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Review article

Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function

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ABSTRACT

Zinc transporter 3 (ZnT3) is the sole mechanism responsible for concentrating zinc ions within synaptic vesicles in a subset of the brain's glutamatergic neurons. This vesicular zinc can then be released into the synaptic cleft in an activity-dependent fashion, where it can exert many signaling functions. This review provides a comprehensive discussion of the localization and function of ZnT3 and vesicular zinc in the central nervous system. We begin by reviewing the fundamentals of zinc homeostasis and transport, and the discovery of ZnT3. We then focus on four main topics. I) The anatomy of the zincergic system, including its development and its modulation through experience-dependent plasticity. II) The role of zinc in intracellular signaling, with a focus on how zinc affects neurotransmitter receptors and synaptic plasticity. III) The behavioural characterization of the ZnT3 KO mouse, which lacks ZnT3 and, therefore, vesicular zinc. IV) The roles of ZnT3 and vesicular zinc in health and disease.

1. Introduction to zinc and ZNT3

That zinc is a critical factor in human health and development has been known at least as far as to the late 1960s, when pioneering work by Prasad et al. (1961) demonstrated that severe zinc deficiency leads to an array of developmental abnormalities and impaired functions. Today, it is known that as much as 10% of the human proteome consists of zinc-binding proteins (Andreini et al., 2006), and it has increasingly come to be understood that zinc fills a diverse set of roles at the cellular level. Biologically, zinc is ubiquitous, is solely found as a divalent cation (Zn^{2+}), and is redox neutral. After iron, it is the most abundant transition metal in the body. In its affinity for binding protein ligands, zinc is second only to copper among the biologically-relevant divalent metals (Maret, 2014).

In the narrower field of neurobiology, zinc is also of considerable interest. One of the more intriguing ideas to emerge from the field of zinc neurobiology is that zinc can act essentially as a neurotransmitter. Such an ability requires that zinc be concentrated in presynaptic terminals and subsequently released into the synaptic cleft in a regulated fashion, where it would bind to and modulate the function of receptors before ultimately being transported out of the cleft or in some other way inactivated. Partial evidence for this idea has been available since the 1980s, when it was shown that activity-dependent release of zinc can occur in the brain (Assaf and Chung, 1984; Howell et al., 1984; Aniksztejn et al., 1987) and that zinc can modulate neuronal function through its effects on receptors (Smart and Constanti, 1983; Peters et al., 1987; Westbrook and Mayer, 1987). But it was not until the discovery and characterization of zinc transporter 3 (ZnT3) in the mid-to-late 1990s (Palmiter et al., 1996; Wenzel et al., 1997) – and the subsequent generation of transgenic mice that lack ZnT3 (Cole et al., 1999) – that the mechanism behind presynaptic concentration and activity-dependent release of zinc was finally elucidated, filling one of the major criteria for zinc to be considered as a neurotransmitter. Experiments conducted in the years since have provided further evidence, and it is now beyond doubt that zinc can serve as a synaptically-released intercellular signal. In the present review, we summarize this research,

Abbreviations: 2-AG, 2-arachidonolylglycerol; Aβ, beta-amyloid; AP-3, adaptor protein 3; BDNF, brain-derived neurotrophic factor; BNST, bed nucleus of the stria terminalis; CaEDTA, calcium-saturated EDTA; ClC-3, chloride channel 3; DCN, dorsal cochlear nucleus; ERK, extracellular signal-regulated kinase; GPR39, G protein-coupled receptor 39; HFS, high-frequency stimulation; IPL, inner plexiform layer; KCC2, potassium-chloride cotransporter 2; KO, knockout; LGN, lateral geniculate nucleus; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MF-CA3, Mossy fiber-CA3; MMP, matrix metalloproteinase; MRE, metal response element; MT, metallothionein; MTF1, metal regulatory element-binding transcription factor-1; pERK, phosphorylated form of ERK1/2; PKA, protein kinase A; SC-CA1, Schaffer collateral-CA1; STEP, striatal-enriched tyrosine phosphatase; TrkB, tropomyosin receptor kinase B; VGLUT1, vesicular glutamate transporter 1; ZIP, Zrt, Irt-like protein; ZnT, zinc transporter

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discussing ZnT3 and zinc signaling in the central nervous system. In particular, we focus on what has proven to be a highly useful tool for understanding the roles of zinc in neurobiology: the ZnT3 knockout (KO) mouse.

1.1. Zinc homeostasis and zinc transport

Zinc is an essential component of all cells due to its structural, catalytic, and signaling functions. The total intracellular concentration of zinc is several hundred micromolar (Krezel and Maret, 2006; Colvin et al., 2008). The vast majority of this zinc is tightly bound within protein structures, but a portion exists as "free zinc". This is an operational definition that describes zinc ions that are not tightly bound by proteins (Maret, 2014). The coordination environment of these free zinc ions is not presently known. They may not exist in an entirely free coordination state (i.e., as hydrated zinc ions) but might instead be bound to low molecular-weight ligands. What is known, however, is that these zinc ions are readily available to serve fast signaling functions.

It is a requirement for normal cellular function that intracellular zinc homeostasis be maintained. The cytosolic free zinc concentration must be high enough that binding sites on zinc metalloproteins remain occupied, but not so high that zinc displaces lower affinity biologically-relevant divalent cations – such as nickel or cobalt – from their respective metalloproteins (Colvin et al., 2010). The destructive effects that zinc can have when its concentration strays too far from the normal physiological level are well-documented. This becomes particularly relevant during pathological events – such as ischemia or severe seizures (Koh et al., 1996; Lee et al., 2000) – that are associated with high extracellular concentrations of glutamate and zinc. Under these conditions, excess zinc can accumulate within cells by entering through calcium-permeable glutamate receptors or voltage-dependent calcium channels (reviewed by Sensi et al., 2011). Damaging effects of zinc can also occur when the ion is liberated from intracellular binding sites during oxidative stress (Figs. 1 and 2).

For normal physiological function, the appropriate concentration of free zinc in the cytosol is in the picomolar range (Colvin et al., 2010), in contrast to the low nanomolar level estimated to be found in the extracellular space (Frederickson et al., 2006a). To maintain cytosolic zinc at the appropriate concentration, a number of mechanisms exist for zinc buffering and muffling, analogous to the way in which the cytosolic concentration of calcium is carefully controlled (Colvin et al., 2010). Various organelles can serve as high-concentration zinc storage compartments. The mechanisms that buffer and muffle zinc in the cytosol include zinc-binding proteins, particularly metallothioneins (MTs), and zinc transporting proteins, which import zinc into and extrude it from the cell, and transfer zinc between the cytosol and organelles.

Two families – consisting of two dozen members in humans – comprise the set of dedicated zinc transporting proteins (reviewed by Kambe et al., 2015). The first is the 14 member Zrt, Irt-like protein (ZIP) family, which is broadly involved in cytosolic import of zinc. The other is the zinc transporter (ZnT) protein family, of which there are 10 members. The ZnT proteins are responsible for extruding cytosolic zinc out of the cell or into intracellular compartments. Extruding zinc from the cell is the function of the ubiquitously-expressed ZnT1. This protein is vital for normal cellular function; its loss results in embryonic non-viability (Andrews et al., 2004). In the brain, ZnT1 concentrates at postsynaptic membranes (Sindreu et al., 2014), where it binds to the GluN2A subunit of NMDA receptors and influences dendritic spine size (Mellone et al., 2015). Transporting zinc into intracellular compartments is the role filled by most of the other ZnTs. Examples include ZnT2, ZnT3, and ZnT4–which transport zinc into endosomes, lysosomes, and secretory vesicles – and ZnT5, ZnT6 and ZnT7, which transport zinc into the Golgi apparatus and *trans*-Golgi network.

Though the structure of the ZnTs is not definitively known, some facts can be inferred from the homologous transporter YiiP – found in *Escherichia coli* – for which the crystal structure has been revealed (Lu and Fu, 2007; Lu et al., 2009). YiiP, along with the ZnT proteins, is a member of the cation diffusion facilitator (CDF) protein family. The ZnTs are predicted to be six transmembrane domain (TMD) proteins with intracellular N- and C-terminal domains (Kambe et al., 2015). TMDs I, II, IV and V form a channel where histidine and aspartate residues provide a zinc-binding site; other zinc-binding sites are present on the C-terminal domain and on the histidine-rich cytosolic loop between TMIV and TMV, which may be involved in sensing zinc levels. The ZnTs function as Zn^{2+}/H^{+} antiporters, exchanging zinc for the protons found in acidified intracellular compartments (Ohana et al., 2009). They form homodimers, with the exception of ZnT5 and ZnT6, which function as heterodimers; ZnT6 on its own cannot transport zinc (Fukunaka et al., 2009). In the case of human ZnT3, homodimers are formed by a covalent bond between tyrosine residues on the C-terminal domain; altering this dimerization alters the intracellular targeting of ZnT3 and its transport capacity (Salazar et al., 2009). Recently, it has been shown that – in addition to forming homodimers – ZnT1, ZnT2, ZnT3, and ZnT4 can form heterodimers, altering their localization and function (Golan et al., 2015).

MTs are cysteine-rich proteins, which gives them ample zinc-binding capacity. Of the four major MT isoforms in the mouse (there are at least a dozen in humans; Li and Maret, 2008), three of them are expressed in the CNS, with MT-I and MT-II being more widely distributed throughout the body, and MT-III being CNS-specific (Masters et al., 1994). Each MT protein can bind up to seven ions of zinc. Interestingly, the MT zinc binding sites differ in their affinity, with four exhibiting a picomolar affinity and three a nanomolar affinity or less (Krezel and Maret, 2007). MTs can thus buffer zinc over a range of concentrations, and can donate zinc ions from their lower-affinity sites to other higher-affinity proteins. Because the cysteine thiol ligands that bind zinc are redox-sensitive, the zinc ions that are buffered by MTs are liable to be released during oxidative stress (Maret and Vallee, 1998). Elevated free zinc can act as an intracellular signal, initiating mechanisms that are protective against oxidative stress or that mediate cell death (reviewed by Aras and Aizenman, 2011). Another fascinating aspect of MT function is that some isoforms are regulated transcriptionally by the cytosolic zinc concentration. This occurs through metal regulatory element-binding transcription factor-1 (MTF1), a cytosolic zinc-sensing protein which, upon binding to zinc, translocates to the nucleus and binds to the metal response element (MRE) present in the regulatory region of some genes, including the genes for MT-I and MT-II (Andrews, 2000). The ZnT1-encoding gene also has an MRE, making it responsive to MTF1 (Langmade et al., 2000). This provides mechanisms by which the cell, in response to elevated, potentially-injurious levels of cytosolic zinc, can increase its capacity for zinc binding and zinc extrusion. ZnT3 is not regulated by MTF1, nor is MT-III, the dominant MT isoform found in neurons.

At the organismal level, zinc homeostasis is maintained by a balance between uptake of zinc from dietary sources and zinc excretion. Dietary zinc uptake, which replenishes about 0.1% of the zinc in the body per day, occurs primarily in the duodenum and jejunum of the small intestine, where enterocytes express ZIP4 on their apical, luminal surfaces and ZnT1 and ZIP5 on their basolateral membranes (Kambe et al., 2015). ZIP4 is upregulated during zinc deficiency, controlling the amount of zinc taken up by these cells (Dufner-Beattie et al., 2003). Mutations in the *Zip4* gene in humans can cause severe zinc deficiency, resulting in a disorder called acrodermatitis enteropathica that is characterized by alopecia, dermatitis, diarrhea, and growth retardation. Postnatal genetic deletion of intestinal ZIP4 reproduces some of these effects in mice, resulting in growth retardation and wasting that progresses to death within about 1–3 weeks, depending on the age at which ZIP4 is deleted (Geiser et al., 2012). Once in the intestinal enterocytes, zinc is exported into the portal blood by ZnT1 (McMahon and Cousins, 1998). If the level of zinc in the blood becomes excessive, ZIP5 may be