Functional interdependence of divergent heavy metal ions, voltage-gated calcium channels and glutamatergic transmission in the central nervous system

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Abstract

Within the last decades, scientists have gained detailed insight into mechanisms of synaptic transmission, hyperexcitability, excitotoxicity and neurodegeneration. These (patho)physiological processes are substantially regulated by voltage-gated Ca²⁺ channels (VGCCs), the glutamatergic system and trace metal ions such as Zn²⁺ and Cu²⁺. Whereas early studies in the field provided significant but only fragmentary insight, recent findings enable us to understand the complex crosstalk between individual voltage- and ligand-gated ion channels entities and divergent heavy metal ions in the brain. Dysbalance in Ca²⁺, Zn²⁺ and Cu²⁺ homeostasis and also the glutamate system may be linked to the pathogenesis of neurodegenerative disorders and hyperexcitability-related disease states, such as Alzheimer’s disease and epilepsy. Starting from hippocampal CA3 mossy fiber terminals, we create an integrative overview of the complex functional and structural interplay of Ca²⁺ channels, the glutamate system and trace metals.

Introduction

Voltage-gated Ca²⁺ channels (VGCCs) are central players in mediating Ca²⁺ influx into living cells. Ten different Ca₂⁻,α subunits of VGCCs complexes have been cloned so far, each exhibiting specific electrophysiological and pharmacological properties [1]. Within the central nervous system (CNS) there is a time- and regional specific distribution pattern of VGCCs that significantly contributes to the establishment of brain eurhythmia. Using electrophysiological, molecular and immunolocalisation techniques we now know that VGCCs are differentially distributed throughout the neurolemma, some of them specifically mediating synaptic transmission and long-term potentiation (LTP). Within the last decade it turned out that within the synaptic fusion machinery also divalent heavy metal, i.e., Zn²⁺ and Cu²⁺ ions play a major role and exert complex effects on both pre- and postsynaptically localized voltage- and ligand-gated ion channels [2]. Importantly, it turned out that glutamate receptors build up a functional triade with specific VGCC entities as well as Zn²⁺ and Cu²⁺ ions and that this functional interdependence is of major relevance for the initiation and perturbation of neural circuitry specific rhythmicity. In consequence, alteration in this sophisticated system is to result in central dysrhythmia leading to aberrant brain excitability or cognitive impairment as observed in dementia. Though we know that the functional interplay between the calcium system, the glutamate system and divergent heavy metal ions severely influences neural excitability it remains unclear how a dysbalance in these systems is to trigger hyperexcitability and ictogenesis.

In this review we provide detailed information on structure and function of VGCCs, their differential distribution within the CNS and their electrophysiological modification by divergent heavy metal ions. Then, we functionally integrate VGCCs and Zn²⁺/Cu²⁺ into the glutamate system to elaborate how these players can interfere and dysfunction contributing to epilepsy or dementia disorders phenotype.

Structural, functional and pharmacological characteristics of voltage-gated Ca²⁺ channels

Voltage-gated Ca²⁺ channels are of central relevance in mediating Ca²⁺ influx into living cells. They can trigger various physiological processes such as excitation-contraction (EC) coupling [3], excitation-secretion (ES) coupling [4], hormone and transmitter release [5,6] and regulation of gene expression [7,8]. Structurally, VGCC complexes are heteromultimeric assemblies composed of a central pore-forming, ion-
conducting Ca$_{-}$- subunit and various auxiliary subunits (α$_{-}$δ$_{-}$, β$_{-}$, and γ$_{-}$) (Figure 1). Ten different Ca$_{-}$- subunits have been cloned which can be differentiated according to their electrophysiological and pharmacological properties into high-voltage activated (HVA) and low-voltage activated (LVA) channels. High-voltage activated Ca$^{2+}$ channels are further subdivided into dihydropyridine (DHP) -sensitive L ("long-lasting")-type Ca$_{1.1}$-1.4 and less DHP-sensitive non-L-type Ca$_{2.1}$-2.3 channels. The LVA T-("transient/tiny") type comprises the Ca$_{3.1}$-3.3 Ca$^{2+}$ channels [1,10]. These channels are activated at rather negative membrane potentials ($V_p = -44$ to $-46$ mV, $\tau = 1-7$ ms at $-10$ mV), exhibit fast inactivation ($V_p = -72$ to $-73$ mV, $\tau = 11-69$ ms at $-10$ mV), and small single-channel conductance (7.5 - 11 pS) [1, 10-14]. By contrast, HVA L- and non-L-type channels are activated at much stronger depolarization (e.g. Ca$_{1.1}$: $V_p = 8 - 14$ mV, $\tau > 50$ ms at $+10$ mV) [15], display higher single-channel conductances (L-type: 13 - 25 pS; Non-L-type: 9 – 20 pS), and prolonged-channel opening compared to T-type channels (Ca$_{1.2}$: $V_{slow} = 1100$ ms; Ca$_{1.3}$: $V_{slow} = 1700$ ms) [1, 16]. One must note, however, that Ca$_{1.3}$ L-type Ca$^{2+}$ channels were also shown to exhibit mid-voltage activating characteristics under special physiological and electrophysiological conditions ($V_p = (-15) - (-37)$ mV, $\tau < 1$ ms at $+10$ mV) [17-21].

Pharmacologically, L-type Ca$^{2+}$-channels are characterized by high sensitivity towards DHPs, e.g. nifedipine (Ca$_{1.1}$: IC$_{50} = 0.15$ nM at $-65$ mV, nitrrendipine, nisoldipine, nicardipine); phenylalkylamines, e.g. verapamil, gallopamil, devapamil (Ca$_{1.2}$: IC$_{50} = 50$ nM at $-60$ mV) and benzoazepines, e.g. diltiazem (Ca$_{1.2}$: IC$_{50} = 33$ µM at $-60$ mV) [18,22-25]. Whereas the first DHPs to be developed exerted both cardiac and vascular effects, next-generation DHPs predominately target vascular smooth muscles leading to relaxation and thus antihypertensive action [26]. An experimental activator of L-type channels is BayK8644, which is not used in clinical applications.

High-voltage activated non-L-type channels which are predominantly engaged in synaptic transmission in the brain are effectively blocked by various snail and spider toxins: Ω-agatoxin IVA, derived from the funnel web spider Agelenopsis aperta targets Ca$_{2.1}$ (IC$_{50} = 1 - 3$ nM), ω-conotoxin GVI A derived from Conus geographus blocks Ca$_{2.2}$ (IC$_{50} < 30$ nM) and ω-conotoxin MVIIIC 2, a toxin from the venom gland of marine snail Conus magnus, targets both Ca$_{2.1}$ (IC$_{50} > 7$ nM) and Ca$_{2.2}$ Ca$^{2+}$-channels (IC$_{50} > 100$ nM) [1,27-30]. For a long time, Ca$_{2.3}$ R-type channels were considered to be unique as they turned out to be resistant to most Ca$^{2+}$-channel blockers outlined above. However, in 1998, the spider peptide toxin SNX-482, derived from the venom of the tarantula Hysterocrates gigas, was shown to be a selective Ca$_{2.3}$ channel inhibitor at low nanomolar concentrations (IC$_{50} = 15-30$ nM) [31]. In addition, Ca$_{2.3}$ turned out to be highly sensitive to Ni$^{2+}$ (IC$_{50} = 27$ µM), a property they share with Ca$_{3.2}$ Ca$^{2+}$-T-type channels (IC$_{50} = 5-10$ µM) [32]. Although these naturally derived toxins are predominantly of experimental interest since they are not applicable in humans, Ca$_{2.1}$-2.3 VGCCs turned out to serve more and more as potential target in epilepsy and pain treatment. Gabapentin, for example, inhibits Ca$_{2.1}$ channels via interaction with the α$_{-}$δ auxiliary subunits (albeit on-selectively), and it can influence epilepsy and pain in humans [33]. Ziconotide (ω-conotoxin MVIIA, i.e. SNX-111), a toxin derived from the marine piscivorous snail Conus geographus, is likely to inhibit Ca$_{2.2}$ Ca$^{2+}$ channels and is a potent drug in humans who turned out to be refractory or non-tolerant to opioids (IC$_{50} = 55$ pm) [29]. For the LVA Ca$^{2+}$ channels, various blockers have been proposed, including the tetraline derivative mibebradil (Ca$_{3.1}$: IC$_{50} = 270$ nM; Ca$_{3.2}$: IC$_{50} = 140$ nM), the scorpion toxin kurtoxin (IC$_{50} = 15$ nM) [34], as well as Ni$^{2+}$-ions (Ca$_{3.1}$: IC$_{50} = 250$ µM; Ca$_{3.2}$: IC$_{50} = 12$ µM; Ca$_{3.3}$: IC$_{50} = 216$ µM) [1,10,34,35]. Recently, azetidones and spiro-azetidines have been described as novel potential blockers of the T-type Ca$^{2+}$ channel Ca$_{3.2}$ for the treatment of neuropathic and inflammatory pain [36]. However, none of these potential T-type blockers has reached clinical application so far.

Diphenylalkylamine derivatives such as flunarizin or cinnarizin exhibit a non-specific blockade on VGCCs. Clinically, these drugs are used for dilatation of cerebral arteries to enhance cerebral blood flow.

Given the important functional implications of VGCCs, it is not surprising that a number of voltage-gated Ca$^{2+}$-channelopathies have been identified so far. An overview of the tissue distribution, pharmacological and functional aberrations of the individual Ca$_{-}$-α subunits is given in Table 1.

It is noteworthy that auxiliary subunits (α$_{-}$δ$_{-1-4}$, β$_{-}$, and γ$_{-}$) are capable of substantially modulating basic electrophysiological and pharmacological properties as well as plasma level expression of the Ca$_{-}$-α subunits [5,37].

Under physiological conditions, Ca$^{2+}$ influx into intact neurons is highly organized in space, frequency and amplitude as the spatiotemporally integrated free cytosolic Ca$^{2+}$ concentration [Ca$^{2+}$_i], contains specific information [38]. Principally, Ca$^{2+}$ can enter the cytosol via the Na$^{+}$/Ca$^{2+}$ exchanger, through release from intracellular stores, e.g. ER and SR, via VGCCs and an armamentarium of other potentially less-specific voltage- and ligand gated cation channels. VGCCs however provide a powerful mechanism to directly link neuronal activity to Ca$^{2+}$ influx. Until buffering mechanisms restore the resting Ca$^{2+}$ levels [39,40], [Ca$^{2+}$_i] regulates critical cellular functions, including channel
modulation, neurotransmitter release and gene transcription. This Ca\(^{2+}\) influx is also supposed to be of central relevance in hyperexcitability and excitotoxicity mediated neurodegeneration. For example, the Ca\(^{2+}\) hypothesis of epileptogenesis claims that alterations in [Ca\(^{2+}\)], may play a crucial role in the development of epilepsy [41-43]. High- and low-voltage activated Ca\(^{2+}\) channels are likely to be predominant mediators of [Ca\(^{2+}\)]\(\text{e}\) elevation during most epileptiform activity [41,44]. In hippocampal neurons, Ca\(^{2+}\) current density was up-regulated during epileptogenesis [45] and inhibition of VGCCs effectively depressed epileptiform activity [46-48]. Ca\(^{2+}\) channels were further shown to be of central relevance in mediating potential epileptiform activity on the cellular electrophysiological level, including phenomena such as afterdepolarization, plateau potentials or exacerbation of low-threshold Ca\(^{2+}\) spikes/rebound firing thus mediating seizure initiation, propagation and kindling [34, 49-53]. Concomitantly, VGCCs exert major effects in excitotoxicity and neurodegeneration by contributing to the devastating pathophysiology of human neuronal diseases associated with neurodegeneration [54, 55] (Table 1). Thus, blockade of VGCCs turns out to be a reasonable approach to pharmacologically interfere with seizure activity, excitotoxicity and neurodegeneration [56-61].

In this context, interaction partners of VGCCs turned out to be most relevant in drug discovery and development. Recently, an exceptional study on quantitative proteomics of Ca\(_2\)-channel nano-environments, using knockout-controlled multiprotein affinity purifications together with high-resolution quantitative mass spectroscopy was carried out to unravel the molecular players in local subcellular signalling [62]. About 200 proteins have been identified that clearly differ in abundance, stability of assembly and preference for the individual Ca\(_2\)-subunits. These potential interaction partners included kinases and phosphatases, cytoskeleton proteins, enzymes, SNAREs, modulators and small GT-Pases, various G-protein coupled receptors, ion channels and transporters, adaptors, extracellular matrix proteins, cytomatrix components, protein trafficking components and additional proteins of yet unknown function.

### The glutamate system and its interaction with VGCCs

One of the most intensively studied CNS structures in terms of neuronal hyperexcitability and excitotoxicity mediated neurodegeneration is the hippocampus, where along with other ion channel entities, VGCCs are highly expressed. Physiological neuronal computation in the brain requires a well-tuned balance between ongoing excitatory transmission and controlled inhibition. Excessive excitatory activity alone results in neuronal damage and cell death through mechanisms known as excitotoxicity [54,55]. The glutamate system and VGCCs are key players in excitotoxicity and hyperexcitability [63]. L-glutamate acts on both metabotropic glutamate receptors (mGluR) and ionotropic glutamate receptors, namely N-methyl-D-aspartate (NMDA)-, α-aminoo-3-hydroxy-5-methyl-4-isoxoazolpropionic acid (AMPA)- and Kainic acid (KA)-receptors. Kainic acid is a well-characterized excitotoxin derived from Dignaea simplex. In the hippocampus, KA induces degeneration predominantly of CA3 pyramidal neurons and hyperexcitability in surviving pyramidal cells thus triggering complex partial seizure activity [64]. Alternatively spliced KA-receptors, e.g. GluR5-7 and KA1-2, are widely distributed in the hippocampal formation on somata, dendrites, neurites and synapses. They exert various effects including regulation of glutamate release [65], modulation of postsynaptic currents [66], and regulation of GABAergic [67] synaptic transmission in the hippocampus [67]. Both NMDA- and KA-receptor activation is further associated with activation of VGCCs due to prolonged depolarisation and Ca\(^{2+}\)-mediated excitotoxicity which might in part be responsible for neuronal cell death [68]. However, the exact mechanisms yet have to be explored. Interestingly, KA-Rs are not only expressed postsynaptically at hippocampal CA3 dendritic spines but also presynaptically at mossy fibre terminals within the stratum lucidum (Figure 2). Whereas single fibre activation causes negligible KA-Rs responses postsynaptically, repetitive activation of mossy fibres results in greatly enhanced KA-R activation. Due to the differences in AMPA- and KA-receptor current kinetics, the resulting excitatory postsynaptic potentials exhibit two components. Interestingly, KA-receptors are activated already by quantal glutamate release into the peripheral cleft. They can induce tonic depolarisation, thereby bringing cells closer to the activation threshold and enhancing the relative importance of single inputs [68]. Presynaptically, KA-receptors act as auto-receptors that sense the glutamate released. These KA-Rs mediated responses exhibit an intriguing multidimensional regulation pattern: 1. dose-dependent effects (Schmitz et al., 2001a; Schmitz et al., 2001b) with a biphasic, bidirectional modulation of glutamate release: low KA concentrations facilitate glutamate release and enhance synaptic currents [69] whereas high KA levels cause inhibition of glutamate release and reduction of...
modulate the amount of presynaptic Ca\textsuperscript{2+} contributing to frequency-dependent facilitation of glutamate release [69].

Both VGCCs and KA-receptors modulate neurotransmission and synaptic long-term plasticity in the hippocampus probably related to the profound excitotoxic effects of KA in this brain region as well [69,72,73]. Interestingly, gene profiling studies using microarray analysis revealed that various neuroproteactants and neurotransmitters are up-regulated in a rat KA seizure model [74], some of which clearly interact with or functionally modulate VGCCs, e.g. hsp70 [75] and neurokinin 1 (NK1) for Ca\textsubscript{v}2.3 Ca\textsuperscript{2+} channels [76] or interfere with other VGCCs. Additionally, other factors such as metallothionein-1 and -2, which indirectly affect VGCCs, e.g. via interference with Zn\textsuperscript{2+}-homeostasis [2] were also up-regulated in the KA seizure model [74]. Up to now little is known about the functional role of VGCCs in neurodegeneration, such as Alzheimer’s disease, although some studies point to a neuroprotective effect of Ca\textsuperscript{2+} channel blockers [34,50,57,77]. In hippocampal neurons, L-type VGCCs, in addition to NMDA-receptors, are known to play a role in excitotoxic processes [78]. Furthermore, mGluR regulation of VGCCs, e.g. N-type channels, was shown to limit NMDA mediated toxicity in the neostriatum [79]. Blocking L- and N-type VGCCs is effective in neuroprotection following traumatic cell injury as well [80].

Recently, Zaman et al. [51] demonstrated that Ca\textsubscript{v}2.3 Ca\textsuperscript{2+} channels and a Ca\textsuperscript{2+}-activated K\textsuperscript{-}channel, i.e. SK2, can form a functional microdomain that mediates slow afterhyperpolarisations (sAHPs) in reticular thalamic nucleus (RTN) neurons following low-threshold Ca\textsuperscript{2+}-spikes. This mechanism is capable of sustaining oscillatory burst activity in RTN neurons and consequently, Ca\textsubscript{v}2.3\textsuperscript{-}mice exhibit reduced burst charges and suppressed sAHP. T-type Ca\textsuperscript{2+} channels per se did not seem to be sufficient to maintain Ca\textsuperscript{2+} levels that can trigger sAHP, the latter however is a prerequisite for repriming T-type Ca\textsuperscript{2+} channels and sustained rebound bursting [51]. Interestingly, this functional scenario of Ca\textsubscript{v}2.3 mediated SK2 activation has previously been described in the CA1 region as part of a functional triad including NMDA-receptors, Ca\textsubscript{v}2.3 and SK2. Similar to RTN neurons, Ca\textsubscript{v}2.3 and SK2 channels induce AHPs that are likely to serve as a negative feedback in regulating synaptic activity and plasticity in dendritic spines [81-83], as well as epileptogenicity [84]. However, the spatio-temporal organisation of Ca\textsuperscript{2+}-channel and the mGluR interaction is rather complex. Studies by Metz et al. [85] suggested that AHPs that typically follow action potentials in CA1 pyramidal neurons are mediated by a Ni\textsuperscript{2+}-sensitive current, the pharmacological and biophysical profile of which clearly resembles Ca\textsubscript{v}2.3 Ca\textsuperscript{2+}-currents. As AHPs are directly related to burst firing in CA1 neurons, Ca\textsubscript{v}2.3 R-type currents are relevant for encoding hippocampal place fields and enhancement of synaptic plasticity. Further studies revealed that group I mGluR1 and group V mGluR5 can dramatically alter firing patterns of CA1 pyramidal neurons via a complex, activity-dependent modulation of Ca\textsubscript{v}2.3 R-type Ca\textsuperscript{2+} channels (Park et al., 2010).

Electrophysiological studies in rat hippocampal CA1 neurons elucidated that VGCCs such as Ca\textsubscript{v}2.3 R-type Ca\textsuperscript{2+} channels can also trigger epileptiform activity by contributing to plateau potential generation following muscarinic M\textsubscript{1}/M\textsubscript{2}-R stimulation via a G\textsubscript{ai11}, phospholipase C (PLC), diacylglycerol (DAG) and protein kinase C (PKC) mediated signalling pathway [52,53]. Consistently, seizure susceptibility studies in Ca\textsubscript{v}2.3\textsuperscript{-}mice exhibited a dramatic resistance to both generalized tonic-clonic and KA-induced hippocampal seizures and increased resistance to KA-induced hippocampal cell loss [86-88]. However, up to now we still lack detailed information on the functional implications of VGCCs in neurodegeneration itself. The molecular chaperone hsp70 functionally interacts with the II-III loop of the Ca\textsubscript{v}2.3 R-type VGCC and thus might also regulate PKC effects on this channel [75]. Besides, KA-receptors also exert metabotropic effects, e.g. via a pertussis-toxin sensitive G-protein coupled signaling pathway including PLC, DAG and PKC thus providing a possible “crosstalk” with pre- and postsynaptically localized VGCCs which are strongly Ca\textsuperscript{2+}- and PKC-regulated [67,89]. It has further been elucidated that mutations in EFHC1, a novel C-terminal interaction partner of the Ca\textsubscript{v}2.3 VGCC, cause juvenile myoclonic epilepsy in...
Figure 3. Physiological interdependence of VGCCs (Ca\textsubscript{2,3}), the glutamate system and divalent heavy metal ions (Zn\textsuperscript{2+}). The hippocampal mossy fiber terminal is an example of the complex interaction of VGCCs with glutamate and Zn\textsuperscript{2+}. Presynaptically, various VGCCs such as Ca\textsubscript{2,1}, Ca\textsubscript{2,2} and Ca\textsubscript{2,3} are expressed. Ca\textsubscript{2,3} channels were shown to be involved in presynaptic long-term potentiation. The mossy fibre terminal represents a glutameric synapse in which glutamate and Zn\textsuperscript{2+} can be released into the synaptic cleft with high local concentrations. Sub- and postsynaptically, glutamate can exert its action directly on ionotropic (NMDA) glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR). Importantly, glutamate can serve as a chelator that reduces the activity of free Zn\textsuperscript{2+} and results in complex modulation of both pre- and postsynaptically localized Ca\textsubscript{2,3} Ca\textsuperscript{2+} channels (modified from [127]).
Zinc and copper – divalent heavy metal ions in the central nervous system

In pharmacology, heavy metal ions are widely used to characterize and distinguish individual components under subtypes of GluRs and VGCCs. Many of these heavy metal ion entities turned out to be of merely theoretical, i.e. pharmacological interest, with two major exceptions: Zn²⁺ and Cu²⁺ ions. Within the last decade, Zn²⁺ emerged as pharmacologically induced generalized tonic-clonic seizure models (4-AP) or hippocampal seizure models (KA, NMDA) versus pharmacologically induced status-like hippocampal seizure models following pilocarpine administration. Calcium influx via Ca_{1.2} L-type and Ca_{2.1} has also been related to neurodegenerative processes. Missense gain-of-function mutations within the Ca_{1.2} α₁-subunit result in the Timothy-syndrome, a multisystem disorder characterized by a plethora of organ dysfunctions including lethal arrhythmias, congenital heart disease, syndactyly, immune deficiency and intermittent hypoglycaemia [92]. Interestingly, the Ca_{1.2} gain-of-function mutations associated with impaired channel inactivation are associated with various neuropsychiatric syndromes, such as autism spectrum disorders, intellectual disability and epilepsy [92,93].

Gain-of-function mutations of neuronal Ca_{2.1} Ca²⁺ channels are known to be involved in the etiopathogenesis of familial hemiplegic migraine type 1(FHM1). The Ca_{2.1} channel is expressed in various brain stem nuclei, the cerebral cortex and terminal ganglia and closely related to the control of nociception. Various missense mutations have been reported for the gene encoding for the pore-forming subunits of Ca_{2.1}. Gain-of-function mutations of Ca_{2.1} channels were studied in detail using FHM knock-in mouse models displaying increased neurotransmitter release from cortical neurons [94,95]. Glutamate release on pyramidal neurons is hypothesized to facilitate and propagate experimentally induced cortical spreading depression (CSD) that underlies the phenomenon of migraine aura [96].

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Furthermore, Zn²⁺ is known to exert effects on epileptic activity and excitotoxicity [102]. The role of Zn²⁺ and Cu²⁺ in epilepsy and excitotoxicity is complex, and partially ambivalent. Whereas a number of studies illustrate that Zn²⁺ is a potential ionic mediator of selective neuronal injury [103-106], others provide strong evidence that Zn²⁺ is a powerful neuroprotector [2,107-110]. Similarly, Zn²⁺ was reported to serve as both a proconvulsant [111] and anticonvulsant [112,113] in humans and various animal models. These findings further support the apparent „Janus“-like behaviour of Zn²⁺ ions in modulating neurodegeneration and seizure susceptibility.

However, most of these prima facie contradictory observations described in the literature are based on differences in voltage- and ligand-gated ion channel expression within various neuronal cell types investigated, e.g. hippocampal interneurons versus pyramidal cells. Following KA-induced limbic seizures, hippocampal interneurons exhibit a dramatic increase in cytosolic Zn²⁺-concentration and cell death which is supposed to be due to mitochondrial dysfunction [105] and activation of specific Zn²⁺-signalling pathways [114]. Hippocampal interneurons were further reported to express Ca²⁺-permeable AMPA-receptors [115], and to release Zn²⁺ from mitochondria and other intracellular stores or metallocin [116], Zn²⁺-levels turned out to be higher in interneurons compared to hippocampal pyramidal cells [117] due to differences in Ca²⁺-AMPA-receptor expression, Ca²⁺-buffering systems and differences in mitochondrial metabolism [118]. Compared to interneurons, CA3 pyramidal cells display only a moderate increase in internal Ca²⁺-levels after KA treatment [117]. Findings of Zn²⁺-release, intracellular Zn²⁺-accumulation and its effects on KA-seizure susceptibility and excitotoxicity are rather divergent as well. Whereas extracellular chelation of Zn²⁺ in one study neither affected hippocampal excitability nor seizure-induced cell death [119], studies by Takeda et al. illustrated that Zn²⁺ can clearly attenuate KA-induced limbic seizure activity and concomitant neurodegeneration in the CA3 region, or induce inverse effects, when being chelated extracellularly [107-110,120]. Thus, by complex modulation of the inhibition – excitation balance, Zn²⁺-homeostasis is crucial for both the induction of and the prevention of hyperexcitability-related seizure development and neurodegeneration.

The molecular targets of Zn²⁺ and Cu²⁺ have been elaborated in detail in the past. Zinc, in particular, was shown to exert important functions in synaptic transmission, e.g. via inhibition of NMDA- and GABA(A)-receptors, modulation of AMPA-receptors, inhibition of the GABA transporter 4 (GAT4), enhancement of Glycine-receptors response and, most importantly, blockade of VGCCs. Using a heterologous expression system with Ca_{1.2}, Ca_{2.3} and Ca_{3.2}, VGCCs were originally reported to be the most sensitive Ca²⁺ channels.
with IC₅₀ values of 10.9 ± 3.4 µM, IC₅₀ = 31.8 ± 12.3 µM and 24.1 ± 1.9 µM, respectively [121]. Inhibition of low-voltage activated Ca²⁺-current by micromolar Zn²⁺ has further been reported in the rat aorta smooth muscle cells, but also in CA1 pyramidal neurons [122]. Importantly, Zn²⁺ can exert distinct and partially opposite effects on Ca₃.1-3.3 T-type Ca²⁺ channels [123]. Whereas Ca₃.2.2 Ca²⁺ channels were blocked by submicromolar Zn²⁺ concentrations (IC₅₀ = 0.78 ± 0.07 µM), Ca₃.1 and Ca₃.3 Ca²⁺ channels turned out to be less sensitive to Zn²⁺ (IC₅₀ = 81.7 ± 9.1 µM and IC₅₀ = 158.6 ± 13.2 µM, respectively). Hence, Zn²⁺ can be used for the pharmacological distinction of different T-type Ca²⁺-channels.

On the electrophysiological level, different Zn²⁺ effects can be explained by subtype-specific modulation of Zn²⁺ acting on multiple binding sites of Ca²⁺ channels and altering gating mechanisms. As a possible allosteric modulator of Ca²⁺ channels, Zn²⁺ is responsible for a shift to more negative potentials of the steady-state inactivation curves of Ca₃.1-3.3 T-type Ca²⁺ channels and the steady-state activation curve for Ca₃.1 and Ca₃.3,3 [123]. Furthermore, inhibitory effects of Zn²⁺ are use-dependent and strongly suggest preferential Zn²⁺ binding to the resting state of T-type Ca²⁺ channels. Inactivation kinetics for Ca₃.1 and Ca₃.3 were significantly slowed, but not for Ca₃.2 VGCCs. Deactivation kinetics of Ca₃.3 were also significantly slowed upon Zn²⁺ exposure whereas Ca₃.1 and Ca₃.2 tail currents remained unaffected. Increased Ca₃.3-mediated Ca²⁺ current was observed after Zn²⁺ application resulting in increased duration of Ca₃.3 mediated action potentials. Consequently, Zn²⁺ can apparently serve as an opener of Ca₃.3 Ca²⁺-channels [123].

Most importantly, Zn²⁺ ions can exhibit not only different modulatory effects on various voltage- and ligand-gated ion channels, but also enter cells via different channels such as VGCCs, AMPA-, NMDA- and KA-receptors, particularly when neurons exhibit repetitive activation/hyperexcitability [2,106,124]. Thus, both Ca²⁺ and Zn²⁺ can serve as synaptic or transsynaptic second messengers with extracellular diffusion i.e. spill over effects at mossy fibre terminals for example, enabling complex heterosynaptic modulation. In line with this, synaptically released Zn²⁺ can effectively inhibit LTP terminals for example, enabling complex heterosynaptic modulation. The authors further demonstrate that Cu²⁺ regulates the voltage dependence of Ca₃.3- mediated Ca²⁺ influx by shifting the voltage-dependence of activation toward more negative membrane potentials [136]. The authors further demonstrate that Cu²⁺ regulates the voltage dependence of Ca₃.3 by affecting gating charge movements. The presence of Cu²⁺ resulted in slowing of gating charges transition into the "ON" position, delaying activation and reducing the voltage sensitivity of the channel. It was further shown that neurotransmitters, such as glutamate and glycine can serve as trace metal chelators by themselves and thus profoundly modulate activity of Ca₃.3 Ca²⁺ channels by influencing their voltage-dependent gating.

Glutamate is released from presynaptic terminals and interferes with receptors on the pre- and postsynaptic membranes, conveying information between interconnected neurons. The spatiotemporal profile of glutamate action on direct and indirect targets is of high importance for proper signal transduction in the brain. Interestingly, glutamate substantially potentiated the activity of Ca₃.3 channels at hyperpolarized potentials by shifting their voltage-dependent activation curve toward more negative voltages. Most importantly, the glutamate effect on Ca₃.3 Ca²⁺ channels was clearly based on the chelating effect and mechanistically distinct from the activation of intracellular signal transduction cascades [137,138]. Glutamate exerts its action on Ca₃.3 VGCCs from the extracellular side and although the trace metal signal transduction cascades [137,138]. Glutamate exerts its action on Ca₃.3 VGCCs from the extracellular side and although the trace metal binding character has been documented before [139,140] it was not considered to be physiologically relevant until now.

Considering the fact that the local concentration of glutamate in the synaptic cleft can transiently reach the millimolar range [141], all glutamate-sensitive postsynaptic channels and receptors should be considered as potential targets. The sensitivity of Ca₃.3 Ca²⁺-channels to glutamate is based on the presence of trace metals in the extracellular milieu. Notably, the concentration of free or loosely bound Zn²⁺ and Cu²⁺ ions, the exact nature of which, remains to be further understood. Given these findings, functional interactions among Zn²⁺, Cu²⁺ and Ca₃.3 R-type channels became the focus of recent research efforts. T-type Ca²⁺ channels are exceptionally sensitive to low concentrations of Ni²⁺, Zn²⁺ and Cu²⁺ [123,131,132]. Nickel, a divalent cation has already been characterized as a selective blocker of Ca₃.3 R-type, but also of Ca₃.2 T-type Ca²⁺-channels. Recently, a key structural motif for Ni²⁺ and Zn²⁺ binding to Ca₃.2 has been attributed to His191 located at the extracellular IS3-IS4 loop [32,132]. Sequence comparison revealed that two His-residues (H179 and H183) are present in the IS3-IS4 region of Ca₃.3 Ca²⁺ channels as well. Following site-directed mutagenesis, electrophysiological studies revealed that both histidines are structural determinants of Ni²⁺ inhibition of the Ca₃.3 R-type Ca²⁺ channel [133].
Cu²⁺ is elevated in the brain [142,143], and the release of these metals from synaptic vesicles in a voltage- and Ca²⁺-dependent fashion has been detected with various techniques [2].

It has never been reported before that the trace amounts of divalent heavy metals that often contaminate extracellular solutions [133,144] can exert tonic inhibitory effects on Ca₂⁺ voltage-dependent gating. Based on the observed shifts in IV-curves and changes in current kinetics in the presence of various Zn²⁺ and Cu²⁺ concentrations, Ca₂⁺ VGCC turned out to be mid-voltage activated in a Zn²⁺ and Cu²⁺ low/ free environment.

It has been estimated that an average HEPES-TEA solution contains 50 nM Cu²⁺, which could be responsible for a 17 mV negative shift in the Ca₂⁺ activation curve. In addition, trace metal chelation also enhanced Ca₂⁺ current inactivation kinetics (Shcheglovitov et al., 2012). These findings are likely to have an impact on our view on Ca₂⁺ VGCCs, requiring a thorough re-assessment of previously reported electrophysiological studies on Ca₂⁺ channels and suggest that a plethora of new (patho)physiological function may exist for this channel entity.

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**Competing interests**

The authors declare no competing interests.

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