New therapeutic targets in Alzheimer’s disease: brain deregulation of calcium and zinc

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The molecular determinants of Alzheimer’s (AD) disease are still not completely known; however, in the past two decades, a large body of evidence has indicated that an important contributing factor for the disease is the development of an unbalanced homeostasis of two signaling cations: calcium (Ca$^{2+}$) and zinc (Zn$^{2+}$). Both ions serve a critical role in the physiological functioning of the central nervous system, but their brain deregulation promotes amyloid-$\beta$ dysmetabolism as well as tau phosphorylation. AD is also characterized by an altered glutamatergic activation, and glutamate can promote both Ca$^{2+}$ and Zn$^{2+}$ dyshomeostasis. The two cations can operate synergistically to promote the generation of free radicals that further intracellular Ca$^{2+}$ and Zn$^{2+}$ rises and set the stage for a self-perpetuating harmful loop. These phenomena can be the initial steps in the pathogenic cascade leading to AD, therefore, therapeutic interventions aiming at preventing Ca$^{2+}$ and Zn$^{2+}$ dyshomeostasis may offer a great opportunity for disease-modifying strategies.

Although the majority of Alzheimer’s disease (AD) cases, referred as sporadic AD (sAD), are idiopathic with no obvious implication of genetic factors, 5% of all the AD patients show a Mendelian pattern of inheritance (familial AD, fAD). In both sAD and fAD, the accumulation of the amyloid-$\beta$ peptide (A$\beta$) and neurofibrillary tangles (NFTs) is a key determinant in AD pathogenesis, the most common form of dementia. A$\beta$, the main constituent of the amyloid plaques, is a 40–42 amino-acid peptide formed by the proteolytic cleavage of the membrane-bound amyloid precursor protein (APP) through the enzymatic activity of the $\beta$- and $\gamma$-secretase complexes. NFTs are intraneuronal aggregates composed of hyperphosphorylated tau (h-tau) protein, a peptide that is involved in stabilizing the microtubular structures of the cytoskeleton. In addition to A$\beta$ dysmetabolism and the appearance of h-tau deposits, a growing body of evidence suggests that the deregulation of brain calcium (Ca$^{2+}$) and zinc (Zn$^{2+}$) homeostasis facilitates the development and progression of AD.1–3

Cytosolic Ca$^{2+}$ levels ([Ca$^{2+}$]) are kept in a low range (~100 nM) compared with the levels present in the extracellular space (2 mM) or what is found inside of intracellular stores (100–500 mM). These low [Ca$^{2+}$], are maintained by the activity of Ca$^{2+}$-binding buffering proteins (e.g., calbindin and parvalbumin), whereas Ca$^{2+}$ extrusion is operated by the Ca$^{2+}$-ATPase pump, and the Na$^{+}$/Ca$^{2+}$ exchanger as well as by the sequestration of the cation in intracellular stores like the endoplasmic reticulum (ER) and mitochondria. Conversely, [Ca$^{2+}$]$_i$ rises are the result of an influx across the plasma membrane via store-operated Ca$^{2+}$ channels (SOCCs), voltage-gated Ca$^{2+}$ channels (VGCCs), ionotropic glutamate receptors (N-Methyl-D-Aspartic acid receptors, NMDARs), z-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor (AMPARs) or the release from intracellular stores. The ER is the largest intracellular store in neurons and loaded with Ca$^{2+}$ at high concentrations (100–500 µM) through the unidirectional pumping of cytosolic Ca$^{2+}$ into the ER lumen, a process operated by the sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) and the Na$^{+}$/Ca$^{2+}$ exchanger (NCX). Dysregulation of calcium homeostasis in neurons and glia is related to the alteration of Ca$^{2+}$ channels and pumps and to the release of pro-inflammatory factors and microglial activation. In addition, Ca$^{2+}$ dyshomeostasis promotes the generation of reactive oxygen species (ROS), free radicals that can cause oxidative stress and neurodegeneration.

The homeostasis of two signaling cations is regulated by calcium and zinc transporters; ZIPs, Zn$^{2+}$-importing proteins; Mfs, mossy fibers; GABA, $\gamma$-Aminobutyric acid; MMPs, matrix metalloproteinases; MAPK, mitogen-activated protein kinase; ZnR, Zn$^{2+}$-sensing receptor; TrkB, tropomyosin-related kinase B; BDNF, brain-derived neurotrophic factor; IMPAC, metal-protein attenuation compounds.

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(SERCA) pump. Ca\(^{2+}\) release from the ER occurs through the activation of two types of Ca\(^{2+}\) receptors: the inositol 1,4,5-trisphosphate (IP3R) and the ryanodine (RyR) receptors. Moreover, capacitative calcium entry (CCE), a process by which the depletion of intracellular Ca\(^{2+}\) stores causes the opening of plasma membrane Ca\(^{2+}\) -permeable channels, has been described in neuronal subpopulations.

Zn\(^{2+}\) is the second most abundant metal in the body after iron (~3 g). Much like [Ca\(^{2+}\)]\(_i\), cytosolic Zn\(^{2+}\) ([Zn\(^{2+}\)]\(_i\)) is kept at low concentrations by the combined activity of Zn\(^{2+}\) transporters (ZnTs), Zn\(^{2+}\) -importing proteins (ZIPs), and by the buffering action of proteins like the metallothioneins (MTs; reviewed in ref. 1). In the central nervous system (CNS), Zn\(^{2+}\) is selectively stored at high levels (~1 mM) in presynaptic vesicles of some, but not all, glutamatergic neurons and co-released with the neurotransmitter.\(^4\) Thus, during intense synaptic activity, Zn\(^{2+}\) can accumulate in the synaptic cleft and enter postsynaptic spines sharing some entry routes that are also employed by Ca\(^{2+}\) (VGCCs, Ca\(^{2+}\) -permeable AMPARs (Ca-ARs) and, with less efficacy, NMDARs\(^1\)). Although Ca\(^{2+}\) and Zn\(^{2+}\) are involved in a variety of CNS functions (neurotransmission, mitochondrial functioning, axonal transport, and a plethora of enzymatic activities), their dysfunctional homeostasis can be a potent trigger for A\(\beta\) oligomerization and NFT formation, as well as overproduction of reactive oxygen species (ROS),\(^1,3\) all of which are factors associated with AD-related neurodegeneration. Further-more, as both Ca\(^{2+}\) and Zn\(^{2+}\) modulate the physiology of synaptic transmission and plasticity, several lines of evidence now reveal that the deregulation of the two ions can help to explain the synaptic dysfunction and cognitive decline associated with AD.

Thus, given the importance of Ca\(^{2+}\) and Zn\(^{2+}\) homeostasis for brain functioning, strategies aimed at restoring the brain balance of these ions are emerging as promising avenues for strategies aimed at restoring the brain function, and/or Ca\(^{2+}\) and Zn\(^{2+}\) homeostasis is supported by studies performed on transgenic mice overexpressing wild-type APP\(^19\) or carrying AD-linked APP mutations.\(^20\) These models show neurons undergoing increased [Ca\(^{2+}\)]\(_i\) levels at rest.\(^20\) However, it should not be overlooked that conflicting results also indicate the absence of a direct effect of AD-linked APP mutations on [Ca\(^{2+}\)]\(_i\) levels and/or Ca\(^{2+}\) release from intracellular stores.\(^21,22\)

Factors linked to sAD are less clear; however, there is evidence to back the idea that some of them are associated with [Ca\(^{2+}\)]\(_i\) perturbation. For instance, the expression of the ApoE4 allele that is associated with sAD promotes a strong increase in [Ca\(^{2+}\)]\(_i\) levels by triggering Ca\(^{2+}\) influx in neurons.\(^23\) Another sAD risk factor is the presence of a polymorphism for the Ca\(^{2+}\) homeostasis modulator 1 (CALHM1) protein, a multipass transmembrane glycoprotein with a large Ca\(^{2+}\) conductance across the plasma membrane.\(^24\) The P86L mutation of the peptide decreases CALHM1-mediated Ca\(^{2+}\) currents, thereby favoring an increase in A\(\beta\) production, which supports the idea that alterations of specific Ca\(^{2+}\) -pools, rather than a global increase of [Ca\(^{2+}\)]\(_i\), may be critical contributing factors for AD development and progression.

But how do [Ca\(^{2+}\)]\(_i\) alterations influence the propagation of the AD-related pathology? The introduction of transgenic animal models as experimental tools has helped to elucidate, at least in part, the complex interactions between AD-linked mutations, ionic deregulation, and the development of A\(\beta\) and tau pathology. For example, transgenic mice expressing

**Ca\(^{2+}\) Dyshomeostasis, Synaptic Dysfunction, and AD**

The idea that the perturbation of Ca\(^{2+}\) homeostasis can have a pivotal role in the cascading events leading to AD was introduced more than 20 years ago,\(^7\) and an impressive amount of evidence obtained from experiments on dissociated cells, brain slices, and, more recently and importantly, on live AD animal models is strongly supporting this hypothesis.\(^8,9\)

The most important evidence for the ‘Ca\(^{2+}\) /AD hypothesis’ came from a large number of studies indicating that fAD cases are associated with mutations of three genes: APP, presenilin-1 (PS1), and presenilin-2 (PS2). Presenilins are transmembrane proteins synthesized in the ER that have been shown to greatly affect ER-Ca\(^{2+}\) dynamics through the activation of the SERCA pump,\(^10\) via IP3R and RyR opening\(^11,12\) as well as by allowing passive Ca\(^{2+}\) leakage from the ER. Notably, all these homeostatic Ca\(^{2+}\) mechanisms are found to be profoundly deregulated by mutant presenilins both in vitro and in vivo\(^10,11,13,14\) substantiating the idea that intraneuronal Ca\(^{2+}\) deregulation can be part of the pathogenic cascade leading to AD. Interestingly, a recent study suggests that PS2 can critically regulate the mitochondrial uptake of Ca\(^{2+}\) that is released from the ER, thereby promoting a potentially harmful cationic overload in the organelles.\(^15\) Presenilins have also been shown to diminish CCE in AD neurons and increase Ca\(^{2+}\) release from intracellular stores, thereby leading to diminished Ca\(^{2+}\) storage.\(^16\) However, another study has reported that AD neurons show impaired CCE along with augmented store-dependent Ca\(^{2+}\) release, as well as increased Ca\(^{2+}\) storage.\(^17\) Regardless of the mechanisms involved, it seems reasonable that a diminished CCE could, in some way, contribute to AD-related neurodegeneration. Besides their functions in Ca\(^{2+}\) homeostasis, PS1 and PS2 may have an additional role in AD, as they are part of the \(\gamma\)-secretase catalytic domain and presenilin mutations accelerate (although with different degrees of activity) the production of ‘aggregation-prone’ forms of A\(\beta\)\(_{1-42}\)-18.

Contrary to what has been demonstrated for presenilins, the role of APP in Ca\(^{2+}\) deregulation is still controversial. Whether or not, independently of A\(\beta\) production, APP can directly modulate Ca\(^{2+}\) homeostasis, is still a matter of debate. Evidence for a direct effects of APP on [Ca\(^{2+}\)]\(_i\) homeostasis is supported by studies performed on transgenic mice overexpressing wild-type APP\(^19\) or carrying AD-linked APP mutations.\(^20\) These models show neurons undergoing increased [Ca\(^{2+}\)]\(_i\) levels at rest.\(^20\) However, it should not be overlooked that conflicting results also indicate the absence of a direct effect of AD-linked APP mutations on [Ca\(^{2+}\)]\(_i\) levels and/or Ca\(^{2+}\) release from intracellular stores.\(^21,22\)

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human mutant APP, generate when aging Aβ plaques in the hippocampus and cortex, ultimately mimicking the AD-like pathology found in patients.

APP processing involves the activation of several Ca^{2+} dependent proteins and therefore it is likely that increased or decreased Ca^{2+} levels may have aberrant effects on APP cleavage. For instance, many studies have shown that the mobilization of different Ca^{2+} pools leads to Aβ production. To date, the global effect of [Ca^{2+}]_{i} rises on Aβ levels is still unclear as some evidence shows that increased [Ca^{2+}]_{i} rises promote the production of the non-amyloidogenic sAPP fragment, but other studies have indicated that [Ca^{2+}]_{i} increases, acting on either the extracellular milieu or intracellular stores, induce a shift toward the amyloidogenic pathway. A critical revision of the most recent studies seems to support the idea that the homeostasis of the ER-Ca^{2+} pool is crucial in controlling the fate of APP processing. In fact, ER overfilling appears to be the first clinical sign of the pathogenic cascade as inhibiting calcineurin with K506 promotes the production of the non-amyloidogenic sAPP fragment, thereby increasing the glutamatergic transmission. Conversely, physiologically elevated Aβ concentrations may negatively affect plasticity by inducing a deficit of NMDAR-mediated excitatory postsynaptic currents as well as a shift toward NMDAR-dependent induction of LTD and synaptic loss. The process is set by Aβ promoting NMDAR endocytosis and, in this view, Ca^{2+} (through calcineurin activation by Aβ) may be considered the intracellular trigger for Aβ-driven synaptic dysfunction. Supporting this hypothesis, a recent study has proposed an intriguing mechanism linking Ca^{2+} deregulation, Aβ dysmetabolism, and synaptic dysfunction.

A large body of evidence also demonstrates the deleterious effects of Aβ in driving further Ca^{2+} deregulation. Many studies have shown that Aβ directly interferes with Ca^{2+} homeostasis and disrupts the activity of ionotropic glutamate receptors, the P/Q or L-type VGCCs, ER-Ca^{2+} stores, and CCE, thereby producing increased [Ca^{2+}]_{i} levels. Recent in vivo experiments employing multiphoton imaging indicate that Aβ is promoting Ca^{2+} deregulation in transgenic AD neurons, and neurites positioned in the proximity of Aβ plaques, a phenomenon that leads to the downstream activation of the Ca^{2+}-dependent protein phosphatase, calcineurin. This phenomenon is a key component of the pathogenic cascade as inhibiting calcineurin with K506 ameliorates dendritic spine density in these animals. Furthermore, several studies indicate that Aβ oligomers can directly induce Ca^{2+} entry through the plasma membrane by promoting the formation of Ca^{2+} permeable channels.

Ionic Deregulation Implication for Synaptic Plasticity

A crucial issue that deserves particular attention is the link between Aβ-driven Ca^{2+} deregulation and synaptic efficacy (long-term potentiation (LTP) and long-term depression (LTD)). Synaptic loss and dysfunction are the most prominent pathological features of AD and these processes best correlate with the development of AD-related cognitive decline. In vitro and in vivo studies have shown that high levels of oligomeric Aβ can negatively interfere with glutamatergic synaptic transmission and plasticity. Several studies have investigated the pathogenic interactions between altered synaptic Ca^{2+} signaling and the aggregation state of Aβ, supporting the idea that Ca^{2+} and Aβ may interact in several different ways to promote synaptic dysfunction.

In the past years, the complex interplay between Ca^{2+}, APP, and Aβ in the modulation of synaptic transmission and plasticity has been the subject of a great number of studies. Through these investigations, it has become clear that Aβ can be secreted in the extracellular space during neuronal activity and that pathologically elevated Aβ concentrations can alter transmission. Physiological levels of endogenous Aβ may act presynaptically as a positive regulator to enhance the release probability of synaptic vesicles of glutamatergic terminals, thereby increasing the glutamatergic transmission. Conversely, pathologically elevated Aβ concentrations may negatively affect plasticity by inducing a deficit of NMDAR-mediated excitatory postsynaptic currents as well as a shift toward NMDAR-dependent induction of LTD and synaptic loss. The process is set by Aβ promoting NMDAR endocytosis and, in this view, Ca^{2+} (through calcineurin activation by Aβ) may be considered the intracellular trigger for Aβ-driven synaptic dysfunction. Supporting this hypothesis, a recent study has proposed an intriguing mechanism linking Ca^{2+} deregulation, Aβ dysmetabolism, and synaptic dysfunction.

According to this study, Ca^{2+} influx through synaptic NMDAR activates at least two pathways that regulate spine density and postsynaptic responses. On one hand, high [Ca^{2+}]_{i} levels obtained upon tetrodotoxin or suprathreshold synaptic stimulation induce LTP by activating the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-dependent pathway. On the other hand, low levels of [Ca^{2+}]_{i}, obtained during low-frequency subthreshold stimulation can induce LTD by activating a calcineurin-dependent pathway (reviewed in ref. 49). Further evidence indicates effects of Ca^{2+} and Aβ on synaptic efficacy and shows that NMDAR-mediated Ca^{2+} influx into active spines is reduced by soluble Aβ oligomers, thereby causing a decrease in spine density via a NMDAR/calcineurin/cofilin-dependent process that leads to LTD. The overall scenario is however more complex as other studies indicate that Aβ oligomers bind to NMDARs and promote excitotoxic Ca^{2+} influx by enhancing NMDAR activation.

Further studies have dissected the link between Ca^{2+}, AMPAR activation, and Aβ-dependent synaptic dysfunction. Acute application of Aβ in the dentate gyrus of rat hippocampal slices has been shown to block the activity-dependent autophosphorylation of CaMKII, thereby leading to reduced phosphorylation and functioning of the GluA1 subunit of AMPARs. Another study has shown that soluble Aβ oligomers selectively cause the loss of surface AMPARs. The study suggests that this detrimental process is driven by Aβ binding to Ca-ARs at the dendritic surface and by the
subsequent activation of calcineurin, an event that leads to the internalization of Aβ/ and AMPARs.44

A growing body of evidence also supports the idea that AD-related neuronal damage may result not only from the actions of synaptically released Aβ, but also derives from the intraneuronal accumulation of the peptide. It is known that elevated intraneuronal Aβ immunoreactivity precedes the appearance of plaques and better correlates with the onset of the synaptic and behavioral abnormalities shown in transgenic AD models.94 Moreover, in neuronal cultures, the depolarization and subsequent Ca2+-mediated transient phosphorylation of both tau and APP induce the accumulation of intraneuronal Aβ.53 However, decreased synaptic activity has also been shown to be detrimental by promoting an increase in intracellular Aβ that is followed by synaptic damage.56,67

As far as tau-dependent pathology, in recent years evidence strongly substantiates the idea that Ca2+- deregulation is also involved in NFTs formation. This phenomenon is particularly relevant given the renewed interest in the pathogenic action of tau pathology in promoting neurodegeneration and cognitive decline in AD.58

It should be remembered that the anatomical distribution of NFTs follows a typical spreading pathway that, compared with the distribution of Aβ pathology, correlates in a better way with the progression of AD symptoms. Recent evidence is in fact starting to question the exact sequence of events previously described in the ‘Aβ amyloid cascade hypothesis’ and suggests that, in fact, Aβ-mediated toxicity is largely modulated (and not in a secondary way) by tau-dependent processes.59–61 For instance, a recent study has shown that some hippocampal cultured neurons exposed to Aβ oligomers relocate tau from axons to cell bodies and dendrites.61 Interestingly, this Aβ-driven dendritic missorting of tau appears to be the key to promote downstream pathogenic events like dendritic Ca2+ rises, changes in tau phosphorylation, loss of spines and mitochondria, and relocation of other cytoskeletal elements, as well as changes in the activity of crucial kinases.61 A similar change in tau distribution and phosphorylation has been shown in neurons exposed to oxidative or excitotoxic stress, suggesting a common signaling cascade that culminates in regional tau pathology and eventually in the breakdown of neuronal architecture.61 Furthermore, recent evidence has linked tau dysmetabolism to Aβ-dependent excitotoxic damage.60,62 According to this model, tau is moving to dendrites, promoting the activation of a Src kinase (Fyn), and leading to the phosphorylation of the GluN2B NMDAR subunit. This process facilitates the interaction of NMDARs with the scaffolding protein PSD-95, increasing the stability of NMDARs within the PSD, thereby coupling the receptors to Aβ-dependent excitotoxic signaling.

The full extent by which tau dysmetabolism mediates synaptic transmission, neuronal degeneration, or both, is actually matter of an exciting current debate.68,63 and new intriguing evidence is emerging. For instance, genetic deletion of tau completely prevents the deleterious effects on LTP elicited by the exposure to either human or rodent Aβ1–42, thereby suggesting that the protein is instrumental for the development of the synaptotoxic effects of Aβ.64 Moreover, tau-induced defects in synaptic plasticity are reversible in transgenic conditional mice after switching off the toxic tau mutant.63 Data linking Ca2+ dyshomeostasis with the neurotoxic effects of a tau fragment of 17 kD are associated with calpain activation.65 Interestingly, another study has shown that [Ca2+]i rises evoked by glutamate or thapsigargin (a SERCA inhibitor) are also able to promote the same cleavage of this ‘17 kD’ fragment, thereby confirming the Ca2+- dependency of the phenomenon.66

Further studies are lending support to the idea that AD-related alterations have an important role in altering glutamate neurotransmission (and indeed, AD patients are now treated with memantine, a low-affinity NMDAR blocker). As described above, a major source for [Ca2+]i rises results from the overactivation of glutamatergic receptors, a phenomenon that occurs in experimental models of AD and triggers robust oxidative stress.59 It is worth noticing that Aβ itself can increase glutamatergic neurotransmission and render neurons more vulnerable to excitotoxicity by interfering with glutamate re-uptake.67

As both Aβ and h-tau further facilitate Ca2+- dyshomeostasis, the process appears to take the shape of an injurious cycle that leads to AD-related synaptic dysfunction and neuronal death. Thus, glutamate and Aβ can operate synergistically to promote oxidative stress and set the stage for a self-perpetuating harmful loop. Oxidative stress can also enhance tau phosphorylation and tau-dependent pathology that further exacerbatess Aβ-driven pathology59 (Figure 1). It should also be noted that, suggesting a feed forward process, recent data indicate that injection of oligomers, obtained from the cortex of AD patients, triggers tau hyperphosphorylation, disrupts the microtubule architecture, and sets in motion neuritic dystrophy.68 Finally, NMDAR activation can also promote the intraneuronal release of Zn2+, thereby suggesting a pathogenic link between the dyshomeostasis of the two cations70 (Figure 2).

Zn2+: Deregulation, Synaptic Functioning, and AD

Like Ca2+, Zn2+ is involved in a wide array of physiological functions1 and, as with Ca2+, the deregulation of intracellular Zn2+ ([Zn2+]i) has an important role in AD. The mammalian brain contains a pool of loosely bound Zn2+ also defined as ‘chelatable Zn2+’.1 Zn2+ -containing neurons form a complex and elaborate associational network that interconnects most of the cerebral cortices and limbic structures and Zn2+ -containing axon terminals are particularly abundant in the neocortex and hippocampus.

Zn2+ is often localized within synaptic vesicles of a subset of glutamatergic axon terminals, although there is also evidence indicating enrichment of Zn2+ at selected inhibitory γ-Aminobutyric Acid (GABA) terminals in the cerebellum and spinal cord.71,72 In the subpopulation of glutamatergic neurons, Zn2+ is transported into small, clear, and round presynaptic vesicles by the neuronal-specific Zn2+ transporter, ZnT3, throughout a process that stores the cation inside the vesicles to bring about additional excitatory transmitter release. Therefore, Zn2+, like Ca2+, can directly regulate presynaptic release of glutamate at glutamatergic nerve endings.

Previous studies showed that sustained stimulation of hippocampal mossy fibers (MFs), a brain structure that shows the strongest Zn2+ amount in the brain, produces a great
increase in synaptic Zn$^{2+}$ levels and more recent evidence has demonstrated that such synaptic Zn$^{2+}$ is a principal factor in modulating neurotransmission.1

Both excitatory ionotropic glutamate receptors (iGluRs) and inhibitory ionotropic receptors (GABA_A) are affected by Zn$^{2+}$.73 The best characterized synaptic Zn$^{2+}$ target is represented by the NMDAR. Zn$^{2+}$ modulates NMDAR properties through a dual mechanism: low micromolar concentrations selectively inhibit NMDAR-mediated currents through a voltage-independent, non-competitive (allosteric) inhibition whereas high Zn$^{2+}$ concentrations (more than 20 μM) are responsible of a voltage-dependent inhibition of the receptor-mediated currents.74–76 The N-Terminal-Domain (NTD) of the NMDAR GluN2A subunit binds Zn$^{2+}$ with high (nanomolar) affinity and this high sensitivity to Zn$^{2+}$ has important consequences. The most relevant is that synaptic Zn$^{2+}$ can exert its modulatory effects not only during fast phasic synaptic release but also in a tonic mode. In other words, under normal resting conditions, the GluN2A-specific Zn$^{2+}$ binding site can be partially occupied by the cation, thereby inhibiting the receptor.75 Interestingly, Zn$^{2+}$ inhibition can also be maximized by the tyrosine kinase src-mediated phosphorylation of the receptor.77 AMPARs are also sensitive to extracellular Zn$^{2+}$,78 however, the phenomenon appears to require higher (micromolar) Zn$^{2+}$ levels.

In addition to ionotropic receptors, synaptically released Zn$^{2+}$ modulates the activity of key synaptic proteins. For instance, Zn$^{2+}$ can interfere with the biochemical activity of proteins like glutamate transporters and matrix metalloproteinases...
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Figure 3 Zn\(^{2+}\) supplementation is beneficial in animal model of AD. (A) Zn\(^{2+}\) supplementation prevents the development of hippocampus-dependent memory deficits in 3 × Tg-AD mice. Three × Tg-AD mice were treated with either water containing 30 p.p.m. of ZnSO\(_4\) or tap water for 11–13 months and tested for the spatial memory version of the MWM. Mice were tested when the platform was removed 1.5 h (to investigate short-term memory) and 24 h (to investigate long-term memory) after the last training trial. Compared with untreated 3 × Tg-AD mice, Zn\(^{2+}\) treatment significantly reduced the latency (time) they employed to reach the point where the platform used to be (Error bars indicate mean ± S.E.M.; \*P < 0.05). (B) Zn\(^{2+}\) supplementation reduces both Aβ and tau pathology in the hippocampus of 3 × Tg-AD mice. Immunohistochemistry shows deposits of intraneuronal Aβ (a–d) and h-tau (f–j) in brain slices from treated and untreated 3 × Tg-AD mice (left column: 20 × magnification; right column: 40 × magnification). Compared with untreated mice (a, b), treated 3 × Tg-AD mice showed a significant decrease of intraneuronal Aβ deposits in the hippocampus of c, d; (b, d) 40 magnification of the hippocampal CA1 area as shown in the rectangle. (e) Quantification of Aβ load as shown in a and c. Compared with untreated mice (f, g), Zn\(^{2+}\) supplementation significantly reduced 3 × Tg-AD mice showed a strong decrease of intraneuronal h-tau immunoreactivity in the hippocampus of (h, i); (g, i) 40 magnification of the CA1 area as shown in the rectangle. (j) Quantification of h-tau levels from f and h. Error bars indicate mean ± S.E.M.; \*P < 0.05. (C) Zn\(^{2+}\) supplementation promotes MMPs activation in 3 × Tg-AD mice. Gelatin zymography indicates that Zn\(^{2+}\) feeding induced a significant increase of MMP-2 and MMP-9 activation in 3 × Tg-AD mice brains. (D) Zn\(^{2+}\) supplementation increases brain BDNF levels. BDNF immunoblotting reveals that Zn\(^{2+}\)-treated 3 × Tg-AD mice showed a fourfold increase in BDNF levels when compared with untreated mice. \*P < 0.05; \**P < 0.01. Error bars indicate mean values ± S.E.M. (modified from ref. 79)

(MMPs) in physiological and pathological conditions (Figure 3).\(^{79}\) Furthermore, a recent study indicates a novel mechanism by which Zn\(^{2+}\) can modulate synaptic functions as the cation is able to activate mitogen-activated protein kinase (MAPK) and CaMKII by acting on a putative Zn\(^{2+}\)-sensing receptor (ZnR).\(^{80}\)

A recent study has also shown that Zn\(^{2+}\) transactivates the tropomyosin-related kinase B (TrkB) receptor (the receptor responsive to the brain-derived neurotrophic factor (BDNF)) and activates BDNF signaling in a neurotrophin-independent manner.\(^{81}\) Furthermore, Zn\(^{2+}\) also affects BDNF signaling by promoting the maturation of pro-BDNF to BDNF throughout the activation of MMPs\(^{82}\) and this phenomenon has been recently validated in animal AD model (Figure 3).\(^{79}\) It is also important to underline that the non-amyloidogenic APP processing is mediated by membrane-anchored Zn\(^{2+}\)-dependent metalloproteinases like the ones belonging to the ADAM family [a disintegrin and metalloproteinases,\(^{83}\)] a phenomenon that suggests that [Zn\(^{2+}\)] might also have a role in regulating the balance between amyloidogenic and non-amyloidogenic pathways.

Finally, intracellular Zn\(^{2+}\) can interfere with neuronal excitability by modulating the activity of the neuronal K\(^+\) transporter, KCCN, a key controller of the neuronal resting membrane voltage potential.\(^{84}\) Thus, the specific localization of Zn\(^{2+}\) at certain glutamatergic synapses, together with its multiple synaptic targets, makes the cation a very attractive candidate as an endogenous modulator of synaptic transmission and plasticity; however, deregulated Zn\(^{2+}\) homeostasis also appears to be a strong promoter of AD development (Figure 4).

Indeed, there are no doubts that a complex alteration of transportation and distribution occurs in the AD brain and that alterations of synaptic Zn\(^{2+}\) are key factors in promoting AD-like pathology. Lending support to this idea, a study has shown that aging is associated with the development of decreased brain levels of ZnT3, a phenomenon that is enhanced in AD brains.\(^{85}\) ZnT3 downregulation leads to a deficit in synaptic Zn\(^{2+}\) that is associated with a strong downregulation of synaptic proteins that are important for memory and learning processes.\(^{85}\) Interestingly, another
study has indicated that ZnT3-KO mice show a reduced activity-dependent induction of Erk1/2 signaling at the MF presynaptic terminals. Such diminished Erk1/2 activity derives from the lack of inhibition of the Zn^{2+}-sensitive MAPK tyrosine phosphatase, thereby suggesting that an increased synaptic release of the cation from MFs on learning might enhance the inhibition of tyrosine phosphatases leading to further presynaptic Erk activation. Consistent with this prediction, ZnT3-KO animals show cognitive deficits.86

A deficit of synaptic Zn^{2+} has also been associated with decreased brain levels of BDNF and other neurotrophins as well as with the development of severe cognitive decline.85 Moreover, data obtained from acute murine hippocampal slices indicate that synaptic Zn^{2+} facilitates the binding of Aβ oligomers to the GluN2B subunit of the NMDAR in MF terminals and interferes with glutamatergic transmission.87 Furthermore, notable studies by Lovell and colleagues have shown alterations in the expression of the Zn^{2+}-dependent proteins that are involved in Aβ clearance (e.g., MMPs) or free radical scavenging (e.g., MTs). Finally, synaptic Zn^{2+} deficiency also leads to neurotoxic activation of NMDARs.

Zn^{2+} also critically modulates amyloid metabolism.1 Indeed, both APP and Aβ specifically bind Zn^{2+}, with APP having a binding site for zinc (K_{D} = 750 nM) in the cysteine-rich region of the APP ectodomain,95 and Aβ exhibiting both a high-affinity binding site (K_{D} = 107 nM; with a 1 : 1 [Zn^{2+}]/Aβ stoichiometry) as well as a lower-affinity binding site (K_{D} = 5.2 μM; with a 2 : 1 stoichiometry).96 Subtle changes in Zn^{2+} and Aβ brain levels appear therefore crucial in determining a shift from a physiology to pathology (Figure 4).

As mentioned, the brain has one of the highest content of Zn^{2+} with an overall concentration of ~150 μM. (ref. 1) Some forebrain neurons store up to 200–600 μM of Zn^{2+} in synaptic vesicles and release ~1–10% of this amount as freely exchangeable Zn^{2+}. However, in a healthy brain, the exchangeable amount of Zn^{2+} in the extracellular fluid is approximately 1–10 nM whereas the free cytosolic [Zn^{2+}±] levels are in the sub-nanomolar range. Aβ is a physiological component of the CSF and normally produced and secreted during cell metabolism at nanomolar concentrations in each microsecond (in vivo).

Very little is known on the exact dynamics of the Aβ intracellular pool. Indeed, although an increasing body of evidence indicates that Aβ accumulates intracellularly, it is still not clear whether this accumulation is the result of a failure of Aβ secretion, or whether secreted Aβ is taken back by the cell to build up intracellular pools.86 While under physiological conditions (especially if one considers the Aβ K_{D} for the higher affinity binding site), [Zn^{2+}±] levels are likely too low to promote significant complex formation with Aβ. However, [Zn^{2+}±], release occurring under oxidative stress might promote sufficient cation levels to initiate Aβ oligomerization. Such ROS-dependent intraneuronal [Zn^{2+}±] rises have been found to be particularly high in AD neurons expressing mutant APP, PS1 and h-tau.97

To date, a precise understanding of how Aβ forms complexes with Zn^{2+} and/or other metal ions is still largely unknown. Even though monomeric Aβ has been shown to form a complex with Zn^{2+}, large oligomeric assemblies seem to be preferentially precipitated upon coordination with the cation.98 The N-terminal region of different Aβ oligomeric populations can access a range of metal-coordination structures,98 thereby leading to an extensive polymorphism.99 It is important to underline that these polymorphic species include antiparallel β-sheet conformations100 increasingly recognized as ‘signature’ structures of toxic Aβ oligomers.101 The neurotoxic fate of these aggregates varies. Depending on their relative stabilities and energy landscapes, some intermediates can be selectively stabilized or precipitated into insoluble, inert fibrils by Zn^{2+} (ref. 100,102). The scenario changes when they get conjugated with metals. Indeed, high [Zn^{2+}]± levels can promote Aβ-induced toxicity both in vitro (at 50 μM)103 and in vivo (at 1 mM),104 when considering equimolar Aβ/Zn^{2+}± concentrations. These findings suggest that high [Zn^{2+}]± rises may preferentially induce a stabilization of small, soluble, toxic intermediates that remain for a longer time in solution without necessarily evolving into fibrils.100 However, a neuroprotective effect can also be seen at lower Zn^{2+}± concentrations (5–0.5 μM; in the case of Aβ/Zn^{2+}± molar ratios of 1 : 0.1 and 1 : 0.01)102,103. In contrast to high Zn^{2+}± levels, low Zn^{2+} concentrations can selectively

![Figure 4](#)

**Figure 4** Alterations of the ‘zinc set point’, implications for AD. The maintenance of brain Zn^{2+} homeostasis is crucial for neuronal functioning. Perturbations of this equilibrium may be a contributing factor in AD development and progression. Excessive Zn^{2+} facilitates Aβ oligomerization as well as overproduction of reactive oxygen species (ROS), thereby promoting synaptic dysfunction and neuronal death. However, Zn^{2+} deficiency may also be deleterious as decreased bioavailability of the cation leads to reduced activation of neuroprotective BDNF signaling. Zn^{2+} deficiency in fact decreases the maturation of pro-BDNF to BDNF and reduces the transactivation of the BDNF receptor, TrkB. Moreover, decreased levels of brain Zn^{2+} can further downregulate the expression and/or the activity of neuroprotective ZnT4, ZnT1, and ZnT6 in the brain of preclinical and early-stage AD patients, suggesting a concurrent causative role of early Zn^{2+}± dyshomeostasis in triggering AD-related pathogenic events.88,89

A strong link between Zn^{2+} and Aβ in AD is also confirmed by several observations: (1) amyloid plaques are highly enriched in Zn^{2+} (ref 90); (2) genetic ablation of synaptic Zn^{2+} prevents Aβ deposition;91 (3) trace concentrations of Zn^{2+}, as well as Cu^{2+}± or Fe^{3+}± are sufficient to induce Aβ nucleation and eventually the fibrilization of the peptide;92,93 (4) Zn^{2+} enhances Aβ aggregation and precipitation during AD progression whereas Zn^{2+} chelators induce a rapid resolubilization of Aβ deposits in post-mortem AD brain samples.94

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**Table 4** Implications of the ‘zinc set point’ regulation in AD. The maintenance of brain Zn^{2+} homeostasis is crucial for neuronal functioning. Perturbations of this equilibrium may be a contributing factor in AD development and progression. Excessive Zn^{2+} facilitates Aβ oligomerization as well as overproduction of reactive oxygen species (ROS), thereby promoting synaptic dysfunction and neuronal death. However, Zn^{2+} deficiency may also be deleterious as decreased bioavailability of the cation leads to reduced activation of neuroprotective BDNF signaling. Zn^{2+} deficiency in fact decreases the maturation of pro-BDNF to BDNF and reduces the transactivation of the BDNF receptor, TrkB. Moreover, decreased levels of brain Zn^{2+} can further downregulate the expression and/or the activity of neuroprotective ZnT4, ZnT1, and ZnT6 in the brain of preclinical and early-stage AD patients, suggesting a concurrent causative role of early Zn^{2+}± dyshomeostasis in triggering AD-related pathogenic events.88,89

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destabilize larger soluble intermediates by lowering their kinetic barriers toward the formation of precipitates.\(^{102}\) This concentration-dependent activity of Zn\(^{2+}\) in the modulation of A\(\beta\)-associated neurotoxicity has been considered a paradox.\(^{105}\)

Besides its activity on A\(\beta\)/Zn\(^{2+}\) also promotes tau aggregation\(^{106}\) and it should be noted that NFT-bearing conditions, tau fibrilization depends on [Zn\(^{2+}\)] levels as low Zn\(^{2+}\) concentrations promoting tau dephosphorylation.\(^{108}\) Finally, tau\(^{2+}\) also exerts a bimodal effect on tau phosphorylation, with high Zn\(^{2+}\) levels inducing tau hyperphosphorylation through the activation of glycogen synthase kinase 3\(\beta\) and low Zn\(^{2+}\) concentrations promoting tau dephosphorylation.\(^{79,108}\)

\[\text{Ca}^{2+} \text{ and Zn}^{2+} \text{ Dyshomeostasis: a Synergy that Produces a Potentially Self-perpetuating Pathogenic Loop}\]

In the complex scenario of AD, all the factors previously mentioned, A\(\beta\)/Zn\(^{2+}\), oxidative stress, tau, glutamate receptor overactivation, Ca\(^{2+}\), and Zn\(^{2+}\) dyshomeostasis may act in concert to promote synaptic loss and, ultimately, neuronal loss (Figure 1). Zn\(^{2+}\) sequestration by A\(\beta\) in extracellular amyloid plaques promotes an over-activation of NMDARs, whereas A\(\beta\) directly activates Ca-ARs and the two phenomena lead to Ca\(^{2+}\) overload, increased superoxide generation from NADPH oxidase and mitochondria as well as nitric oxide (NO) production from Ca\(^{2+}\)-dependent activation of NO synthase (NOS). Ca\(^{2+}\)-dependent production of reactive oxygen and nitrosative species (ROS and RNS) then mobilize Zn\(^{2+}\) from MTs leading to toxic Zn\(^{2+}\) concentrations. These Zn\(^{2+}\) rises impair mitochondrial function, promote further ROS generation,\(^{109}\) and release pro-apoptotic factors. ROS-driven Zn\(^{2+}\) mobilization may then promote intraneuronal A\(\beta\)/Zn\(^{2+}\) aggregation. A\(\beta\) may amplify the damaging results of this vicious cycle by increasing oxidative stress, reducing glutamate reuptake and inducing the activation of NMDARs and Ca-ARs. Furthermore, glutamate-driven mitochondrial Ca\(^{2+}\) overload mobilizes Zn\(^{2+}\) from these organelles,\(^{79}\) thereby increasing toxic cytosolic Zn\(^{2+}\) levels. PS1 and APP mutations may enhance these pathways by contributing to Ca\(^{2+}\) dyshomeostasis via increased Ca\(^{2+}\) release from the ER-mitochondrial network.\(^{51,110}\) In that respect, NMDAR overactivation, Ca\(^{2+}\) overload, and the resultant increased superoxide generation may all favor and increase tau hyperphosphorylation.

All these processes, set in motion at the level of synaptic spines, may be the \textit{primum movens} of synaptic dysfunction, neuronal deafferentation, and death.

\[\text{Ionic Dyshomeostasis as Potential Therapeutic Target}\]

Consistent with the idea that AD may involve a chronic deregulation in the homeostasis of Ca\(^{2+}\) and Zn\(^{2+}\), several therapeutic strategies have aimed at restoring physiological cationic signaling in an effort to counteract the development and progression of the disease. For example, recent evidence points to a possible therapeutic role for Ca\(^{2+}\)-induced chelation, as the cation may have an important role in stabilizing the \(\gamma\)-secretase complex, as confirmed by recent observations showing that EGTA and EDTA reduce its activity.\(^{111}\)

Furthermore, targeting NMDAR-mediated Ca\(^{2+}\) influx has produced a promising anti-AD drug. Memantine, an uncompetitive and moderate-affinity NMDAR antagonist, protects neurons against A\(\beta\)-related toxicity,\(^{112}\) reduces AD-like pathology in transgenic mice,\(^{113}\) and appears to be an effective, safe, and well-tolerated treatment for moderate-to-severe AD.\(^{114}\) Great attention has also been paid toward the activity of antagonists of a specific NMDAR subunit. Ifenprodil and Ro 25–6981, two compounds that selectively target NMDAR containing the GluN2B subunit protect neurons from A\(\beta\)-induced synaptic plasticity impairment.\(^{115}\) Furthermore, EVT-101 (Evotec AG, Hamburg, Germany; http://www.evotec.com/), a newly developed GluN2B antagonist, has been shown to improve the cognitive performance of AD patients and has less side effects when compared with memantine.

In the past few years, efforts have also been made to address AD-related Zn\(^{2+}\) dyshomeostasis. To date, the most promising class of drugs developed for counteracting Zn\(^{2+}\) deregulation are the so called metal-protein attenuation compounds (MPACs). MPACs aim at removing Zn\(^{2+}\) from A\(\beta\) and relocate the cation to sites where it can be beneficial, for instance the TrkB receptor or the MMPs, thereby increasing BDNF signaling. Among the MPACs, the lipophilic quinoline derivative, PBT2, has shown the most promising results by serving as metal exchanger and ionophore. In AD murine models, PBT2 restores cognition, strongly reduces interstitial A\(\beta\)/

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supplementation appear to be linked to the action of the cation in increasing BDNF levels and by its activity in counteracting age-dependent mitochondrial dysfunction,79 (Figure 3).

In summary, a better understanding of the complex network linking the AD-dependent deregulation of these two cations poses great challenges yet also offers the potential for the development of effective disease-modifying drugs.

Conflict of Interest
The authors declare no conflict of interest.

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