Eur Biophys J (2008) 37:301–314
DOI 10.1007/s00249-007-0230-7

REVIEW

Trace metals in the brain: allosteric modulators of ligand-gated receptor channels, the case of ATP-gated P2X receptors

J. Pablo Huidobro-Toro · Ramón A. Lorca · Claudio Coddou

Received: 30 August 2007 / Revised: 10 October 2007 / Accepted: 11 October 2007 / Published online: 31 October 2007

Abstract Zinc and copper are indispensable trace metals for life with a recognized role as catalysts in enzyme actions. We now review evidence supporting the role of trace metals as novel allosteric modulators of ionotropic receptors: a new and fundamental physiological role for zinc and copper in neuronal and brain excitability. The review is focussed on ionotropic receptor channels including nucleotide receptors, in particular the P2X receptor family. Since zinc and copper are stored within synaptic vesicles in selected brain regions, and released to the synaptic cleft upon electrical nerve ending depolarization, it is plausible that zinc and copper reach concentrations in the synapse that profoundly affect ligand-gated ionic channels, including the ATP-gated currents of P2X receptors. The identification of key P2X receptor amino acids that act as ligands for trace metal coordination, carves the structural determinants underlying the allosteric nature of the trace metal modulation. The recognition that the identified key residues such as histidines, aspartic and glutamic acids or cysteines in the extracellular domain are different for each P2X receptor subtype and may be different for each metal, highlights the notion that each P2X receptor subtype evolved independent strategies for metal coordination, which form upon the proper three-dimensional folding of the receptor channels. The understanding of the molecular mechanism of allosteric modulation of ligand-operated ionic channels by trace metals is a new contribution to metallo-neurobiology.

Keywords Zinc · Copper · Nucleotide receptors · P2X receptors · Trace metal modulation · Ionic channel gating

J. P. Huidobro-Toro (✉) · R. A. Lorca · C. Coddou
Laboratorio de Nucleótidos,
Centro de Regulación Celular y Patología, JV Luco,
Instituto Milenio MIFAB, Departamento de Fisiología,
Facultad de Ciencias Biológicas,
Pontificia Universidad Católica de Chile, Santiago, Chile
e-mail: jphuid@bio.puc.cl

Metallo-biology: from nutritional studies to understanding the three-dimensional topology of structural complexes of proteins and trace metals

During the last century, scientists recognized the indispensable nature of minor concentrations of iron, iodine, zinc, cobalt and copper as components of hormones, prosthetic enzyme groups, transport proteins and vitamins. Other metals like manganese, selenium and molybdenum were also incorporated in the list of essential nutrients although their role has been less intensively investigated. Since the blood concentrations of these elements are about 4–5 orders of magnitude less than that of sodium, potassium, chlorine, calcium or phosphorous, collectively these nutrients have been termed trace metals, referring to their low concentrations in blood and tissues. In support of the body requirements of these metals, a variety of well-controlled nutritional studies have established that animal diets deficient in these metals cause characteristic syndromes for each essential nutrient. If metal deprivation was persistent, death invariably followed, highlighting their essential role for life.

The molecular significance and the essential role of trace metals in life, has been clarified within the past 50 years. It was recognized that trace elements are obligatory components of a variety of enzymes and vitamins. For example, iron is a necessary element for a multiplicity of heme-proteins such as hemoglobin, the electron transport system of

the mitochondria, microsomal enzymes involved in drug metabolism, and a variety of other enzymes with or without a prosthetic heme group, as in nitrogenase (Nelson and Cox 2005). In the brain, zinc and copper are metal catalysts required for the action of various enzymes, including superoxide dismutase, tyrosine and tryptophan hydroxylases, alcohol dehydrogenase among other CNS enzymes (Jornvall 1994; Richardson et al. 1975). Moreover, zinc plays a role in the regulation of gene expression by binding to extended DNA sequences through bonding to the famous zinc-finger motifs in proteins (Miller et al. 1985; Nelson and Cox 2005). Other zinc-containing enzymes are of paramount interest for tissue growth, development, and maintenance of the nervous system (Cho et al. 1994; Freemont 1993; Somers et al. 1994). In addition, zinc and copper are stored in nerve termini, apparently playing a role as atypical brain messengers.

Essential metal nutrients, including iodine and cobalt are required for the synthesis of thyroxine and vitamin B_{12}, respectively. While most trace metals such as iron, zinc, copper and molybdenum, are loosely bonded to proteins through electrostatic interactions, forming classical metal-coordination complexes, cobalt, iodine and selenium are amongst the few trace metals covalently linked to organic molecules. Cobalt is bound to the four pyrrole nitrogens of cobalamin, and via a C–C bond to the 5′-deoxyadenosyl unit of the corrin core of vitamin B_{12}; iodine is bonded to the aromatic ring of tyrosine to form tri- and tetraiodothyronine; and selenium is covalently attached to glutathione peroxidase.

Over the past 20 years, structural biologists have provided convincing three-dimensional maps of protein domains for trace metal binding sites of brain related proteins, establishing a solid structural basis for the action of trace metals in neuronal excitability. The use of sophisticated techniques based on studies of the physico-chemical properties of metal-protein interactions has allowed solid progress in this area, establishing the field of metallo-neurobiology. Several speakers at the *Metals and Membranes in Neuroscience Symposium* (Barnham, Grobner, Hill and Separovic) focused on β-amyloid and prion proteins due to their special interest in two major neurodegenerative diseases, and showed impressive progress in the determination of metal actions at the molecular and structural level. The modeling of the interaction of trace metals with these proteins received special attention; we believe that this area of research has benefited from the rapid progress in physical/chemical instrumentation. In this regard, our interest and contribution to understanding the role of trace metals as allosteric modulators of ionic channels is relevant to this Special Issue, highlighting the contribution of our laboratory to metallo-neurobiology. This review is centered to a large extent on the modulator role of trace metals, and particularly zinc and copper, in brain ionic channels. Within the vast family of ligand-gated ion channels, we have limited our efforts to the understanding of the role of trace metals in the biology of purinergic P2X receptors, as endogenous allosteric modulators of these channels.

Zinc and copper homeostasis in the brain

Zinc and copper are indispensable elements for brain excitability; zinc is the second most abundant trace metal in the body after iron. In the brain, zinc concentrations range from 100 to 150 μM, most of which is associated with zinc metalloenzymes, transcription factors and metallothionein proteins, a set of small proteins rich in cysteines with high capacity to chelate trace metals (Cousins 1983). Copper ranks as the third most abundant trace metal in the body; although present at low levels in a variety of neurons and peripheral tissues, its highest concentration is found in the liver. There are increasing and compelling arguments in favor of the notion that both zinc or copper can also function as novel signalling molecules, with evidence of release from synaptic terminals and a measured action on a wide range of membrane proteins, including ionic channels. As such, these ions may be predicted to have relevant modulator roles in neuronal circuits and, therefore, alter brain excitability.

Zinc bioavailability and brain uptake

Since these trace metals are incorporated into the body from the diet, selective transporters participate in their absorption from the gastro intestinal tract to the blood, and from the blood into the brain (Frederickson et al. 2000, 2005). In the blood plasma, the circulating concentration of these metals is around 15 μM; almost all the zinc is loosely bound to plasma proteins, including albumin and free amino acids such as L-histidine. Following metal uptake to the blood, its passage through the brain barrier and cerebrospinal fluid is unrestricted; neuronal uptake relies and depends on the functioning of metal transporters. The brain has the highest zinc content of the body, approx 100–150 μM, a value about 10-fold higher than that found in the plasma (Choi and Koh 1998; Frederickson 1989; Kozma et al. 1981). The zinc content varies within the different brain areas, which might be interpreted to indicate selective regional expression of these metal transporters (Frederickson et al. 2000). In the nervous system, the most relevant zinc transporters are ZnT3 and ZnT4 (Palmiter and Huang 2004). These proteins are involved in the metal transport into synaptic vesicles; the metal storage site relevant and consistent with its putative role as a neuronal messenger and a modulator of synaptic activity and neuronal excitability. In the brain, 90% of the

total zinc is bound to metalloproteins; only approximately 10% of the metal is stored in synaptic vesicles, where the metal is found either loosely bound or free. Several studies have illustrated that the vesicle-stored metal may be released upon nerve terminal electrical depolarization (Aniksztejn et al. 1987; Assaf and Chung 1984; Howell et al. 1984), which has been interpreted in favor of its role as a novel and atypical brain messenger.

Copper absorption and brain levels

Less is known about the biological life cycle of copper and its brain bioavailability, although it is generally assumed that the mechanisms that govern the biology of zinc and copper are much alike. Like zinc, copper accumulates in the brain after its absorption from the distal small intestine as a dietary component. The oral metal absorption varies considerably depending on a variety of factors, including the form of copper in the diet, and other constituents of the diet. In the plasma, roughly 80–95% of copper is bound to ceruloplasmin, a blood protein with high affinity and selective sites for copper binding (Hellman and Gitlin 2002). The metal passage from the blood to the brain implies crossing the brain hemato encephalic barrier, a process that likely involves metal transporters. The copper concentration in the cerebrospinal fluid is around 70 μM; much of this copper is protein bound, with only a minor 10% available for storage in nerve intracellular compartments. Copper transporters were initially identified in yeasts, since these cells were used as a paradigm to understand copper homeostasis (Dancis et al. 1994; Zhou and Thiele 2001). Subsequently, a putative transmembrane transport protein for copper was identified in mammalian and human cells, termed hCtr1 (Zhou and Gitschier 1997). Moreover, from the protein databank a second copper transport protein, designated hCtr2, was also identified (Zhou and Gitschier 1997). In mammals, the copper-transporting ATPases, ATP7A and ATP7B, play a key role in copper regulation, a process that is essential for central nervous system development, liver function, connective tissue formation and many other physiological processes, and the malfunction of these proteins is associated with Menkes and Wilson diseases (Lutsenko et al. 2007). In contrast to zinc, an oxido-redox system is associated with copper transport since, at least in yeast, the transporter operates with Cu(I), while Cu(II) is the active form of copper participating as a modulator of neuronal activity, which implies that Cu(I) must be oxidized to Cu(II) to have a meaningful biological role in enzymes or channels.

The extracellular copper content that may equilibrate with neurons and nerve endings is in the range of 0.2–2 μM. However, and in keeping with the analogy to the bioavailability of brain zinc, the concentration of copper is remarkably larger in the synaptic cleft where copper may reach concentrations bordering 200–300 μM. As with zinc, these values may be even more elevated in pathology, like neurodegenerative diseases.

Great progress has been achieved in the understanding of the intracellular handling of copper. A series of proteins were identified to be involved in the transfer of copper from the cell surface to their intracellular targets; these proteins were named “chaperones”, since they bind copper that had been transferred across the plasma membrane and transfer it to the copper intracellular targets, denoting their protective function. Although these chaperones were initially identified and sequenced from yeast, their mammalian counterparts soon emerged. Three main mammalian chaperones have been identified. The chaperone ATX_1 is a yeast protein that delivers copper to a Cu-ATPase in the Golgi apparatus (Lin et al. 1997). COX17 is a phylogenetically conserved protein that transfers copper to cytochrome oxidase in the mitochondria (Glerum et al. 1996). Finally, a third chaperone is involved in the delivery of copper to superoxide dismutase (Culotta et al. 1997). Altogether, this information indicates that the intracellular copper concentration is largely protein bound with only a minuscule fraction of the metal in its free form, suggesting that the free metal does not exceed the attomolar range (Rae et al. 1999).

An atypical role of trace metals in synapses: metal release from brain nerve endings

A range of experimental techniques ranging from histochemical staining, imaging of zinc by fluorescent dyes, electron microscopy and atomic absorption spectroscopy, concur to unambiguously demonstrate the presence of zinc atoms in the brain. There is consensus that the metal is not uniformly distributed, for example, the grey mater has a denser concentration of the metal compared to the white matter. Histochemical staining of zinc in the brain showed that the highest concentration of the metal is detected in forebrain regions including the hippocampus, amygdala, striatum and neocortex (Slomianka et al. 1990).

Upon closer ultra structural examination of the metal location, high zinc concentrations were located almost exclusively within nerve ending vesicles where the metal is accumulated by the metal ZnT3 transporter (Palmiter and Huang 2004). This observation gave rise to the notion that a pool of free or loosely bound zinc may be stored within the synapse in vesicles as if it were ready for neuronal release. In support for the vesicular storage of zinc, several authors concluded that zinc is co-stored with either glutamate or GABA (Beaulieu et al. 1992; Frederickson 1989; Wang et al. 2002), the main brain excitatory or inhibitory transmitter,

respectively. The putative co-localization of glutamate and zinc gave rise to the term "gluzinergic" neurons (Frederickson et al. 2000), highlighting that both the transmitter plus the nutrient may be the transmitter messengers acting at these synapses. In analogy to zinc, copper is also differentially distributed in the brain with a high preference for certain brain nuclei such as the hippocampus, olfactory bulb and locus coeruleus (Ono and Cherian 1999; Sato et al. 1994). Since in these neurons copper is associated with glutamate or catecholamines, the term "glucupergic" neurons was coined to parallel the gluzinergic nerve endings (Mathie et al. 2006).

The finding of zinc in glycinergic brain synapses (Birinyi et al. 2001) is intriguing. It broadens the distribution of zinc to include the transmission mediated by glycine, suggesting that the glycine receptor might also be under metal modulation. This raises the question of whether presynaptic ZnT3 transporters accumulate the metal in glycine nerve terminals or, alternatively, the overflow of zinc released from neighboring glutamatergic synapses may contribute as a source of zinc stored presynaptically. Experiments with transgenic mice lacking these transporters ($ZnT3^{-/-}$) will help to clarify this and related questions on the homeostasis of zinc by different nerve terminals.

The synaptic terminations of the mossy fiber terminals in the hippocampus contain high concentrations of zinc in synaptic vesicles. The extracellular release of zinc occurs from small presynaptic vesicles upon drug-induced seizures, electrical depolarization or neuronal activity (Takeda et al. 2003). Moreover, the releasable pool of zinc depends on the extracellular content of calcium (Assaf and Chung 1984; Howell et al. 1984), matching the criterion that zinc is released to the synaptic cleft in a calcium-dependent manner.

There is general consensus that glutamatergic, GABA and even glycine nerve terminals may contain high concentrations of zinc in their synaptic vesicles. Since trace metals modulate the several ionic channels operated by these transmitters, the hypothesis was raised that the stored metals in synaptic vesicles could act as novel and atypical neural messengers (Barañano et al. 2001). Nitric oxide or carbon monoxide gases are also included in this list due to their atypical properties. In contrast to classical transmitters, these gases are not stored in vesicles and appear to act on demand, depending on their synthesis availability (Barañano et al. 2001). In addition, further physiological relevance is assigned to nitric oxide and carbon monoxide since these compounds do not act through traditional receptors on postsynaptic membranes but on post-junctional enzymes to alter neuronal metabolism, including the synthesis of cGMP (Boehning and Snyder 2003; Dawson and Snyder 1994). Based on these findings, it has been therefore assumed for some time that synaptic zinc is loosely bound and readily released during nerve activity to act as a post-junctional synaptic modulator. Recent investigations, using fluorometric imaging, has been interpreted to indicate that the presynaptically stored zinc is not in a free-form, but bound tighter to macromolecules in the vesicles (Kay 2006). This novel finding has prompted alternative roles for the synaptic zinc. We hope further developments will resolve these issues and temper the release hypothesis with novel proposals accounting for the kinetics of presynaptic metal release.

Trace metals are allosteric modulators of ligand-gated ionic channels

Proteins are well known trace metals targets. Zinc and copper may directly bind target proteins via ionic bond interactions with amino acid residues such as the sulfhydryl group of cysteines, the imidazole of histidines, carboxylic acid residues of aspartate or glutamic acid residues. Copper, but not zinc, in addition to electrostatic bonding, may oxidize sulfhydryl groups or generate free radicals, which can profoundly alter protein structure and function (Ali et al. 2003). Therefore, the modulator role of copper is more complex than zinc and may involve additional mechanisms of action.

This review focused on the modulator role of trace metals on ligand-gated ionic channels, with particular reference to the P2X nucleotide receptor family. Therefore, we will limit its scope only to ligand-gated ionic channels, although it is well recognized that trace metals also affect other membrane channels, including most specially the family of voltage-gated channels. The interaction of trace metals with these channels is purposely omitted; recent and well-documented reviews are devoted to these channels (Mathie et al. 2006). Trace metals also modulate G protein-coupled receptors; only a brief review of these receptors will be included since they have been less studied in this respect than the ionic receptors.

Glutamate receptors

There is consensus that glutamate is the main excitatory brain transmitter. Two major families of membrane ionic channels are activated by glutamate: the NMDA and the AMPA/kainate receptors. Both receptors are permeable to sodium and in varying degrees also to calcium. Twenty years ago, a first study demonstrated that zinc potently modulated gluatamate-gated currents at either type of receptors. The action of zinc at the NMDA receptor is apparently related to the metal interaction with two separate mechanism(s), each attributed to different metal binding sites on the receptor subunits that form the NMDA ionic

channel (Legendre and Westbrook 1990; Williams 1996). Zinc inhibits NMDA-evoked currents, acting at a voltage-independent site on the NR2A subunit and at a voltage-dependent site on the NR2B subunit (Paoletti et al. 1997). By contrast, the action of zinc at the AMPA/kainate receptors is biphasic, while micromolar concentrations increase the receptor activity, millimolar concentrations decrease it (Rassendren et al. 1990). Minor variations to this general picture have recently emerged in view of the description of a zinc-insensitive population of AMPA/kainate receptors, a finding possibly attributed to the varying subunit composition expressed by individual receptors.

Copper also inhibits the NMDA and AMPA/kainate receptors with affinities of 30 and 5 μM, respectively (Trombley and Shepherd 1996; Weiser and Wienrich 1996).

$GABA_A$ receptors

GABA is a main brain inhibitory transmitter; the $GABA_A$ receptor is chloride selective ion channel that in adult neurons causes membrane hyperpolarization due to the entrance of chloride ions into neurons. A major drawback in the understanding of the action of trace metals with this receptor is related to the heterogeneity of receptor channel subunit composition; different $GABA_A$ receptor subunit combinations have varying sensitivities to the inhibitor modulation by trace metals. For example, the $\alpha_1\beta_3$ variant of the receptor, which is not the most abundant brain receptor population, is much more sensitive to zinc inhibitor modulation, while other subunit receptor combinations are remarkably less sensitive to the zinc inhibitor modulation. The most abundant brain $GABA_A$ receptors contain in addition γ subunits; however, this receptor combination is markedly less sensitive to the modulator action of zinc. Moreover, copper has proved to inhibit the activity of $GABA_A$ receptors; its half maximal inhibition (IC_{50}) is 20 μM (Trombley and Shepherd 1996). Therefore, both trace metals, although with varying affinities, are inhibitor modulators of the various brain combinations of the $GABA_A$ receptor.

Glycine receptors

This is also a CNS inhibitory receptor closely related to the $GABA_A$ receptor channel, which is permeable to chloride ions. Glycine receptors are modulated in a biphasic manner by zinc. Low micromolar metal concentrations facilitate the glycine-evoked currents, while larger concentrations result in inhibition of the glycine-gated currents (Bloomenthal et al. 1994). Site-directed mutagenesis has helped to identify key amino acid residues responsible for both the zinc-induced potentiation and inhibition (Harvey et al. 1999; Laube et al. 2000).

Nicotinic receptors

As with the $GABA_A$ receptors, zinc has different actions on the nicotinic ionic channels, depending on the subunit composition of heteromeric nicotinic receptors; for example, zinc exerts a biphasic modulation of nicotinic receptors composed of $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and $\alpha_4\beta_4$ receptors, while inhibiting the currents mediated by $\alpha_3\beta_2$ receptors (Hsiao et al. 2001). The homomeric α_7 receptor, the predominant CNS receptor involved in the psychotropic action of nicotine, is inhibited by zinc in a concentration-dependent and voltage-independent manner, suggesting an allosteric action by the metal (Palma et al. 1998).

G protein coupled receptors

Trace metal modulation of metabotropic receptors has been studied to less extent than for ionotropic receptors. In despite of this, a zinc-induced inhibition has been described in the A1 adenosine receptor (Rosati and Traversa 1999). The same effect for zinc has been demonstrated in the purinergic $P2Y_2$ receptor, whereas the $P2Y_4$ receptor is resistant to this metal (Wildman et al. 2003). In the case of the $P2Y_1$ receptor, copper inhibits the activity of this receptor at concentrations >30 μM, while zinc has no effect (see Fig. 1). The β2 adrenergic receptor is also modulated by zinc, a histidine residue has been identified as critical for zinc modulation (Swaminath et al. 2003). In the $5\text{-}HT_{2A}$ serotonin receptor, copper slightly inhibits the receptor activity, while zinc has no effect (Huidobro-Toro and Acuña 1998).

Nucleotide receptors as effectors for extracellular purines and pyrimidines

The recognition that extracellular nucleotides, such as adenosine 5′-triphosphate (ATP) and related compounds including UTP, ADP, UDP, CTP and most recently sugar nucleotides such as UDP-glucose and UDP-galactose, are signalling molecules (Abbracchio et al. 2003; Burnstock 2006; Chambers et al. 2000) has prompted increased interest in understanding their roles in physiology and pathology. Cell signalling mediated by nucleotides is exerted by a set of at least 15 distinct cell membrane receptors, which bind selectively either purines or pyrimidines and trigger different intracellular cascades to account for their physiological actions. During the past 20 years, the receptors for this extended family of ligands have been classified into two main groups: the adenosine and the nucleotide receptor families (Ralevic and Burnstock 1998). While the former recognizes both natural and synthetic adenosine nucleosides, the latter receptors are activated exclusively by di- or

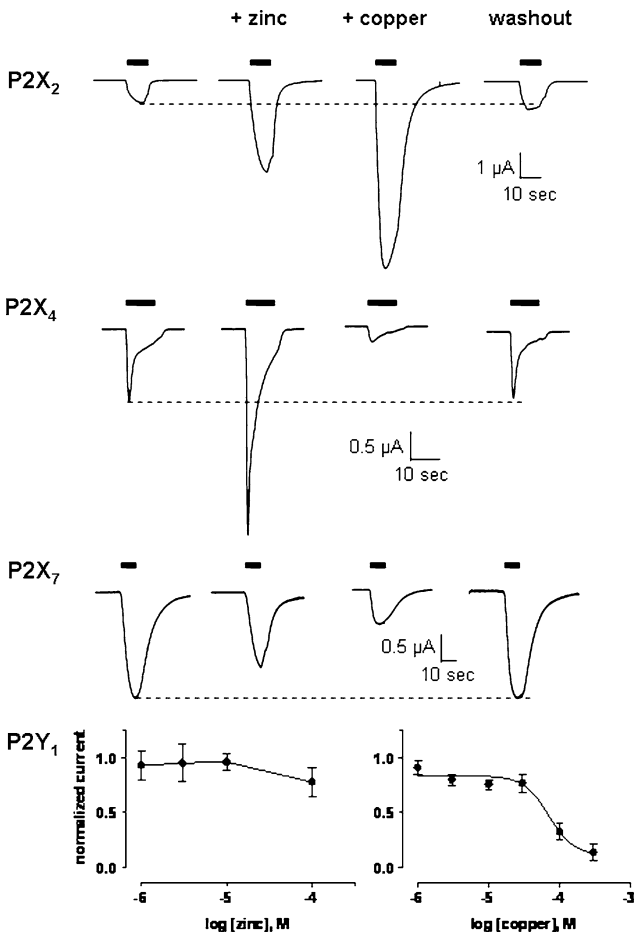

Fig. 1 Representative ATP-gated currents elicited by the $P2X_2$, $P2X_4$, $P2X_7$ or the $P2Y_1$ receptors and their modulation by zinc or copper. Separate *Xenopus laevis* oocytes were injected with cDNA coding for each P2X receptor subtypes or the $P2Y_1$ receptor; 48 h later, currents were measured by the two electrode voltage-clamp technique; using the ATP EC_{50} for each receptor (*solid line* on top of the recording: 30 μM for $P2X_2$, 10 μM for $P2X_4$ and 600 μM for $P2X_7$ receptor). The receptor-gated currents were examined by a 10-s application of ATP alone or in oocytes preincubated for 1 min with either 10 μM zinc or 10 μM copper. The modulator effect of each metal is reversible as indicated by the recordings following extensive metal washout. While the P2X receptors are markedly modulated by these metals, the *lower panel* shows that the activation of the $P2Y_1$ receptor with 1 μM ATP is essentially insensitive up to 100 μM zinc. Copper exhibits a concentration-dependent inhibition with a half-inhibitor potency of 70.2 ± 20.4 μM. The current amplitudes were normalized against the current obtained with 1 μM ATP without metals. *Symbols* indicate mean values; *bars*, the S.E.M. ($n = 4$–6 per data point in the $P2Y_1$ receptor curves)

tri-phosphorylated nucleotides derived from adenosine, uridine, cytosine or synthetic products bearing a bi- or tri-phosphorylated chain (Ralevic and Burnstock 1998). Three subtypes of adenosine receptors are known, one bearing a splice variant, termed the adenosine A_{2A} and A_{2B} receptors (Collis and Hourani 1993; Stone 1991). The nucleotide receptors belong to two major classes: the P2X and the P2Y receptors. The former class comprises seven clones of non-selective cationic channels while the latter is composed of eight receptors each of which is coupled to one of several trimeric G-proteins, belonging to the GPCR family (Ralevic and Burnstock 1998). Both types of nucleotide receptors are widely distributed in the body, both in the nervous system, epithelial lining, bone and blood cells (Ralevic and Burnstock 1998).

Nucleotides, but particularly, purine nucleotides have been recognized to play increasing roles in brain excitability, sensory signalling and a variety of peripheral actions such as pain transmission, urinary voiding, platelet aggregation, vasodilatation, fertility, bronchial airway inflammation and sensory modulation including taste. Transgenic mice lacking nucleotide receptors are available (Cockayne et al. 2000; 2005; Mulryan et al. 2000; Sim et al. 2006; Solle et al. 2001) opening novel opportunities to study the involvement of these extracellular signals in cellular processes, unveiling their participation in animal physiology in health and disease.

P2X receptors: general principles of allosteric modulation

A recurrent theme, common to other ionic channel receptors, refers to the mode of action of zinc or copper as modulators since their action depends to a large extent on the P2X receptor subunit composition and to a lesser extent on the metal concentration. There are two main effects of these metals: either potentiation or inhibition of the ATP-evoked currents, effects observed in concentrations ranging from 0.3 to 100 μM. For example, while zinc inhibited in a concentration-dependent manner the $P2X_1$ receptor activity (Wildman et al. 1999), zinc or copper potentiated the $P2X_2$ receptor activity (Lorca et al. 2005; Xiong et al. 1999). The $P2X_3$ receptor is also potentiated by zinc (Wildman et al. 1999), whereas no information is available for copper on this receptor. However, contrary to the observations in the $P2X_2$ or $P2X_3$ receptors, both zinc and copper inhibit the $P2X_7$ receptor-gated currents (Virginio et al. 1997). An exceptionally interesting case is the $P2X_4$ receptor since this purinoceptor is potentiated by zinc but inhibited by copper (Acuña-Castillo et al. 2000; Soto et al. 1996), demonstrating a more complex pattern of modulation.

A representative set of electrophysiological tracings obtained from *Xenopus laevis* oocytes injected with different P2X receptors show the selective and reversible modulator action of zinc and copper and highlights the actions of these metals on the best characterized P2X receptors (Fig. 1). The tracings also show that each P2X receptor subtype has different electrophysiological properties including the ATP potency and desensitization rate. Consonant with

the effects shown for a single metal concentration (Fig. 1), we consistently have observed that the facilitator mode of zinc action in the $P2X_2$ and $P2X_4$ receptors is due to a reversible and parallel leftward displacement of the ATP concentration-response curve (Acuña-Castillo et al. 2000; Coddou et al. 2003; Lorca et al. 2005). The zinc mode of action suggests that the binding of this metal to the facilitator site increases the receptor affinity for ATP. The allosteric modulation must somehow change the receptor conformation resulting in a larger channel conductance. The inhibitor modulator role of copper, at least in the $P2X_4$ and $P2X_7$ receptors follows the pattern of a non-competitive inhibition, suggesting that the allosteric binding of copper alters the receptor conformation in such a way that as a final result it reduces the receptor efficacy for ATP, which is ultimately linked to a decreased channel-evoked current (Acuña-Castillo et al. 2000; 2007). The copper-induced inhibition of the $P2X_4$ receptor activity is mediated by Cu(II) since the challenge with reduced copper (Cu(I)), using ascorbic acid as a reducing agent, failed to inhibit the ATP-evoked currents. While 5 μM Cu(II) inhibited the 10 μM ATP-induced current 48.3 ± 6.7%, the application of 5 μM Cu(I) resulted in 5.5 ± 6.6% inhibition ($n = 7$, $P < 0.01$; R. A. Lorca, unpublished work).

To examine and identify the role of critical amino acid residues on trace metal modulation of P2X receptors, site-directed mutagenesis has proved an extraordinarily useful tool. This molecular biology strategy makes possible to generate single amino acid mutants, which can be expressed in a heterologous systems as *Xenopus* oocytes (see recordings in Fig. 1) or HEK cells and the metal modulation can be measured using electrophysiological techniques and hence is possible to determine the importance of the particular residue that has been mutated. Using this approach we have identified specific amino acids that exert a key role on metal modulation, concluding that these residues are part of specific allosteric sites that coordinate trace metals, rather than an indirect effect resulting for the possible interaction between trace metals and ATP.

The $P2X_2$ receptor

Since both zinc and copper facilitate the ATP-gated currents in a similar manner, the hypothesis was raised that both trace metals may interact at a common metal binding site on the extracellular domain of this receptor. Therefore, it was anticipated that receptor mutants that modified the zinc effect should likewise modify in a similar manner the effect of copper. This appeared to be the case, since with rare exceptions most of the mutants examined showed parallelism between the receptor activity with zinc or copper. Because imidazole groups are commonly involved in the coordination of zinc or copper, we reasoned that single mutations of extracellular histidines should help us to identify which of the nine histidines are indispensable for modulation by the two metals. Out of the nine extracellular histidines, His-120 and His-213 were reported as playing a key role for the modulator action of both zinc and copper potentiation (Fig. 2); the single substitution of each of these two histidine residues rendered the respective receptor mutants resistant to the action of both metals altogether (Clyne et al. 2002; Lorca et al. 2005). Moreover, most recent reports indicate that these two residues are supposedly in close contact between adjacent subunits of the receptor trimer, forming an intersubunit binding site for zinc, and probably also for copper (Nagaya et al. 2005). Intersubunit metal binding sites had been previously demonstrated for the cyclic nucleotide channel (Gordon and Zagotta 1995). Additional to His-120 and His-213, several residues have been implicated in copper and zinc modulation. These residues (His-192, His-245 and His-319) seem to be only partially involved in metal modulation, indicating a role in stabilization of the metal binding site (Lorca et al. 2005).

Moreover, single channel recordings of the $P2X_2$ receptors expressed in stably transfected HEK 293 cells demonstrated that copper increased $P2X_2$ channel-receptor gating in a cooperative manner (E. Leiva-Salcedo, personal communication). A plausible interpretation of this result suggested that the action of trace metals might be exerted at several levels of the receptor topology. The simplest interaction might only involve each subunit, implying that the trace metals may bind at a metal site within each subunit, or through an intra subunit interaction as depicted in Fig. 3. A second level of interaction might occur within the receptor subunits or, as shown in Fig. 3, inter subunits. But the most attractive possibility refers to inter-channel interactions, as showed diagrammatically in Fig. 3, accounting for the cooperativity of the $P2X_2$ receptor channel, an interaction that appeared to be greatly favoured by copper or zinc. This last challenging possibility remains to be further examined experimentally, but there is evidence showing cooperative interaction between $P2X_2$ receptors when activated by ATP (Ding and Sachs 2002).

To assess the specificity of the receptor metal binding site and to characterize whether other metals might also interact at the trace metals modulator site, other divalent metals were also examined. The $P2X_2$ receptor activity is potentiated by cadmium, mercury, nickel, palladium and cobalt in concentrations ranging from 10 to 100 μM; platinum had no effect on the $P2X_2$ receptor (Lorca et al. 2005), revealing some degree of metal specificity for the $P2X_2$ receptor. Moreover, the magnitude of the ATP-current potentiation was also different for each metal, with copper being the most effective, since 10 μM elicited up to a 25-fold increase in the 10 μM ATP evoked currents.

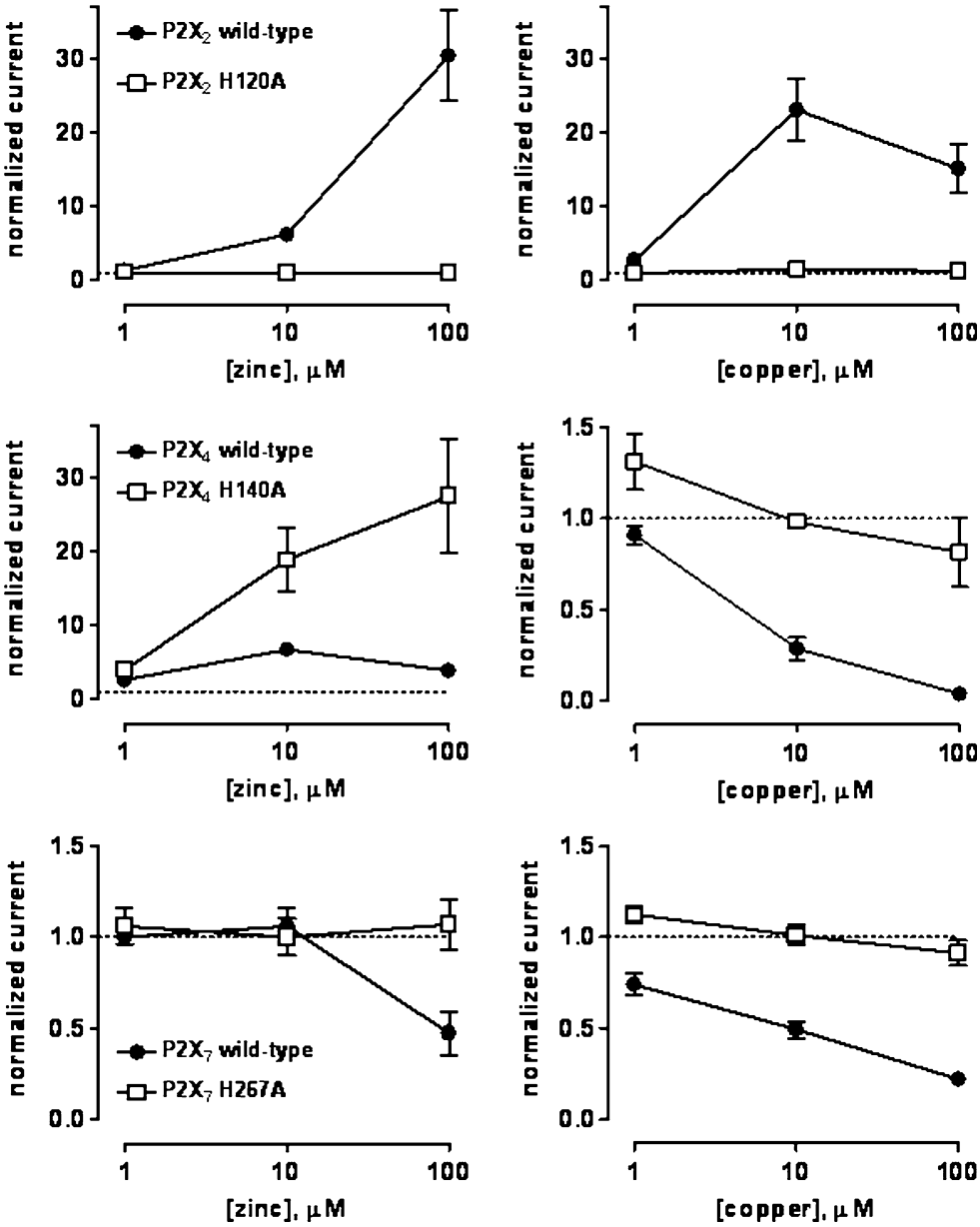

Fig. 2 Zinc and copper concentration-response curves in wild-type and selected receptor mutants of the $P2X_2$, $P2X_4$ and $P2X_7$ receptors. Comparative trace metal concentration-response curves in wild-type and receptor mutants with marked effects on the modulator action of either zinc or copper. Each of the mutants selected replaced an extracellular histidine for an alanine and obliterated the action of zinc and copper, or strongly increased the zinc-potentiation as in the case of the mutant H140A for the $P2X_4$ receptor. The current amplitudes were normalized against the current obtained with ATP without metals

The study of mercury, a metal that causes permanent deterioration of cognitive brain function, was of particular interest, because the inhibitory modulation of mercury was not abrogated in several receptor mutants rendered insensitive to copper. We therefore reasoned that copper and mercury must act at separate and distinct sites. On the way to better define the action of mercury, we examined chimeric $P2X_{4/2a}$ or $P2X_{4/2b}$ receptors (J. P. Huidobro-Toro, unpublished work). The extracellular domains of these receptors chimera corresponded to the $P2X_4$ receptor, while the transmembrane and intracellular domains are of $P2X_2$ receptor phenotype. Moreover, there is a splice variant of the $P2X_2$ receptor, which lacks a 69 amino acid segment towards the carboxy terminal end of the receptor. Preliminary data showed that mercury interacts and labels an intracellular cysteine of the $P2X_{2a}$ splice variant of the $P2X_2$ receptor. Apparently the sulfhydryl group of Cys-430 of the $P2X_{2a}$ receptor acts as an intracellular redox sensor since hydrogen peroxide increases the ATP-gated currents in a concentration-dependent manner. Consistent with an intracellular site for mercury and the peroxide, the action of both mercury and hydrogen peroxide was abrogated in the $P2X_2$ mutant C430A, an indication that an intracellular cysteine must be involved in the mercury inhibitory modulation (C. Coddou, unpublished work), allowing us to propose that the intracellular Cys-430 plays a pivotal role in receptor activity, acting likely as an intracellular redox sensor.

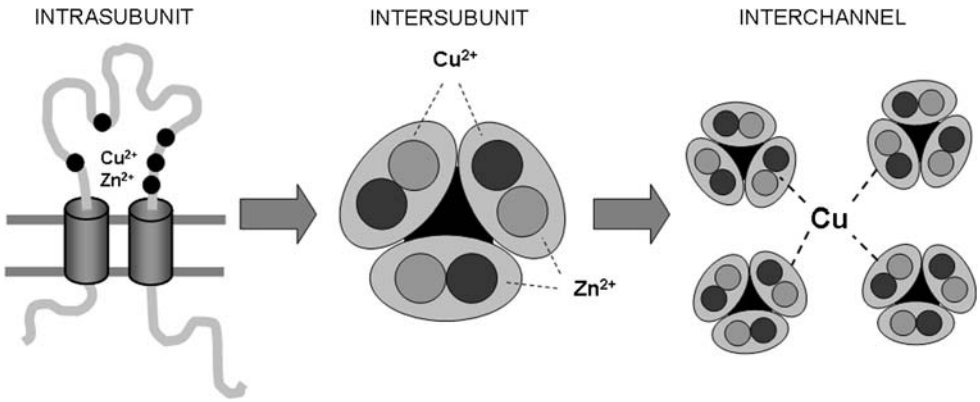

Fig. 3 Schematic representations of different putative levels of trace metal interactions with the P2X receptors. Heuristic diagrams show three theoretical possibilities on the mode of zinc or copper interactions to modulate P2X receptor activity. The simplest interaction might only imply metal binding to ligand receptor amino acid residues forming metal modulator sites restricted to each of the subunits comprising the functional receptor channel trimer. A second plausible type of interaction might consider metal binding sites with amino acid ligands within the different subunits of the functional trimer receptor channel. Finally, a third possible mode of interaction of these metals could imply metal coordination with amino acid residues of adjacent channels, implying cooperative interactions between functional channels in the cell membrane surface, as might occur in metal-triggered receptor clustering. Obviously, these models also suggest alternative intermediate possibilities implying multiple modes of trace metal interactions with the identified amino acid, which act as ligands for either zinc and/or copper binding at their modulator sites. In these schemes, *circles* represent each receptor subunit membrane domain, referring to the two transmembrane domains of the P2X receptor subunits; *ovals* represent the receptor subunit. The functional receptor channel is represented by a *trimer*

The $P2X_4$ receptor

As mentioned previously, the $P2X_4$ receptor is exceptionally interesting and different from the rest of the P2X receptor members since zinc and copper have opposite effects on the ATP-gated currents (Acuña-Castillo et al. 2000), a feature that denotes it as an ideal candidate to dissect whether the receptor contains two separate allosteric sites for zinc and copper. Alternatively, these metals might act in a different mode at a common allosteric metal recognition site.

Regarding extracellular histidines, a key amino acid as a ligand for both zinc and copper at the $P2X_2$ receptor, this receptor contains only three extracellular imidazole groups. Of these three imidazoles, only His-140 has demonstrated to be critical for the copper-induced inhibition (Fig. 2; Coddou et al. 2003). In addition to the copper-resistance of the H140A mutant, a significant increase of zinc-potentiation was observed changing the metal concentration-response curve from a bell shape in the wild-type receptor to a sigmoidal curve. A plausible interpretation of these results implies that the $P2X_4$ receptor has two separate and distinct allosteric sites for metal modulation. One is an inhibitor site that binds copper with high affinity and zinc at concentrations above 10 μM. The other allosteric site is a facilitator site that accounts for the zinc-induced potentiation. Since zinc binds to both sites with different affinities, the final effect observed at the wild-type $P2X_4$ receptor is a biphasic bell-shaped curve. Recent findings from our laboratory confirmed the existence of these two distinct and independent allosteric sites within the $P2X_4$ receptor. A cysteine (Cys-132) very close and in the vicinity of His-140, is key for zinc potentiation and therefore must be part of the facilitator site. Moreover, the C132A mutant was resistant to the metal-induced potentiation, but instead was consistently inhibited by zinc. These results were not observed in the C126A mutant, confirming the specificity of Cys-132 for zinc potentitation and suggest that this residue might have its sulfyhydryl group in a free form to account for the zinc effects. We also identified an additional residue (Asp-138) that is part of the inhibitory site (C. Coddou, unpublished work). The $P2X_4$ receptor may have a third allosteric site for divalent metals in addition to the extracellular facilitator and inhibitory sites, since mercury, a metal that mimics the action and potency of copper in this receptor, strongly inhibits the copper-resistant H140A mutant (Coddou et al. 2005). The localization of this putative third allosteric site remains unknown.

As with the $P2X_2$ receptor, we also examined whether other metals also interacted at the two modulator sites of the $P2X_4$ receptor. We observed that cadmium mimicked the action of zinc, while mercury elicited a copper-like inhibition, although its action was not related to His-140 (Coddou et al. 2005). Cobalt also augmented the ATP-evoked current in an irreversible manner, while manganese, barium, lead and nickel had no effect on the $P2X_4$ receptor activity (Acuña-Castillo et al. 2000; Coddou et al. 2005).

The $P2X_7$ receptor

Like other members of the P2X family, the $P2X_7$ receptor is modulated by divalent cations, including calcium and

magnesium (North 2002). In contrast to other family members, the $P2X_7$ receptor is inhibited by copper and zinc (Coddou et al. 2002; Virginio et al. 1997; Watano et al. 2002); copper being at least 20-fold more potent than zinc (Acuña-Castillo et al. 2007). Protons also modulate this receptor (Acuña-Castillo et al. 2007; Virginio et al. 1997), a feature in common with other P2X receptors that will not be covered in this review. Keeping in mind that extracellular histidines are ligands for trace metals and, therefore, essential for trace metal coordination, we addressed the relevance of the extracellular histidines for the action of zinc or copper. Out of the six histidines of the extracellular $P2X_7$ receptor domain, three were identified as part of the copper and zinc modulator sites. Of these residues, His-267 plays a pivotal role for both copper and zinc modulation (Fig. 2; Acuña-Castillo et al. 2007). In contrast, His-130 is involved only in copper inhibition while His-219 plays a more preponderant role for zinc but not for copper modulation (Acuña-Castillo et al. 2007).

Of particular relevance, was to address the mechanism by which magnesium inhibits the $P2X_7$ receptor function, an issue considered a hallmark in the physiology of this receptor. His-130 together with His-201 proved essential for the inhibitor modulator role of this divalent metal (Acuña-Castillo et al. 2007). Magnesium inhibition was previously attributed to the complexation of ATP with magnesium to form ATP^{4-}, which was supposed to be the $P2X_7$ receptor agonist. However, in our view, the lack of magnesium inhibition in the H130A and the H201A mutants evidenced unambiguously that this metal binds to an allosteric site in the receptor extracellular domain and not to ATP itself (Acuña-Castillo et al. 2007).

Therefore, we hypothesized that the action of magnesium is due to its recognition of a novel allosteric metal binding site, which includes His-130 and His-201 as key amino acids.

Physiological implications for the role of trace metals as allosteric modulators of multiple ionic channels

Altogether, the previous section concurs to indicate that the concentration of zinc and copper that markedly modulate ATP-gated responses, at least in-vitro, are within the range of physiological concentrations that may be found within the synapse. This conclusion is of particular relevance for $P2X_2$, $P2X_4$ and $P2X_7$ nucleotide receptors subtypes, which as summarized, are amongst the most sensitive receptor channels to trace metal modulation since they require the lowest metal concentrations to profoundly modify the ATP-evoked currents. We have repeatedly reported that zinc or copper within 1–10 μM, notoriously modify P2X receptor-evoked currents (Acuña-Castillo et al. 2000; 2007; Coddou et al. 2002; 2003; Lorca et al. 2005), an indication that these receptors may be persistently modulated by the basal concentration of trace metals permanently found in the synapse. However, upon nerve depolarization zinc or copper may be released from their storing vesicles to the synaptic cleft (Anikszteljn et al. 1987; Assaf and Chung 1984; Barañano et al. 2001; Howell et al. 1984; Kardos et al. 1989), allowing the accumulation of 200–300 μM, at the synaptic cleft. At this large metal concentration, glutamate and $GABA_A$, nicotinic or glycine receptors may also be modulated. Therefore, we deem plausible to propose as a working hypothesis that trace metals acting at the synapse as allosteric transmitter modulators, are one of the physiological factors that may influence directly brain excitability in health and disease.

Structural consequences

Independent of the precise identification of all the amino acids involved as ligands for divalent metals in the P2X family of receptors, the current experimental evidences allows us to draw conclusions on whether the trace metal coordination sites are conserved in the primary sequence of the P2X receptor family. We hope that this feature will be completed within a few years in an effort to encompass the research led by several laboratories contributing to metalloneurobiology. The identification and comparison of the postulated amino acid residues involved in trace metal binding are key to understanding the structural determinants required for trace metal coordination at the $P2X_2$, $P2X_4$ and $P2X_7$ receptor. Figure 4 represents a schematic diagram comparing key amino acids identified for trace metal binding. We conclude on the paramount, but not unique, role played by extracellular histidines in these proteins to bind zinc and particularly copper. These residues are not all evolutionary conserved in the primary sequences of the P2X receptor members (North 2002), in contrast to the well conserved 10 cysteines present in all P2X receptors, which apparently play a structural role in receptor channel conformation. The variations in the amino acids observed to participate in trace metal modulation might indicate separate adaptations to trace metals depending on the tissue selectivity for regional subunit receptor expression.

A most remarkable example of this condition is exemplified by the mode of zinc binding to the $P2X_2$ versus the $P2X_4$ receptor. While the histidine imidazole groups are required as zinc ligands at the $P2X_2$ and $P2X_7$ receptors, no imidazole groups are involved as zinc ligands at the $P2X_4$ receptor (Coddou et al. 2003). Instead, we raised the proposal that Cys-132 is key as a zinc ligand but is not indispensable for copper coordination (C. Coddou, unpublished

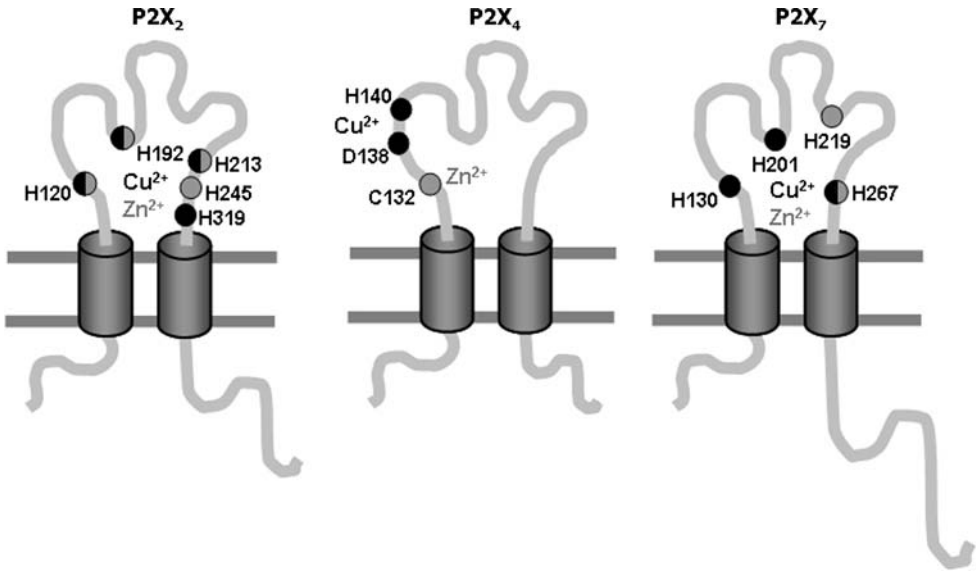

Fig. 4 Schematic comparative topology maps depicting identified key amino acids indispensable for metal binding sites in the $P2X_2$, $P2X_4$ and $P2X_7$ receptors. Linear schematic representation of P2X receptors depicting essential amino acids involved in the modulator site(s), which contribute to the metal coordination. It is obvious that in spite of the 40–50% identity in their primary amino acid sequences, the metal binding site(s) particularly, for zinc, is not evolutionary conserved. For example, while the $P2X_2$ receptors binds zinc by two imidazole groups identified as His-120 and His-213, this metal does not require imidazole groups for zinc binding at the $P2X_4$ receptor, but a sulfhydryl group contributed by Cys-132. For the $P2X_4$ receptor a single extracellular histidine is critically required for the binding of copper, while in the $P2X_2$ and $P2X_7$ receptor, at least two histidines are involved in copper coordination. *Black circles* represent critical amino acids identified in the coordination of copper; *grey circles* denote amino acids involved in zinc coordination. *Half black and grey circles* represent amino acids that bind both metals

work). Likewise, and consonant with these findings for trace metals, the extracellular histidines sensitive to changes in pH are different from those involved in copper coordination, an observation common for the $P2X_2$, $P2X_4$ and $P2X_7$ receptors. Therefore, it might be expected that the several P2X receptors evolved different binding pockets for trace metal coordination, each involving the interaction with separate and independent amino acids. Until the three-dimensional protein folding is firmly established, it is as yet not possible to assess whether amino acids in separate receptor subunits or even different functional channels are involved in trace metal action.

Perspectives and future developments

A challenging feature that is urgently required demands the development of in vivo studies replacing the current in vitro protocols by more physiological meaningful approaches using either neuronal cell cultures or brain slices. These experiments should assess whether our observations in *Xenopus laevis* oocytes are relevant to neuronal excitability. In this regard, a most interesting avenue is the recent report of transgenic mice lacking the zinc modulator sites of a mutant glycine receptor channel (Laube et al. 2000). These animals develop a characteristic phenotype, evidencing the critical role of trace metals in neuronal excitability and, therefore, in behavior. The mutations identified as critical for trace metal modulation in vitro provide a range of possible targets to develop transgenic mice to assess the modulator role of zinc or copper in purinergic brain neurotransmission.

A second issue critically relevant to underscore the role of trace metals is the quantification of trace metals released from brain synaptic vesicles. Even though the literature is plagued with reports assuming the relevant role of trace metals to neuronal events, recent reports cast doubts on the interpretation that significant concentrations of zinc are released to the synapse upon electrical nerve ending depolarization. The measurement of the metals using fluorescent dyes and physical techniques to image the metal indicate that only a minuscule fraction of the stored metal might be released. Moreover, the fraction of zinc released is not in a free form since apparently the metal might be complexed with an as yet unidentified macromolecule (Kay and Toth 2006). Altogether, considerable research is urgently required to resolve the question of synaptic zinc mobilization and its synaptic role. Even more challenging are parallel experiments assessing the role and mobilization of synaptic copper.

A further clinically relevant aspect refers to the functional significance of trace metals in diseased states

characterized by dietary deficits in zinc or copper. Animal studies with dietary deficiencies, conditioning therefore reduced brain uptake and particularly the storage of synaptic trace metals, are urgently required. Carefully controlled animal studies, linking behavior to brain levels of zinc or copper will serve to better define the functional role of these metals at the cellular and molecular levels. Studies using brain slices, particularly assessing the role of these metals in long-term potentiation (Doreulee et al. 1997; Goldschmith et al. 2005; Vogt et al. 2000), will also provide useful models to better analyze the molecular basis of trace metal action at the synapse and their influence in brain excitability. The recent work of R. A. Lorca (unpublished work) highlights the potential of dissecting the role of purinergic mechanisms in long-term potentiation responses by using zinc or copper as brain transmitter modulators. Brain compensatory mechanisms adjusting to the dietary deficiency in these metals, will also provide relevant information about the molecular basis of brain homeostasis to trace metal depletion and their molecular consequences at the synapse.

Final remarks

The role of zinc or copper as novel and atypical brain messengers and transmitter modulators of ligand-gated receptor channels, is an exciting and fruitful field of neuroscience that will grow with the revolutionary success of molecular biology, electrophysiology and neurochemistry to understand brain functioning. The metallo-neuroscience of zinc and copper, although as yet imperfect and incomplete, will mature within a few years to provide spectacular new avenues to understand how a broad variety of dietary components regulate and participate in the intricate mechanisms of the brain and behavior.

Acknowledgments C. Coddou and R. A. Lorca are CONICYT graduate student fellowship awardees. Partially funded by FONDAP grant 13980001; the Millennium Institute for Applied and Fundamental Biology, MIFAB, and the International Copper Association also contributed with grants to the Center development.

References

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 24:52–55

Acuña-Castillo C, Morales B, Huidobro-Toro JP (2000) Zinc and copper modulate differentially the P2X4 receptor. J Neurochem 74:1529–1537

Acuña-Castillo C, Coddou C, Bull P, Brito J, Huidobro-Toro JP (2007) Differential role of extracellular histidines in copper, zinc, magnesium and proton modulation of the P2X7 purinergic receptor. J Neurochem 101:17–26

Ali FE, Barnham KJ, Barrow CJ, Separovic F (2003) Copper catalysed oxidation of amino acids and Alzheimer's disease. Lett Peptide Sci 10:405–407

Aniksztejn L, Charton G, Ben-Ari Y (1987) Selective release of endogenous zinc from the hippocampal mossy fibers in situ. Brain Res 404:58–64

Assaf SY, Chung SH (1984) Release of endogenous Zn2+ from brain tissue during activity. Nature 308:734–736

Barañano DE, Ferris CD, Snyder SH (2001) Atypical neural messengers. Trends Neurosci 24:99–106

Beaulieu C, Dyck R, Cynader M (1992) Enrichment of glutamate in zinc-containing terminals of the cat visual cortex. Neuroreport 3:861–864

Birinyi A, Parker D, Antal M, Shupliakov O (2001) Zinc co-localizes with GABA and glycine in synapses in the lamprey spinal cord. J Comp Neurol 433:208–221

Bloomenthal AB, Goldwater E, Pritchett DB, Harrison NL (1994) Biphasic modulation of the strychnine-sensitive glycine receptor by Zn2+. Mol Pharmacol 46:1156–1159

Boehning D, Snyder SH (2003) Novel neural modulators. Annu Rev Neurosci 26:105–131

Burnstock G (2006) Historical review: ATP as a neurotransmitter. Trends Pharmacol Sci 27:166–176

Clyne JD, LaPointe LD, Hume RI (2002) The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH. J Physiol 539:347–359

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature 407:1011–1015

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP (2005) P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. J Physiol 567:621–639

Coddou C, Villalobos C, Gonzalez J, Acuña-Castillo C, Loeb B, Huidobro-Toro JP (2002) Formation of carnosine-Cu(II) complexes prevents and reverts the inhibitory action of copper in P2X4 and P2X7 receptors. J Neurochem 80:626–633

Coddou C, Morales B, Gonzalez J, Grauso M, Gordillo F, Bull P, Rassendren F, Huidobro-Toro JP (2003) Histidine 140 plays a key role in the inhibitory modulation of the P2X4 nucleotide receptor by copper but not zinc. J Biol Chem 278:36777–36785

Coddou C, Lorca RA, Acuña-Castillo C, Grauso M, Rassendren F, Huidobro-Toro JP (2005) Heavy metals modulate the activity of the purinergic P2X4 receptor. Toxicol Appl Pharmacol 202:121–131

Collis MG, Hourani SM (1993) Adenosine receptor subtypes. Trends Pharmacol Sci 14:360–366

Cousins RJ (1983) Metallothionein-aspects related to copper and zinc metabolism. J Inherit Metab Dis 6(Suppl 1):15–21

Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD (1997) The copper chaperone for superoxide dismutase. J Biol Chem 272:23469–23472

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, Livi GP (2000) A G protein-coupled receptor for UDP-glucose. J Biol Chem 275:10767–10771

Cho Y, Gorina S, Jeffrey PD, Pavletich NP (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 265:346–355

Choi DW, Koh JY (1998) Zinc and brain injury. Annu Rev Neurosci 21:347–375

Dancis A, Yuan DS, Haile D, Askwith C, Eide D, Moehle C, Kaplan J, Klausner RD (1994) Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. Cell 76:393–402

Dawson TM, Snyder SH (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 14:5147–5159

Ding S, Sachs F (2002) Evidence for non-independent gating of P2X2 receptors expressed in *Xenopus* oocytes. BMC Neurosci 3:17

Doreulee N, Yanovsky Y, Haas HL (1997) Suppression of long-term potentiation in hippocampal slices by copper. Hippocampus 7:666–669

Frederickson CJ (1989) Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol 31:145–238

Frederickson CJ, Suh SW, Silva D, Frederickson CJ, Thompson RB (2000) Importance of zinc in the central nervous system: the zinc-containing neuron. J Nutr 130:1471S–1483S

Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462

Freemont PS (1993) The RING finger. A novel protein sequence motif related to the zinc finger. Ann N Y Acad Sci 684:174–192

Glerum DM, Shtanko A, Tzagoloff A (1996) Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem 271:14504–14509

Goldschmith A, Infante C, Leiva J, Motles E, Palestini M (2005) Interference of chronically ingested copper in long-term potentiation (LTP) of rat hippocampus. Brain Res 1056:176–182

Gordon SE, Zagotta WN (1995) Subunit interactions in coordination of Ni2+ in cyclic nucleotide-gated channels. Proc Natl Acad Sci USA 92:10222–10226

Harvey RJ, Thomas P, James CH, Wilderspin A, Smart TG (1999) Identification of an inhibitory Zn2+ binding site on the human glycine receptor alpha1 subunit. J Physiol 520(Pt 1):53–64

Hellman NE, Gitlin JD (2002) Ceruloplasmin metabolism and function. Annu Rev Nutr 22:439–458

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308:736–738

Hsiao B, Dweck D, Luetje CW (2001) Subunit-dependent modulation of neuronal nicotinic receptors by zinc. J Neurosci 21:1848–1856

Huidobro-Toro JP, Acuña C (1998) Copper inhibits the brain P2X4 ATP receptor and the 5-HT2 receptor responses expressed in *Xenopus laevis* oocytes. Naunyn Schmiedebergs Arch Pharmacol 358(S1):377

Jornvall H (1994) The alcohol dehydrogenase system. EXS 71:221–229

Kardos J, Kovacs I, Hajos F, Kalman M, Simonyi M (1989) Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. Neurosci Lett 103:139–144

Kay AR (2006) Imaging synaptic zinc: promises and perils. Trends Neurosci 29:200–206

Kay AR, Toth K (2006) Influence of location of a fluorescent zinc probe in brain slices on its response to synaptic activation. J Neurophysiol 95:1949–1956

Kozma M, Szerdahelyi P, Kasa P (1981) Histochemical detection of zinc and copper in various neurons of the central nervous system. Acta Histochem 69:12–17

Laube B, Kuhse J, Betz H (2000) Kinetic and mutational analysis of Zn2+ modulation of recombinant human inhibitory glycine receptors. J Physiol 522(Pt 2):215–230

Legendre P, Westbrook GL (1990) The inhibition of single N-methyl-D-aspartate-activated channels by zinc ions on cultured rat neurones. J Physiol 429:429–449

Lin SJ, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC (1997) A role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. J Biol Chem 272:9215–9220

Lorca RA, Coddou C, Gazitua MC, Bull P, Arredondo C, Huidobro-Toro JP (2005) Extracellular histidine residues identify common structural determinants in the copper/zinc P2X2 receptor modulation. J Neurochem 95:499–512

Lutsenko S, Barnes NL, Bartee MY, Dmitriev OY (2007) Function and regulation of human copper-transporting ATPases. Physiol Rev 87:1011–1046

Mathie A, Sutton GL, Clarke CE, Veale EL (2006) Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol Ther 111:567–583

Miller J, McLachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. Embo J 4:1609–1614

Mulryan K, Gitterman DP, Lewis CJ, Vial C, Leckie BJ, Cobb AL, Brown JE, Conley EC, Buell G, Pritchard CA, Evans RJ (2000) Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. Nature 403:86–89

Nagaya N, Tittle RK, Saar N, Dellal SS, Hume RI (2005) An intersubunit zinc binding site in rat P2X2 receptors. J Biol Chem 280:25982–25993

Nelson DL, Cox MM (2005) Lehninger principles of biochemistry, 4th edn. Freeman HW & Co, New York

North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067

Ono S, Cherian MG (1999) Regional distribution of metallothionein, zinc, and copper in the brain of different strains of rats. Biol Trace Elem Res 69:151–159

Palma E, Maggi L, Miledi R, Eusebi F (1998) Effects of Zn2+ on wild and mutant neuronal alpha7 nicotinic receptors. Proc Natl Acad Sci USA 95:10246–10250

Palmiter RD, Huang L (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch 447:744–751

Paoletti P, Ascher P, Neyton J (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci 17:5711–5725

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284:805–808

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

Rassendren FA, Lory P, Pin JP, Nargeot J (1990) Zinc has opposite effects on NMDA and non-NMDA receptors expressed in *Xenopus* oocytes. Neuron 4:733–740

Richardson J, Thomas KA, Rubin BH, Richardson DC (1975) Crystal structure of bovine Cu,Zn superoxide dismutase at 3 A resolution: chain tracing and metal ligands. Proc Natl Acad Sci USA 72:1349–1353

Rosati AM, Traversa U (1999) Mechanisms of inhibitory effects of zinc and cadmium ions on agonist binding to adenosine A1 receptors in rat brain. Biochem Pharmacol 58:623–632

Sato M, Ohtomo K, Daimon T, Sugiyama T, Iijima K (1994) Localization of copper to afferent terminals in rat locus ceruleus, in contrast to mitochondrial copper in cerebellum. J Histochem Cytochem 42:1585–1591

Sim JA, Chaumont S, Jo J, Ulmann L, Young MT, Cho K, Buell G, North RA, Rassendren F (2006) Altered hippocampal synaptic potentiation in P2X4 knock-out mice. J Neurosci 26:9006–9009

Slomianka L, Danscher G, Frederickson CJ (1990) Labeling of the neurons of origin of zinc-containing pathways by intraperitoneal injections of sodium selenite. Neuroscience 38:843–854

Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA (2001) Altered cytokine production in mice lacking P2X(7) receptors. J Biol Chem 276:125–132

Somers W, Ultsch M, De Vos AM, Kossiakoff AA (1994) The X-ray structure of a growth hormone-prolactin receptor complex. Nature 372:478–81

Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, Stuhmer W (1996) P2X4: an ATP-activated ionotropic receptor cloned from rat brain. Proc Natl Acad Sci USA 93:3684–3688

Stone TW (1991) Receptors for adenosine and adenine nucleotides. Gen Pharmacol 22:25–31

Swaminath G, Lee TW, Kobilka B (2003) Identification of an allosteric binding site for Zn2+ on the beta2 adrenergic receptor. J Biol Chem 278:352–356

Takeda A, Hirate M, Tamano H, Oku N (2003) Zinc movement in the brain under kainate-induced seizures. Epilepsy Res 54:123–129

Trombley PQ, Shepherd GM (1996) Differential modulation by zinc and copper of amino acid receptors from rat olfactory bulb neurons. J Neurophysiol 76:2536–2546

Virginio C, Church D, North RA, Surprenant A (1997) Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. Neuropharmacology 36:1285–1294

Vogt K, Mellor J, Tong G, Nicoll R (2000) The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron 26:187–196

Wang Z, Danscher G, Kim YK, Dahlstrom A, Mook Jo S (2002) Inhibitory zinc-enriched terminals in the mouse cerebellum: double-immunohistochemistry for zinc transporter 3 and glutamate decarboxylase. Neurosci Lett 321:37–40

Watano T, Matsuoka I, Kimura J (2002) Inhibitory effects of metals on ATP-induced current through P2X7 receptor in NG108-15 cells. Jpn J Pharmacol 89:296–301

Weiser T, Wienrich M (1996) The effects of copper ions on glutamate receptors in cultured rat cortical neurons. Brain Res 742:211–218

Wildman SS, King BF, Burnstock G (1999) Modulation of ATP-responses at recombinant rP2X4 receptors by extracellular pH and zinc. Br J Pharmacol 126:762–768

Wildman SS, Unwin RJ, King BF (2003) Extended pharmacological profiles of rat P2Y2 and rat P2Y4 receptors and their sensitivity to extracellular H+ and Zn2+ ions. Br J Pharmacol 140:1177–1186

Williams K (1996) Separating dual effects of zinc at recombinant N-methyl-D-aspartate receptors. Neurosci Lett 215:9–12

Xiong K, Peoples RW, Montgomery JP, Chiang Y, Stewart RR, Weight FF, Li C (1999) Differential modulation by copper and zinc of P2X2 and P2X4 receptor function. J Neurophysiol 81:2088–2094

Zhou B, Gitschier J (1997) hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci USA 94:7481–7486

Zhou H, Thiele DJ (2001) Identification of a novel high affinity copper transport complex in the fission yeast *Schizosaccharomyces pombe*. J Biol Chem 276:20529–20535