal ions on channel gating (Elinder and Arhem, 2004; Mathie et al., 2005).

So far, most of the reported studies have emphasized the inhibitory role of these cations, but our results suggest that for these and other neurons, depending of their concentrations, copper and zinc may be either excitatory or inhibitory agents.

## 1. Experimental procedures

All experiments conformed to the guidelines set by the National Scientific and Technological Commission and the Ethics Committee of the Faculty of Sciences, University of Chile and were done in conditions that minimized animal suffering.

The Chilean toad *Caudiververa caudiververa* was used as the source of biological tissue. Animals, obtained from the university's animal facility, were anaesthetized by cooling in ice, after which they were killed and pithead before dissecting out their olfactory epithelia.

## 2. Extracellular recording

The extracted epithelia were kept at 4 °C in extracellular saline solution containing (in mM) 115 NaCl, 2.5 KCl, 1 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 3 glucose, 10 HEPES, pH 7.6. The upper bone layer was removed in order to make accessible the tissue to the recording electrode. A piece of bone containing the epithelium was glued to the bottom of a 3 ml plastic chamber and bathed with external saline.

The extracellular activity was recorded with a Wood's metal electrode (low melting point alloy purchased from Cerrometal Products, CO, USA) using a glass pipette as a mold. The electrode tip diameter was around 2  $\mu m$  and it was coated with Platinum Black in order to reach an impedance of 100 k $\Omega$  (at 1 kHz). For each recording the electrode was inserted in a piece of epithelium and moved until a spot with a single firing neuron was identified. A spike was defined when the standard deviation of the amplitude of voltage recordings was over a threshold corresponding to a signal to noise ratio of at least four units. Spikes were considered as originating from a single neuron when the standard deviation of their amplitude varied within the same interval as the standard deviation of noise. Multiunit recordings were discarded. Recordings were amplified with a differential amplifier (Warner Instrument Corp., CT, USA, Model DP-301), AC filtered (low pass 300 Hz, high pass 1000 Hz), monitored in an oscilloscope and stored using the Fetchex routine from pClamp 6 program. The acquired data were analyzed using routines made on the Igor Pro 5.0 software (Wavemetrics Corp., OR, USA).

For each neuron four series of 30 s recordings were made, i.e. 2 min in total for each experimental condition. First, the basal firing rate was measured, then a mechanical control was done to check that solution change did not affect firing rate. Recordings in the presence of different concentrations of

chloride salts of the divalent cations were done afterwards. Finally, reversibility was tested at the end of the experiment by perfusing 10 ml of divalent-free buffer solution through the chamber. Each experiment was repeated four times. Statistical significance was evaluated with respect to the values obtained after the mechanical control (Buffer labeled column in the graphs). Divalent cations solutions were freshly prepared every day. In the experiments with DEPC, 1 mM of the compound was applied to the epithelia for 10 min and then washed for an hour before recording neuronal activity.

### 3. Macroscopic current measurements in dissociated olfactory neurons

Whole cell current recordings were conducted by the patch-clamp technique on mechanically dissociated olfactory receptor neurons as previously described (Delgado and Labarca, 1993). Neurons were placed in the experimental chamber (500  $\mu$ l) and exposed to the same saline solution described above. The experimental chamber was perfused with six volumes of external solution supplemented with DTT or copper chloride at the concentration indicated for each experiment. For total current measurements, the patch clamp pipette was filled with a saline solution containing (in mM) 125 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 2 EGTA, 4 HEPES, pH 7.6. For inward current measurements, KCl was replaced by CsCl. Resistances of the patch pipettes used ranged from 2 to 4 M $\Omega$ . The current records were made with an Axoptach 200 B amplifier (Axon Instruments, Foster City, CA). Pulse protocols were generated by a computer through a D/A convert (Scientific Solution, Solon, Ohio 44139) using pClamp6.0 routines (Axon). The data were acquired at a sampling rate of 10 KHz, stored in a PC and analyzed using the Clampfit routine from pClamp6. The currents were evoked by a family of depolarizing pulses increasing in 10 mV steps starting from a holding potential of -80 mV. Pulses were separated by a 2 s interval.

#### 3.1. Materials

DEPC, DTT and HEPES, were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. All inorganic salts were of analytical grade and were purchased from Merck KGaA, Darmstadt, Germany.

## 4. Results

### 4.1. The spontaneous firing rate in the olfactory epithelia increases and then decreases as a function of concentrations of copper and zinc

**Fig. 1(A)** shows representative traces of extracellularly recorded potentials obtained in a piece of undissociated olfactory epithelia bathed in normal external solution and in the saline containing 0.05 or 1  $\mu$ M Cu<sup>2+</sup>. In the case shown, the basal spontaneous firing rate increased from 0.9 to 3.2 Hz after the addition of 0.05  $\mu$ M Cu<sup>2+</sup>. A further increase in copper concentration to 1  $\mu$ M caused a decrease from the initial rate

to 0.4 Hz. **Fig. 1(B)** summarizes the results of four independent experiments in which the epithelia were exposed to four different copper concentrations, ranging from 10 nM to 1  $\mu$ M. We controlled that mechanical changes induced by chamber perfusion did not affect the basal firing rate (Buffer). The decrease in firing after exposing the epithelia to 1  $\mu$ M Cu<sup>2+</sup> could not be reverted by taking away the ion (Wash). The differential effects of nano and micromolar Cu<sup>2+</sup> concentrations on neuronal firing frequency suggest that this ion is interacting with two different binding sites: a high affinity site involved in the activation and a low affinity site related to inhibition. **Fig. 1(C)** illustrates the effect of Zn<sup>2+</sup> on neuronal spontaneous firing rate. Qualitatively, this metal ion presented the same biphasic effect as copper, but at much higher concentrations. **Fig. 1(D)** shows the average of four experiments where neurons were exposed to six different zinc concentrations ranging from 0.1 to 500  $\mu$ M. The activating effect of Zn<sup>2+</sup> was observed at 1  $\mu$ M, a concentration that for copper was already inhibitory. As was the case for Cu<sup>2+</sup>, the inhibitory effect of Zn<sup>2+</sup> was irreversible since washout did not return neuronal firing rate to the basal level.

### 4.2. Each cation potentiates the effect of the other one

Given that copper and zinc could be co-released during synaptic activity, it is important to evaluate what would be their effect on excitability when both are present simultaneously. To test if these cations influence each other we repeated the measurements described in **Fig. 1**, in the presence of fixed low concentrations of the second cation. **Fig. 2(A)** shows a summary of four independent experiments where the effect of increasing concentrations of Cu<sup>2+</sup> were evaluated in the presence of two different nanomolar Zn<sup>2+</sup> concentrations. **Fig. 2(B)** depicts the equivalent experiment for Zn<sup>2+</sup> at two different low nanomolar Cu<sup>2+</sup> concentrations. From the results, it is clear that, at the concentrations used, each ion potentiates the effect of the other one by shifting the concentration response curve to the left.

### 4.3. Neuronal firing rate is also increased by other divalent metal ions

Although the chemical properties and coordination geometry of Cu<sup>2+</sup> and Zn<sup>2+</sup> are different, both present a biphasic effect in the firing rate of olfactory neurons. To test if this would be the case for other divalent cations, we also evaluated the effect of cobalt and nickel. **Fig. 3(A)** shows a summary of four experiments where the firing rate was measured in the presence of increasing extracellular Co<sup>2+</sup> concentrations. A significant increase in firing rate was found for Co<sup>2+</sup> concentrations higher than 10  $\mu$ M, with a maximum effect at 100  $\mu$ M. A slight decrease in firing rate respect to the maximum was observed at 1 mM but the rate was still significantly higher than control. The effect of Co<sup>2+</sup> at 1 mM was only partially reversed to basal values by washing.

**Fig. 3(B)** shows the effect of increasing concentrations of Ni<sup>2+</sup> in the spontaneous firing rate of olfactory neurons. We found an increase in firing rate when the epithelia was exposed

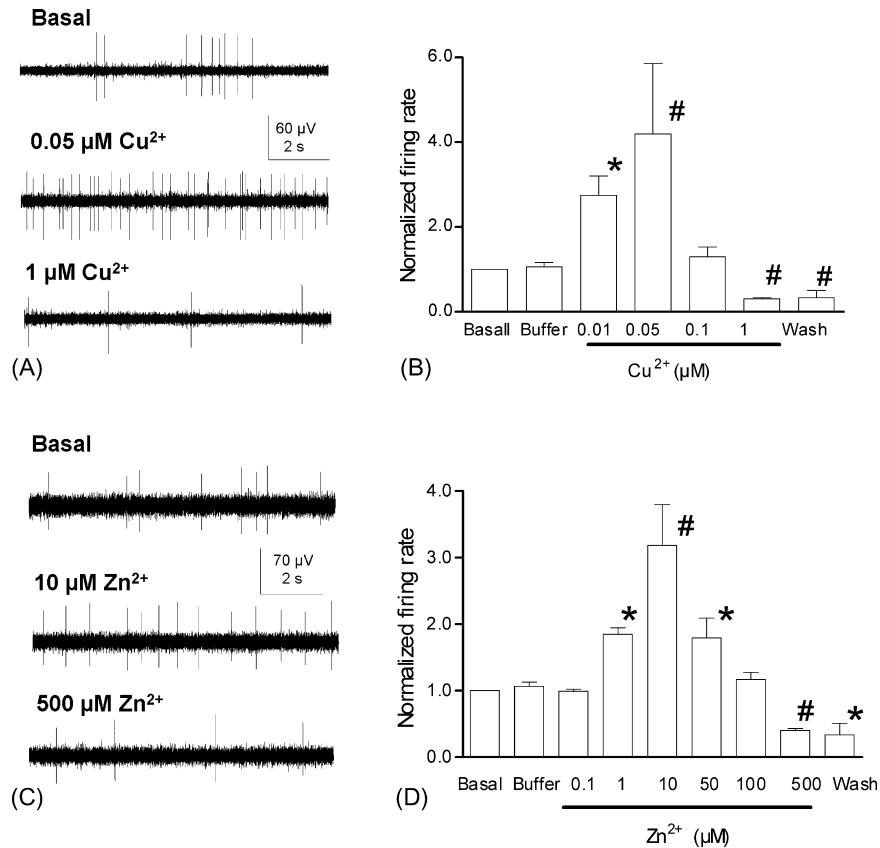


Fig. 1. Effect of copper and zinc in the spontaneous firing rate of olfactory epithelium neurons. (A) Ten seconds extracellular recordings from a single neuron in the undissociated epithelia with normal external solution (Basal) or after successive additions of Cu<sup>2+</sup> to the final concentrations indicated in the figure. (B) Average of four (0.01 and 0.05 μM Cu<sup>2+</sup>) and five (0.1 μM Cu<sup>2+</sup>) independent experiments after the addition of copper. (C and D) Same conditions as described for A and B but with Zn<sup>2+</sup> added to the external solution. Normalized firing rate values represent the ratio between the spontaneous firing rates obtained in the presence and absence (Basal) of divalent cations. Error bars represent S.E.M. (paired t-test, \*p < 0.05; #p < 0.01).

to concentrations over 100 μM Ni<sup>2+</sup> (*n* = 4). After washing from the highest concentration tested (1 mM), the firing rate decreased to values not significantly different from the initial one, i.e., the effect of niquel was reversible. In summary, it seems that cobalt and nickel can also interact with the higher affinity activating site and, at the concentrations tested, cobalt can also interact with the lower affinity inhibitory site.

#### 4.4. Copper and zinc seem to decrease neuronal firing rate by different mechanisms

Since the inhibitory effect of micromolar Cu<sup>2+</sup> on neuronal firing rate was irreversible we considered the possibility that this metal ion could have induced an oxidative change in key residues in some of the ion channels involved in action potential firing. In order to assess the possible involvement of thiol groups in the modulation of neuronal firing activity we tested the effect of the SH reducing agent DTT by measuring its effect on firing frequency. Fig. 4(A) shows that DTT by itself from 1 to 10 mM causes an increase in neuronal firing rate suggesting that the redox state (tone) of some sulfhydryl groups can regulate neuronal firing frequency. Concentrations of DTT of 30 mM or higher apparently damaged the epithelia (*n* = 4).

To establish if copper inhibition was mediated by a redox change of sulfhydryl groups we investigated if DTT could

revert the effect of micromolar Cu<sup>2+</sup>. Fig. 4(B) shows a plot of the firing rate as function of Cu<sup>2+</sup> concentration in the absence and in the presence of DTT (*n* = 4). The increase in firing rate induced by 0.01–0.1 μM Cu<sup>2+</sup> is not affected in the presence of DTT. Nevertheless, the reduction in firing rate triggered by 1 μM Cu<sup>2+</sup> is not seen with DTT present in the external solution. As found for copper, the firing rate in the presence of activating Zn<sup>2+</sup> concentrations is the same with or without DTT (Fig. 4(C)). For Zn<sup>2+</sup> concentrations that decreased the firing rate, with respect to the maximum value, 1 mM DTT induced a further reduction of it to values below the basal one. Not being Zn<sup>2+</sup> an oxidizing agent, we did not expect that DTT could reverse this inhibition. Additionally, we did not expect that zinc and DTT together could cause such a decrease in cell excitability. It is known that DTT and zinc bind strongly (*K*<sub>D</sub> of 10<sup>-10</sup> M at pH 9.2; Cornell and Crivaro, 1972; Paoletti et al., 1997) so, in the presence of 1 mM DTT, we expected that at all the Zn<sup>2+</sup> concentrations used, the firing rate would be the same with or without DTT. So far, we do not have an explanation for the effect of zinc at concentrations higher than 50 μM in the presence of DTT. Nevertheless, the reversal of the inhibitory effect of micromolar Cu<sup>2+</sup> by DTT strongly suggests that the inhibition of firing rate triggered by copper is a redox-mediated process.

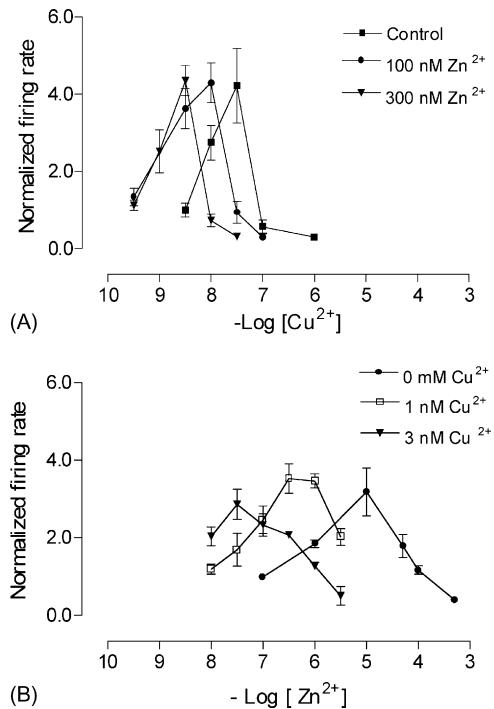


Fig. 2. Effect of co-application of Cu<sup>2+</sup> and Zn<sup>2+</sup> in the spontaneous firing rate of the olfactory neurons. Plots show the mean of four independent experiments. (A) Firing rate of neurons exposed to different Cu<sup>2+</sup> concentrations in the presence of the indicated concentrations of Zn<sup>2+</sup>. (B) Firing rate of neurons exposed to different Zn<sup>2+</sup> concentrations in the presence of the indicated Cu<sup>2+</sup> concentrations. Normalized firing rates were measured as described for Fig. 1. Error bars represent S.E.M. Dotted lines connect the points of the respective concentration response curves.

#### 4.5. Reversibility of the effect of copper and zinc on firing rate when acting over the higher affinity sites

The results shown in Fig. 1 strongly suggest the existence of two different modulatory sites to which copper and zinc have access. We described them as “high and low” affinity sites, and for both, the affinity for Cu<sup>2+</sup> is much higher than for Zn<sup>2+</sup>. The experiments with copper in the presence of DTT suggest that the higher affinity site is not redox-sensitive and therefore, the effects of divalent cations after binding to this site could be reversible. Fig. 5(A) and (B) show that the effect of 0.01 and 0.05 μM Cu<sup>2+</sup> in the spontaneous firing rate of the olfactory neurons is reversed after washing. The same is observed in Fig. 5(C) and (D) for 1 to 100 μM Zn<sup>2+</sup>.

#### 4.6. DEPC effect on copper- or zinc-induced changes in firing rate

Considering that histidine residues are found in consensus copper-binding motifs (Aitken, 1999) we tested the effect of DEPC in the copper and zinc induced changes in firing rate of olfactory neurons; DEPC is a compound that selectively alkylates histidines (Leonard et al., 1970). Fig. 6(A) shows ten seconds recordings for DEPC pre-treated epithelia in control solution (basal DEPC) and after the successive addition of 0.05 and 1 μM Cu<sup>2+</sup>. In these DEPC-treated epithelia, copper did not

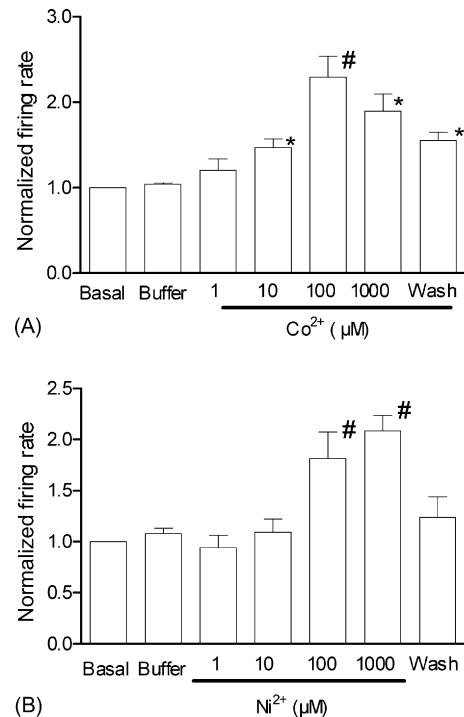


Fig. 3. Spontaneous firing rate of olfactory epithelium neurons as a function of Co<sup>2+</sup> (A) or Ni<sup>2+</sup> (B) concentrations in the external buffer. Normalized firing rates were measured as described for Fig. 1. Values are the mean of four experiments. Error bars represent S.E.M. (paired t-test, \*  $p < 0.05$ ; #  $p < 0.01$ ).

activate much firing at 0.05 μM nor inhibited it at 1 μM. (compare with the recordings in Fig. 1(A)). Fig. 6(B) shows the analysis of four independent experiments in the absence and in the presence of DEPC. Fig. 6(C) and (D) shows the equivalent experiment in the presence of zinc. In this case, the recordings from the DEPC pre-treated epithelia are not different from the ones in its absence (see Fig. 1), i.e., neuronal firing rate increased by exposures to 10 (M zinc and decreased for exposures to 500 (M zinc).

Given that many ion channels are modulated by pH (Takahashi and Copenhagen, 1996; Carlin, 2005; Jensen et al., 2005) including sodium channels (Woodhull, 1973; Brodwick and Eaton, 1978) we tested the effect of decreasing pH in the extracellular solution. The pH values tested were: 7.6 (basal), 7.1, 6.6, 6.1, 5.6 and 5.1 (four independent determinations for each value). We found no significant changes in the normalized firing rate for that pH range (data not shown).

So far we have described an activating and a blocking effect of copper and zinc on the firing rate of these neurons and to our knowledge, the activating effect had not been previously described. In principle, an increase in excitability can occur if the balance between inward and outward currents changes. The changes could be due to increases in the amplitude and/or kinetics of either inward or outward currents. Since we found that exposure of cells to DTT also increased firing rate, it becomes important to check which is the effect of DTT alone or together with copper at concentrations that activate or inhibit firing on the ionic currents.

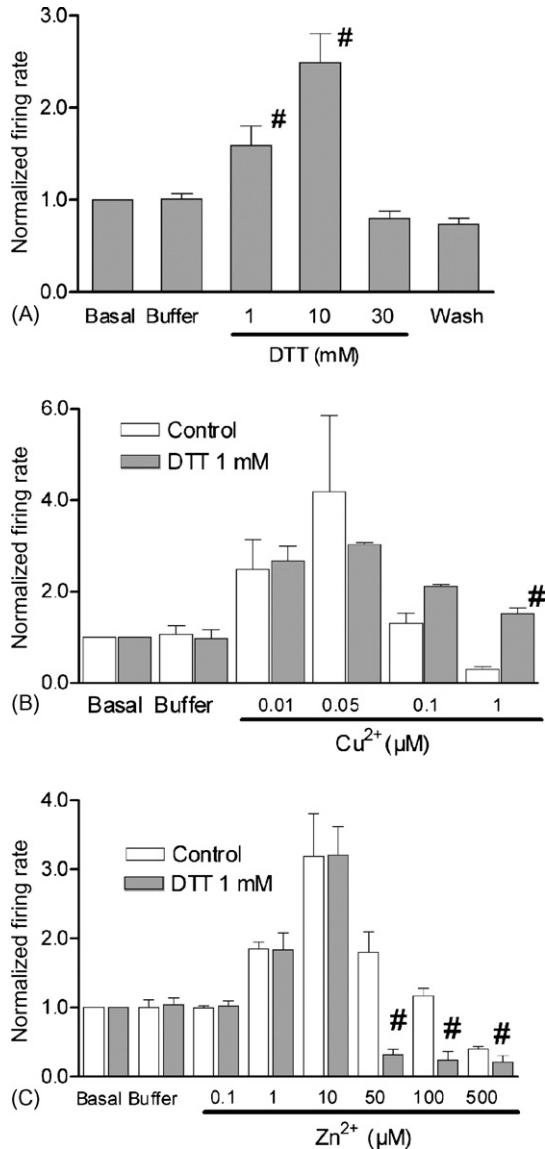


Fig. 4. DTT alters the effects of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  on firing rates from olfactory neurons. (A) The DTT effect on firing frequency was tested by adding it to the external buffer at the indicated concentrations. Values represent the mean of four independent experiments. (B) Average of four independent experiments after the addition of four different  $\text{Cu}^{2+}$  concentrations, in the absence (filled columns) and in the presence (open columns) of DTT. Values in the absence and presence of DTT were normalized with respect to their own basal firing rates. (C) Normalized firing rate determined in the presence of 1 mM DTT at the indicated zinc concentrations. Error bars represent S.E.M. (paired *t*-test,  ${}^{\#}p < 0.01$ ).

#### 4.7. Effects of DTT and copper on macroscopic currents

Fig. 7(A) shows the effect of DTT and DTT plus copper on three families of total current records obtained under voltage clamp conditions. Currents obtained under control condition are shown on the upper panel and the effects of 1 mM DTT and 1 mM DTT plus 0.1  $\mu\text{M}$  copper ions are shown in the middle and lower panels respectively. In the presence of 1 mM DTT the kinetics of both, inward and outward currents are slightly faster but also the outward current increases in amplitude. This is shown in the current–voltage curve for the outward current (Fig. 7(D)). To

check if the change in kinetics is due to an affect of DTT over the inward current or it is just due to the fact that the amplitude of the outward current is greater we recorded only the inward currents by replacing KCl by CsCl in the patch pipette. The inward currents recorded under control condition, in the presence of 1 mM DTT and in the presence of DTT plus 0.1  $\mu\text{M}$  or 1  $\mu\text{M}$  copper are shown in the first, middle and lower panels in Fig. 7(B) and (C), respectively. DTT by itself causes a slight decrease in the time to peak of the inward currents (Fig. 7(E) and (F)). The addition of 0.1  $\mu\text{M}$  copper causes a slight increase in the current amplitude and a further decrease in the time to peak of the inward current (inset lower group of currents, Fig. 7(B) and (E)). However, in the presence of 1  $\mu\text{M}$  copper, the inward currents do not change with respect to the currents recorded just in the presence of DTT (inset lower group of currents (Fig. 7(C)) and (F)).

## 5. Discussion

In the present study we have shown that the divalent metal cations copper and zinc activate the spontaneous firing rate of olfactory epithelial cells when added in the nanomolar and low micromolar range, respectively. In contrast, at higher concentrations, they inhibit firing. The effects of copper and zinc are important for neuronal physiology because both ions are liberated to the synaptic space during normal neuronal activity and the effects we describe occur within the concentration range that has been estimated they reach in the synaptic cleft (Hopt et al., 2003). Because we also found that copper and zinc potentiates the effect of the other one making it more efficient in its activating and inactivating effects, it seems evident that the “basal firing potential” of neurons around release sites of copper and zinc would be affected by them.

A simple explanation for the capacity of these ions to increase basal firing rate is that they could trigger a depolarization leading the membrane potential closer to firing threshold. To our knowledge, there is no evidence that copper or zinc in the nanomolar or low micromolar concentration range could cause membrane depolarization. The inhibitory effect of  $\sim 3 \mu\text{M}$  copper on the resting potassium channel TASK-3 could result in an increase of membrane excitability (Gruss et al., 2004) but for olfactory epithelial neurons exposed to this concentration range of copper we find a decrease of excitability. Alternatively, divalent cations could somehow alter the gating parameters of one or more conductance(s) in a way that the cells become more excitable.

Elinder and Arhem (2004) have recently summarized the currently accepted models that may explain the divalent ion effects on ion channel gating. They proposed four main mechanisms: (a) unspecific screening of negative fixed surface charges that changes the electric field near the channel voltage sensor, (b) binding of the ions to fixed charges and an associated electrostatic modification of the voltage sensor, (c) binding of the ions to the channel surface that causes slowing of the opening and closing rates (d) a voltage dependent binding and pore blockade. Also, combinations of these mechanisms have been proposed. However, for copper ions, these authors did not consider the possibility of redox induced changes. Given that

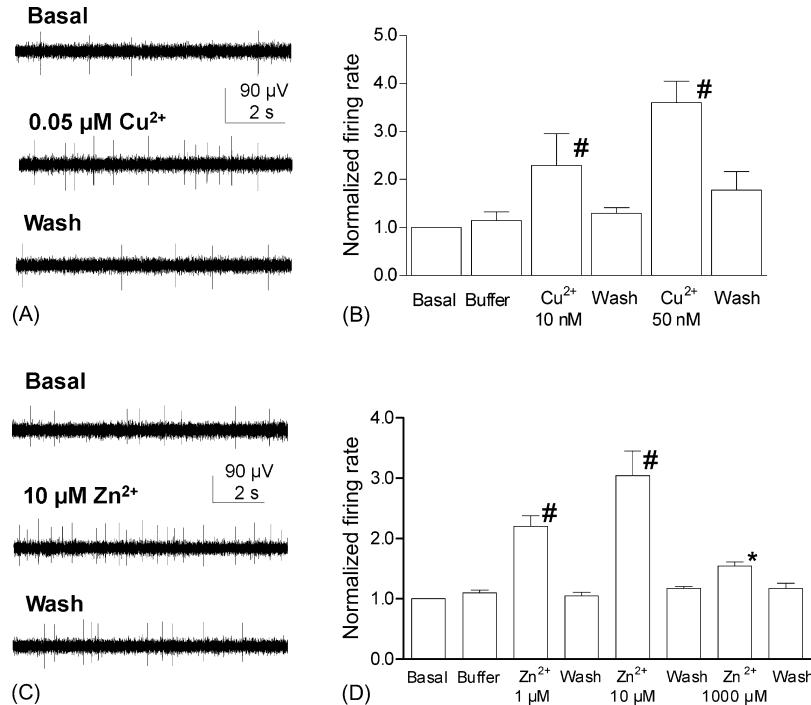


Fig. 5. The increase in neuronal firing rate induced by Cu<sup>2+</sup> and Zn<sup>2+</sup> is reversible. (A) Ten seconds recordings under basal condition, after the addition of 0.05 μM copper and after washing copper from the external solution. (B) Mean of four experiments in the presence of 0.01 and 0.05 μM copper. (C and D) Same conditions for (A and B) but with zinc in the external solution at the indicated concentrations. Error bars represent S.E.M. (paired *t*-test, \**p* < 0.05; #*p* < 0.01).

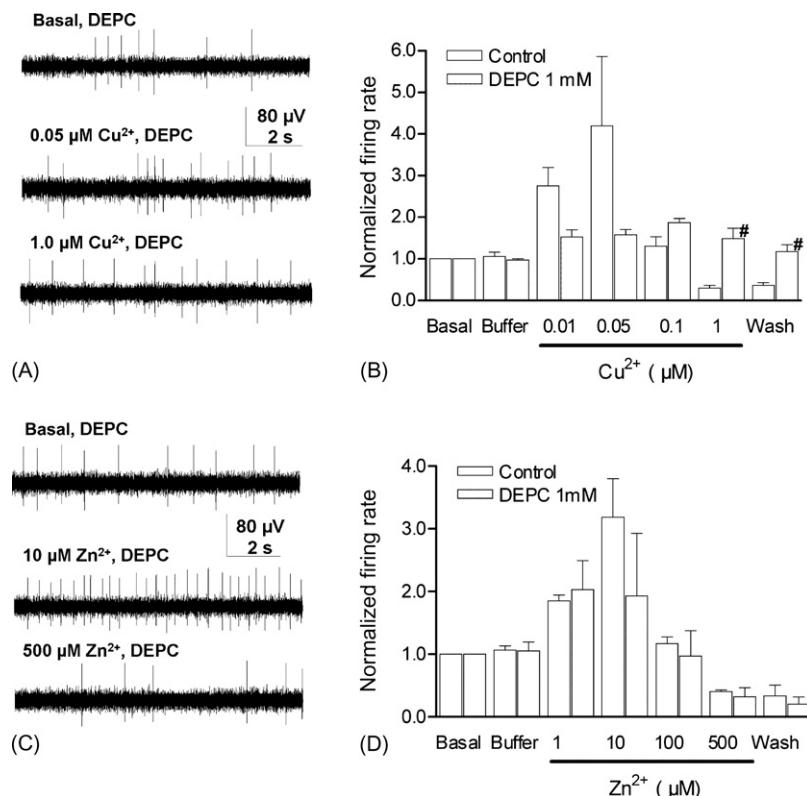


Fig. 6. Effect of DEPC on copper- and zinc-induced firing rate changes of olfactory neurons. Epithelia were exposed to 1 mM DEPC for 10 min and then allowed to recover for 1 h before making the recordings. (A) Ten seconds recordings from a single neuron in an epithelia previously exposed to DEPC (Basal DEPC) and after the addition of 0.05 and 1 μM copper to the external solution. (B) Average of four independent experiments after the addition of four different Cu<sup>2+</sup> concentrations, in the absence (filled columns) and in the presence (open columns) of DEPC. Values in the absence and presence of DEPC were normalized respect to their own basal firing rates. (C) Same conditions as described for (A) but when Zn<sup>2+</sup> 10 and 500 μM were added to the external solution. (D) same as (B) but with zinc in the external solution. Error bars represent S.E.M., (paired *t*-test, #*p* < 0.01).

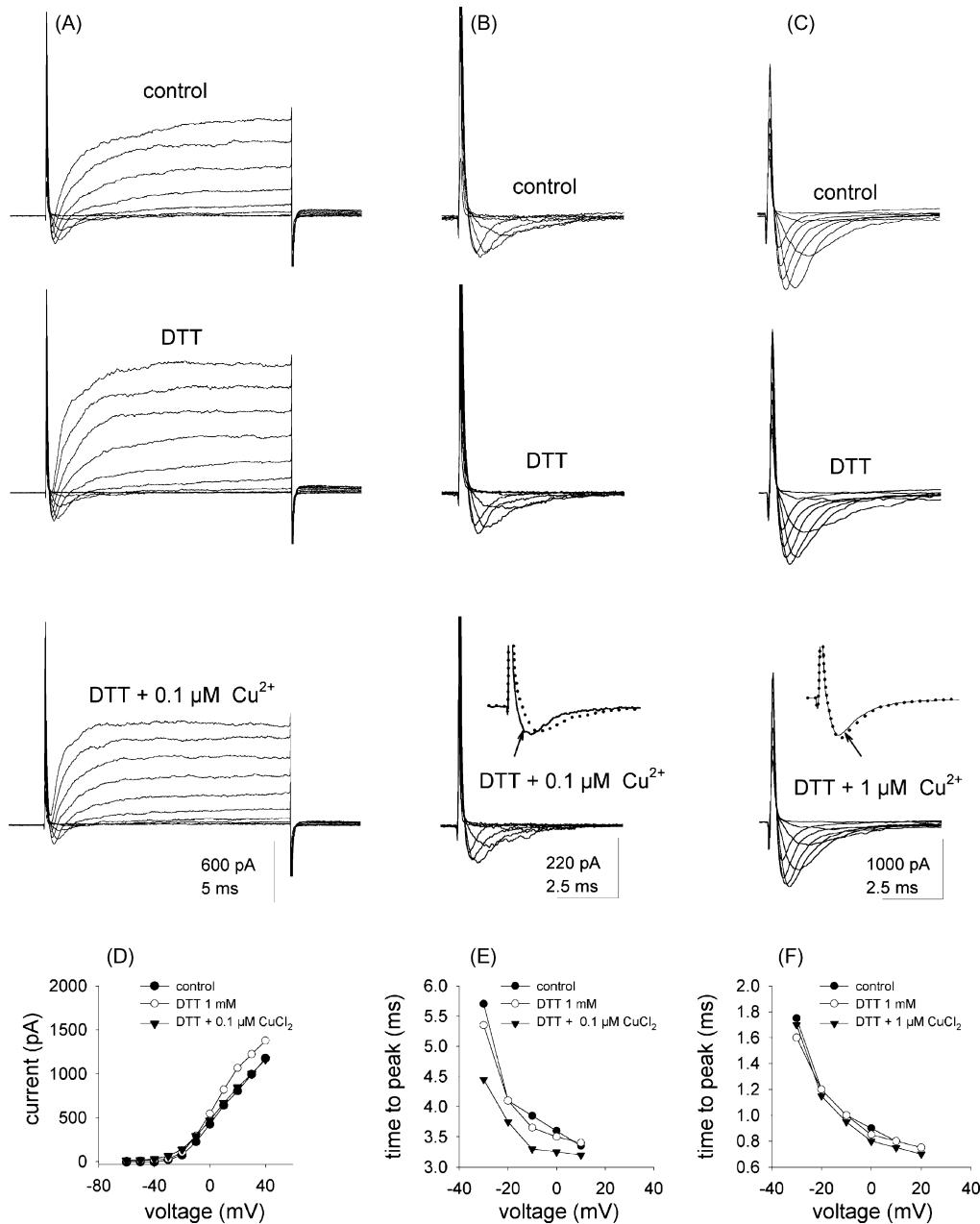


Fig. 7. Effect of DTT and copper on total and inward currents. Total (A) or inward currents (B and C) recorded under voltage clamp conditions from three different cells representative of three independent measurements for each condition. (A) Current families recorded under control condition (first panel), in the presence of 1 mM DTT (middle panel) and with 1 mM DTT and 0.1  $\mu$ M Cu<sup>2+</sup> (lower panel). The inset in the lower set of currents in (B) and (C) shows the superimposed inward currents obtained for a depolarizing pulse to -20 mV in the presence of 1 mM DTT (dotted line) and in the presence of copper (continuous line) at 0.1 and 1  $\mu$ M Cu<sup>2+</sup> respectively. (D) current–voltage curve for the outward currents shown in (A), (E) and (F), time to peak vs. voltage curves for recordings shown in (B) and (C).

Cu<sup>2+</sup> has oxidative properties, such mechanism is highly probable, and it has been recently considered as a possible mechanisms of its action over proteins (Mathie et al., 2005) or to explain a possible role of copper in Alzheimer disease (Opazo et al., 2003; Bishop and Robinson, 2004). Considering those mechanisms and our results, we think it is reasonable to discard a general surface charge screening for the activating effects of nanomolar copper on sodium currents. Given that the effect of copper is reversible and not affected by the reducing agent DTT, we think that most probably Cu<sup>2+</sup> binds to high affinity site(s) triggering a change in channel gating.

The concentration range in which we found copper and zinc activating and inactivating effects on firing frequency coincides with the range in which these cations modulate the inward sodium current in dissociated olfactory neurons (Delgado et al., 2006). Since the sodium conductance is more sensitive to copper and zinc concentrations than the calcium and potassium conductances, we think this is an indication that the firing rate changes produced by Cu<sup>2+</sup> and Zn<sup>2+</sup> are mainly related to changes in the inward sodium channel gating properties. The effect of 1 mM DTT on the outward and inward currents suggests that, in these neurons, the sodium and potassium

channels have redox sensitive sites that modulate their gating properties. Interestingly, the activating and blocking effect of copper on the inward current is not masked by the presence of DTT.

To confirm the proposal that copper increases neuronal firing rate by altering the gating of sodium channels we considered the model for activation of this channel described for these cells in Madrid et al., 2003. When the values for activating and inactivating time constants of the sodium current obtained from control conditions were used (without considering a change in voltage dependence) the model shows firing of single action potentials but when we used the values obtained for cells exposed to 100 nM copper, the model shows firing of multiple action potentials. This copper concentration triggered a 46% decrease in the activation and a 17% decrease in the inactivation time constant (Delgado et al., 2006).

The differential effects of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  at low and high concentrations could be explained considering that sodium channels have at least a high and a low affinity modulatory site for divalent cations. The high affinity site would be involved in the action potential firing stimulation and the low affinity site would be related to action potential inhibition. Our results with cobalt and nickel suggest that both sites can bind different divalent cations but with different affinities. On the other hand, when  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  are together, the activating as well as the inactivating effect of copper and zinc become potentiated. Concentrations that for each cation were not sufficient to cause a change in firing rate, became effective in the presence of the other cation. We consider this as a hint that sodium channels could have more than two sites where divalent cations would exert channel modulation and then, the binding of one cation to its site could increase the affinity of the other metal ion for its own site(s).

On the other hand, the reversion of the inhibitory effect of  $\text{Cu}^{2+}$  on neuronal firing rate by DTT hints that sulfhydryl groups are present in the low-affinity inhibitory site. The involvement of thiol groups in the gating of inward and outward conductances is supported by the results shown in Fig. 7 and also from results of a previous study carried out in our laboratory (Morera et al., 2003). We showed that micromolar  $\text{Cu}^{2+}$  added to the extracellular side of the high-conductance calcium activated potassium ( $\text{BK}_{\text{Ca}}$ ) channel induced a time and concentration dependent decrease of its open time probability ( $P_o$ ). The decrease in  $P_o$  was not reverted by washing, however, channel activity was restored by external DTT, suggesting that copper could participate in direct or indirect oxidation of sulfhydryl groups involved in the  $\text{BK}_{\text{Ca}}$  channel gating mechanism. In that study we compared the effect of  $\text{Cu}^{2+}$  with,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Cd}^{2+}$ , divalent cations of similar ionic radii, but with different redox potentials. Neither of them caused significant decrease of channel activity, hinting again that the effect of  $\text{Cu}^{2+}$  on  $\text{BK}_{\text{Ca}}$  channel activity is redox-mediated.

Histidines and cysteines have been proposed like important amino acids in the coordination of copper and zinc (Richardson et al., 1975; Vallee and Auld, 1990). Our results with DTT and DEPC suggest that for copper, histidines could play an important role in its coordination in the high and low affinity sites and also that a cystein forms part of the low affinity site.

On the other hand, histidines do not seem to participate in coordination of zinc. The alignment of the complete amino acid sequence (BLAST p <http://www.ncbi.nih.gov>; Jorge Vera, personal communication) for ten different species including amphibian and mammalian sodium channels revealed the presence of highly conserved histidine residues in the extracellular loop between transmembrane segments 1 and 2 of the second repeat. Assuming the *Caudiververa* sodium channel is similar to the ones used for the alignment, those histidines could be the target(s) affected by DEPC. It is also possible that the target of copper and or zinc ions could be the modulatory beta subunit. Most probably, the molecular mechanisms associated to neuronal modulation by copper and zinc are different.

We do not have an explanation for the effect of zinc at high concentration. Its inhibitory effect, which is observed at much higher concentration than for copper, apparently occurs by a molecular mechanism different than a redox modification since it is not affected by DTT.

In this simple model system, we showed that at very low concentrations, copper and zinc modulate neuronal firing frequency. Therefore, one could predict that in a more complex system this effect should also be present. Interestingly, we have found that the biphasic effects herein described are also observed, in the same concentration range, in rat hippocampal slices exposed to these two cations. In the slice preparation, some synaptic parameters are also affected (Maureira et al., 2006). The epithelial model system has the advantage of allowing an easier characterization of the role of these ions on voltage-gated channels in the absence of neurotransmitter activated conductances.

Our results support the notion that copper and zinc could be normal regulators of the excitable state of neuronal cells. Therefore, this physiological role has to be considered before designing therapies that use copper chelating agents to treat pathologies either in animal models like autoimmune encephalomyelitis (Offen et al., 2004) or in humans like for Wilson's disease (Brewer, 2005). With some chelating agents there is a further "drug-induced" neurological deterioration whose basis could be explained by the sensitivity to low levels of copper of certain neurons (Brewer, 2005).

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