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Functional interdependence of divalent 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 Ca^{2+} channels (VGCCs), the glutamatergic system and trace metal ions such as Zn^{2+} and Cu^{2+}. 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 divalent heavy metal ions in the brain. Dysbalance in Ca^{2+}, Zn^{2+} and Cu^{2+} 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 Ca^{2+} channels, the glutamate system and trace metals.

Abbreviations: $[Ca^{2+}]_i$: free cytosolic Ca^{2+} concentration; AD: Alzheimer's disease; 4-AP: 4-aminopyridine; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS: central nervous system; DAG: diacylglycerole; DHP: dihydropyridine; EC: excitation-contraction; ER: endoplasmatic reticulum; ES: excitation-secretion; HVA: high-voltage activated; KA: kainic acid; LVA: low-voltage activated; LTP: long-term potentiation; mGluR: metabotropic glutamate receptor; NMDA: N-methyl-D-aspartate; PKC: protein kinase C; PLC: phospholipase C; RTN: reticular thalamic nucleus; SR: sarcoplasmatic reticulum; VGCC: voltage-gated Ca^{2+} channels

Introduction

Voltage-gated Ca^{2+} channels (VGCCs) are central players in mediating Ca^{2+} influx into living cells. Ten different Ca_v-α_1 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.* Zn^{2+} and Cu^{2+} 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 Zn^{2+} and Cu^{2+} 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 divalent 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 divalent heavy metal ions. Then, we functionally integrate VGCCs and Zn^{2+}/Cu^{2+} 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 Ca^{2+} channels

Voltage-gated Ca^{2+} channels are of central relevance in mediating Ca^{2+} 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-

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conducting Ca_v-α_1 subunit and various auxiliary subunits (α_2-$\delta_{(1-4)}$, $\beta_{(1-4)}$ and $\gamma_{(1-8)}$) (Figure 1). Ten different Ca_v-α_1 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 $Ca_v1.1$–1.4 and less DHP-sensitive non-L-type $Ca_v2.1$–2.3 channels. The LVA T-("transient/tiny") type comprises the $Ca_v3.1$-3.3 Ca^{2+} channels [1,9,10]. These channels are activated at rather negative membrane potentials (V_a = (-44) – (-46) mV, τ_a = 1 - 7 ms at -10 mV), exhibit fast inactivation (V_h = (-72) – (-73) mV, τ_h = 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. $Ca_v1.1$: V_a = 8 – 14 mV, τ_a > 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 ($Ca_v1.2$: τ_{slow} = 1100 ms; $Ca_v1.3$: τ_{slow} = 1700 ms) [1, 16]. One must note, however, that $Ca_v1.3$ L-type Ca^{2+} channels were also shown to exhibit mid-voltage activating characteristics under special physiological and electrophysiological conditions (V_a = (-15) – (-37) mV, τ_a <1 ms at +10 mV) [17-21].

Pharmacologically, L-type Ca^{2+}-channels are characterized by high sensitivity towards DHPs, e.g. nifedipine ($Ca_v1.1$: IC_{50} = 0.15 nM at -65 mV, nitrendipine, nisoldipine, nicardipine); phenylalkylamines, e.g. verapamil, gallopamil, devapamil ($Ca_v1.2$: IC_{50} = 50 nM at -60 mV) and benzothiazepines, e.g. diltiazem ($Ca_v1.2$: 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

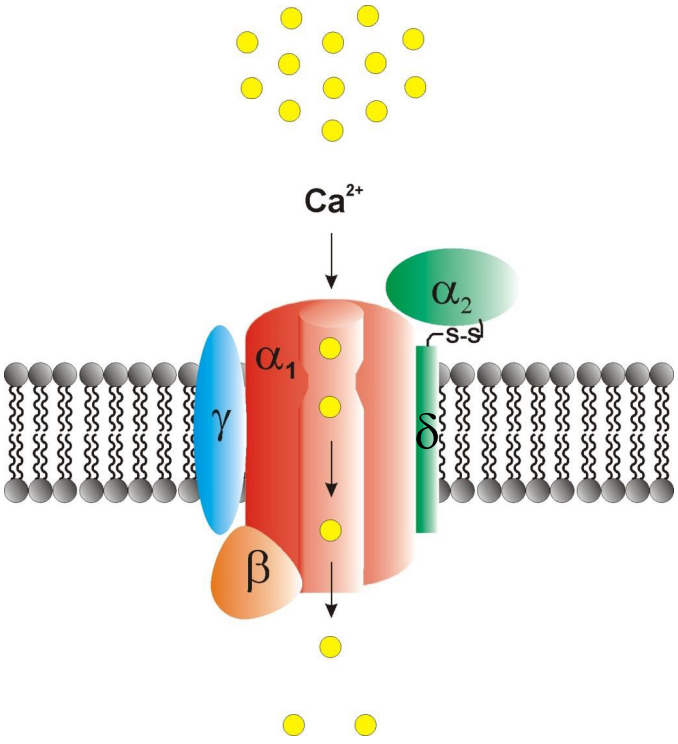

Figure 1. Structure of heteromultimeric voltage-gated Ca^{2+} channels complexes. The pore-forming Ca_v-α_1 subunit is a four domain macromolecular protein that can harbour extracellularly located trace metal binding sites. Various auxiliary subunits, *i.e.* the $\alpha_2\delta$, β and γ subunit, are capable of modulating basic electrophysiological and pharmacological properties of the Ca_v-α_1 subunit. Mutations in both pore-forming and auxiliary subunits are related to voltage-gated Ca^{2+} channelopathies (Table 1) (adapted from [145]).

[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 predominately 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 $Ca_v2.1$ (IC_{50} = 1 - 3 nM), ω-conotoxin GVIA derived from Conus geographus blocks $Ca_v2.2$ (IC_{50} < 30 nM) and ω-conotoxin MVIIC 2, a toxin from the venom gland of marine snail Conus magnus, targets both $Ca_v2.1$ (IC_{50} > 7 nM) and $Ca_v2.2$ Ca^{2+} channels (IC_{50} > 100 nM) [1,27-30]. For a long time, $Ca_v2.3$ 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 $Ca_v2.3$ channel inhibitor at low nanomolar concentrations (IC_{50} = 15-30 nM) [31]. In addition, $Ca_v2.3$ turned out to be highly sensitive to Ni^{2+} (IC_{50} = 27 µM), a property they share with $Ca_v3.2$ 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, $Ca_v2.1$-2.3 VGCCs turned out to serve more and more as potential target in epilepsy and pain treatment. Gabapentin, for example, inhibits $Ca_v2.1$ channels *via* interaction with the α_2-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 $Ca_v2.2$ 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 mibefradil ($Ca_v3.1$: IC_{50} = 270 nM; $Ca_v3.2$: IC_{50} = 140 nM), the scorpion toxin kurtoxin (IC_{50} = 15 nM) [34], as well as Ni^{2+}-ions ($Ca_v3.1$: IC_{50} = 250 µM; $Ca_v3.2$: IC_{50} = 12 µM: $Ca_v3.3$: IC_{50} = 216 µM) [1,10,34,35]. Recently, azetidinones and spiro-azetidines have been described as novel potential blockers of the T-type Ca^{2+} channel $Ca_v3.2$ 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_v-α_1 subunits is given in Table 1.

It is noteworthy that auxiliary subunits ($\alpha_2\delta_{1-4}$, β_{1-4} and γ_{1-8}) are capable of substantially modulating basic electrophysiological and pharmacological properties as well as plasma level expression of the Ca_v-α_1 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

Table 1. Pathophysiological implications of voltage-gated Ca^{2+} channels in channelopathies. VGCCs are subdivided into HVA L- and Non-L-type and LVA Ca^{2+} channels based on activation characteristics and the pharmacological inhibition profile (modified from [145].

Ca_v-α_1	Pharmacology	Tissue affected	Syndromes associated
$Ca_v1.1$	Dihydropyridne, Benzothiazepine, Phenylalkylamine, TaiCatoxin, Calciseptine Calcicludine, FS-2	skeletal muscles	Hypokalemic periodic paralysis type 1 (HypoPP1), malignant hyperthermia type 5 (MHS5)
$Ca_v1.2$		ubiquitary	Timothy syndrome (LQT8, epilepsy)
$Ca_v1.3$		ubiquitary	not known
$Ca_v1.4$		retina	x-linked conginital stationary night blindness 2 (xCSNB2), X-linked cone-rod dystrophy type 3 (CORDX3)
$Ca_v2.1$	ω-Agatoxin IVA	CNS	Absence-epilepsy, episodic ataxia type 2, spinocerebellar ataxia type 6, familial hemiplegic migraine, Lambert-Eaton myastenie-Syndrome
$Ca_v2.2$	ω-Conotoxin GVIA	CNS/PNS	Lambert-Eaton myastenie-syndrome
$Ca_v2.3$	SNX-482, Ni^{2+}	CNS/PNS	not known
$Ca_v3.1$	Mibefradil, Kurtoxin, Ni^{2+}	CNS/PNS	not known
$Ca_v3.2$		CNS/heart	Absence-epilepsy (CAE), Autism spectrum disorders (ASD)
$Ca_v3.3$		CNS	not known

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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 burst firing thus mediating seizure initiation, propagation and kindling [34, 49-53]. Concomitant, 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_v2 channel nano-environments, using knockout-controlled multiepitope 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_v2 subunits. These potential interaction partners included kinases and phosphatases, cytoskeleton proteins, enzymes, SNAREs, modulators and small GTPases, 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-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and Kainic acid (KA)-receptors. Kainic acid is a well-characterized excitotoxin derived from Digenea 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 (*gabazincergic*) 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

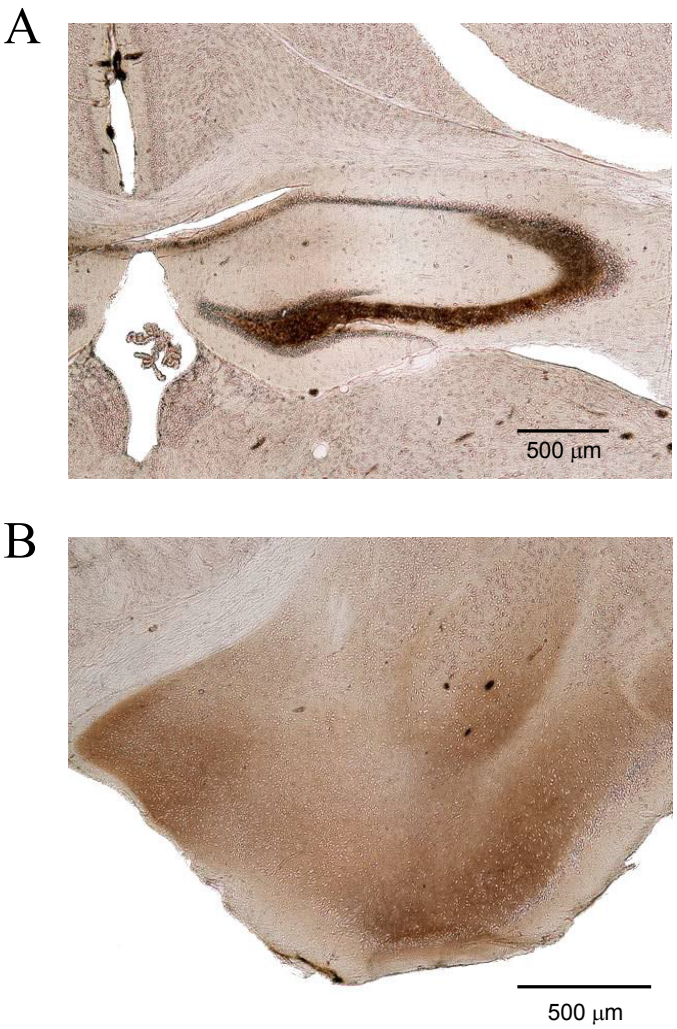

Figure 2. Distribution of Zn^{2+} in the normal mouse brain. TIMM staining of a coronal cryosection of a mouse brain (C57BL/6). Note that Zn^{2+} accumulations are predominantly localized in the hippocampus (A), amygdala (B), striatum and cortex. Hp, hippocampus, CA, cornu ammonis, DG, dentate gyrus, bar: 500 µm.

synaptic current. *2. time-dependent effects* of KA-receptors mediated action with early stimulatory effects on neurotransmission and late depressing effects. There is intense and on-going discussion on how KA-receptors mediate these multiple spatiotemporal presynaptic effects. Low concentrations might result in only small depolarisations, whereas higher concentrations cause presynaptic inhibition of neurotransmission due to ionotropic action of KA-receptors and strong depolarisations that inhibit Na^+- and Ca^{2+}-channels [69-71]. Recent studies clearly indicate that presynaptic KA-receptors substantially contribute to long-lasting afterdepolarisations. They can, therefore, modulate the amount of presynaptic Ca^{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 neuroprotectants 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 $Ca_v2.3$ Ca^{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^{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^{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 $Ca_v2.3$ Ca^{2+} channels and a Ca^{2+}-activated K^+-channel, *i.e.* SK2, can form a functional microdomain that mediates slow afterhyperpolarisations (sAHPs) in reticular thalamic nucleus (RTN) neurons following low-threshold Ca^{2+}-spikes. This mechanism is capable of sustaining oscillatory burst activity in RTN neurons and consequently, $Ca_v2.3^{-/-}$ mice exhibit reduced burst charges and suppressed sAHP. T-type Ca^{2+} channels per se did not seem to be sufficient to maintain Ca^{2+} levels that can trigger sAHP, the latter however is a prerequisite for repriming T-type Ca^{2+} channels and sustained rebound bursting [51]. Interestingly, this functional scenario of $Ca_v2.3$ mediated SK2 activation has previously been described in the CA1 region as part of a functional triad including NMDA-receptors, $Ca_v2.3$ and SK2. Similar to RTN neurons, $Ca_v2.3$ 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^{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^{2+}-sensitive current, the pharmacological and biophysical profile of which clearly resembles $Ca_v2.3$ Ca^{2+}currents. As AHPs are directly related to burst firing in CA1 neurons, $Ca_v2.3$ 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 $Ca_v2.3$ R-type Ca^{2+} channels (Park *et al.,* 2010).

Electrophysiological studies in rat hippocampal CA1 neurons elucidated that VGCCs such as $Ca_v2.3$ R-type Ca^{2+} channels can also trigger epileptiform activity by contributing to plateau potential generation following muscarinic M_1/M_3-R stimulation *via* a $G_{q/11}$, phospholipase C (PLC), diacylglycerole (DAG) and protein kinase C (PKC) mediated signalling pathway [52,53]. Consistently, seizure susceptibility studies in $Ca_v2.3^{-/-}$ 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 $Ca_v2.3$ 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^{2+}- and PKC-regulated [67,89]. It has further been elucidated that mutations in EFHC1, a novel C-terminal interaction partner of the $Ca_v2.3$ VGCC, cause juvenile myoclonic epilepsy in

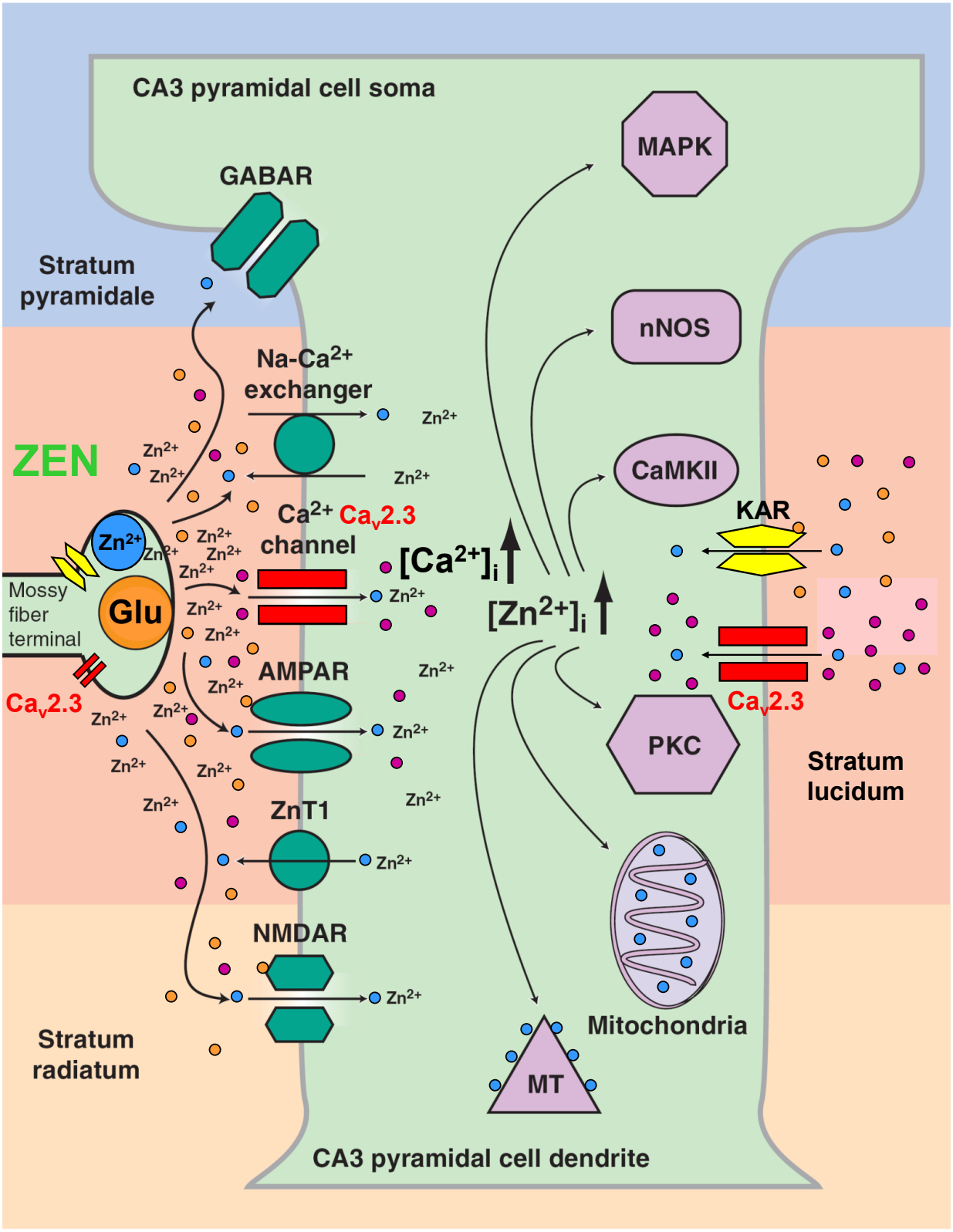

Figure 3. Physiological interdependence of VGCCs ($Ca_v2.3$), the glutamate system and divalent heavy metal ions (Zn^{2+}). The hippocampal mossy fiber terminal is an example of the complex interaction of VGCCs with glutamate and Zn^{2+}. Presynaptically, various VGCCs such as $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ are expressed. $Ca_v2.3$ channels were shown to be involved in presynaptic long-term potentiation. The mossy fibre terminal represents a gluzincerigc synapse in which glutamate and Zn^{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^{2+} and results in complex modulation of both pre- and postsynaptically localized $Ca_v2.3$ Ca^{2+} channels (modified from [127]).

humans, indicating that $Ca_v2.3$ is likely to be involved in neuronal apoptotic processes related to excitotoxicity [90]. A critical role of $Ca_v2.3$ in neuronal degeneration is supported by the observation that antiepileptic drugs known to block $Ca_v2.3$, e.g. topiramate and lamotrigine, also exert strong neuroprotective effects [87]. Other VGCCs have been implicated in the pathogenesis of epilepsies and neurodegeneration as well. $Ca_v3.2$ T-type Ca^{2+} channels have been related to hippocampal hyperexcitability and neuronal cell death in the rat pilocarpine model of limbic seizures [49,91]. The individual contribution of $Ca_v2.3$ R-type and $Ca_v3.2$ T-type Ca^{2+}-channels to hippocampal hyperexcitability and neurodegeneration is likely to be dependent on the different experimental paradigms that have been used, e.g. *acute*, 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* $Ca_v1.2$ L-type and $Ca_v2.1$ has also been related to neurodegenerative processes. Missense gain-of-function mutations within the $Ca_v1.2$ α_1-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 $Ca_v1.2$ 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 $Ca_v2.1$ Ca^{2+} channels are known to be involved in the etiopathogensis of familial hemiplegic migraine type 1(FHM1). The $Ca_v2.1$ 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 $Ca_v2.1$. Gain-of-function mutations of $Ca_v2.1$ 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].

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 and 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: Zn^{2+} and Cu^{2+} ions. Within the last decade, Zn^{2+} emerged to be one of the most important heavy metal ions within the CNS, to the extent that is was sometimes referred to as "the calcium of the twenty-first century" [2]. Some forebrain neurons, *i.e.* zinc-enriched neurons (ZEN) exhibit extremely high Zn^{2+}-concentrations, e.g. glutamatergic (*gluzinergic*) neurons of the amygdala, the cerebral cortex and particularly the hippocampus. Zinc-storing vesicles, so called zincosomes exist in both GABAergic (*gabazinergic*) neurons, e.g. in the cerebellum and glutamatergic (*gluzinergic*) neurons, e.g. in granule cell mossy fibres boutons [2] with Zn^{2+}-transporters such as ZnT_3 and ZnT_4 being responsible for the strong vesicular Zn^{2+}-enrichment of up to 350 μM [2, 97]. Plasma Zn^{2+}-concentrations are about 15 μM, with free Zn^{2+} in the nanomolar range [97,98]. In the brain, about 90 % of Zn^{2+} is bound to metalloproteins and 10% in vesicles [97]. Like Zn^{2+}, Cu^{2+} also accumulates in the CNS, predominantly in adrenergic and glutamatergic (*glucupergic*) neurons in the hippocampus, the olfactory bulb and the locus coeruleus [99-101]. Normal extracellular Cu^{2+}-concentrations are in the range of 0.2 – 1.7 μM [98] and are frequently increased in neurodegenerative processes such as Alzheimer's disease, then reaching levels of up to 200 – 400 μM. Both divalent trace metals, Zn^{2+} and Cu^{2+}, are implicated in a range of neurological disease states in humans that are characterized by alterations in neuronal excitability and/or neurodegeneration. For example, there is strong evidence of a pathogenic Zn^{2+}- and Cu^{2+}-interaction with amyloid-β in Alzheimer's disease. As an established pathogenetic process, pathological accumulation of Cu^{2+} can lead to Wilson's disease, *i.e.* hepatolenticular degeneration. In addition, alterations of Zn^{2+}-metalloproteins, such as superoxide dismutase or metallothionein, can cause amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) [2].

Furthermore, Zn^{2+} is known to exert effects on epileptic activity and excitotoxicity [102]. The role of Zn^{2+} and Cu^{2+} in epilepsy and excitotoxicity is complex, and partially ambivalent. Whereas a number of studies illustrate that Zn^{2+} is a potential ionic mediator of selective neuronal injury [103-106], others provide strong evidence that Zn^{2+} is a powerful neuroprotector [2,107-110]. Similarly, Zn^{2+} 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 Zn^{2+} 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 Zn^{2+}-concentration and cell death which is supposed to be due to mitochondrial dysfunction [105] and activation of specific Zn^{2+}-signalling pathways [114]. Hippocampal interneurons were further reported to express Ca^{2+}-permeable AMPA-receptors [115], and to release Zn^{2+} from mitochondria and other intracellular stores or metallothioneins [116]. Zn^{2+}-levels turned out to be higher in interneurons compared to hippocampal pyramidal cells [117] due to differences in Ca^{2+}-AMPA-receptor expression, Ca^{2+}-buffering systems and differences in mitochondrial metabolism [118]. Compared to interneurons, CA3 pyramidal cells display only a moderate increase in internal Ca^{2+}-levels after KA treatment [117]. Findings of Zn^{2+}-release, intracellular Zn^{2+}-accumulation and its effects on KA-seizure susceptibility and excitotoxicity are rather divergent as well. Whereas extracellular chelation of Zn^{2+} in one study neither affected hippocampal excitability nor seizure-induced cell death [119], studies by Takeda *et al.* illustrated that Zn^{2+} 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, Zn^{2+}-homeostasis is crucial for both the induction of and the prevention of hyperexcitability-related seizure development and neurodegeneration.

The molecular targets of Zn^{2+} and Cu^{2+} 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 $Ca_v1.2$, $Ca_v2.3$ and $Ca_v3.2$, VGCCs were originally reported to be the most sensitive Ca^{2+} channels

with IC_{50} values of 10.9 ± 3.4 μM, IC_{50} = 31.8 ±12.3 μM and 24.1 ± 1.9 μM, respectively [121]. Inhibition of low-voltage activated Ca^{2+}-current by micromolar Zn^{2+} has further been reported in the rat aorta smooth muscle cells, but also in CA1 pyramidal neurons [122]. Importantly, Zn^{2+} can exert distinct and partially opposite effects on $Ca_v3.1$-3.3 T-type Ca^{2+} channels [123]. Whereas $Ca_v3.2$ Ca^{2+} channels were blocked by submicromolar Zn^{2+} concentrations (IC_{50} = 0.78 ± 0.07 μM), $Ca_v3.1$ and $Ca_v3.3$ Ca^{2+} channels turned out to be less sensitive to Zn^{2+} (IC_{50} = 81.7 ± 9.1 μM and IC_{50} = 158.6 ± 13.2 μM, respectively). Hence, Zn^{2+} can be used for the pharmacological distinction of different T-type Ca^{2+} channels.

On the electrophysiological level, different Zn^{2+} effects can be explained by subtype-specific modulation of Zn^{2+} acting on multiple binding sites of Ca^{2+} channels and altering gating mechanisms. As a possible allosteric modulator of Ca^{2+} channels, Zn^{2+} is responsible for a shift to more negative potentials of the steady-state inactivation curves of $Ca_v3.1$-3.3 T-type Ca^{2+} channels and the steady-state activation curve for $Ca_v3.1$ and $Ca_v3.3$ [123]. Furthermore, inhibitory effects of Zn^{2+} are use-dependent and strongly suggest preferential Zn^{2+} binding to the resting state of T-type Ca^{2+} channels. Inactivation kinetics for $Ca_v3.1$ and $Ca_v3.3$ were significantly slowed, but not for $Ca_v3.2$ VGCCs. Deactivation kinetics of $Ca_v3.3$ were also significantly slowed upon Zn^{2+} exposure whereas $Ca_v3.1$ and $Ca_v3.2$ tail currents remained affected. Increased $Ca_v3.3$ mediated Ca^{2+} current was observed after Zn^{2+} application resulting in increased duration of $Ca_v3.3$ mediated action potentials. Consequently, Zn^{2+} can apparently serve as an opener of $Ca_v3.3$ Ca^{2+} channels [123].

Most importantly, Zn^{2+} 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 Ca^{2+} and Zn^{2+} 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 Zn^{2+} can effectively inhibit LTP presynaptically at the mossy fiber synapse [125]. These findings directly corroborate the critical role of $Ca_v2.3$ R-type Ca^{2+}-channels, serving as a Zn^{2+} target in presynaptic LTP [73,121,126].

$Ca_v2.3$ channel activity is not only affected by Zn^{2+}. The channel itself, in turn, is likely to regulate cellular Zn^{2+}-influx in an excitability dependent fashion thereby controlling intracellular Zn^{2+}-signalling. Zn^{2+}-ions significantly influence signal transduction factors, such as PKC, CaMKII, mitogen-activated protein kinase (MAPK) and probably PKA [127]. PKC activity, for example, is modulated by both Ca^{2+} and Zn^{2+} ions in a dose dependent and interactive fashion. At low Ca^{2+}-levels (5 μM), Zn^{2+} enhances PKC activity, whereas high Ca^{2+} (> 50 μM) results in a Zn^{2+}-mediated inhibition of PKC. As PKC functionally interacts with VGCCs such as $Ca_v2.3$, these findings suggest a further regulatory mechanism *via* $[Ca^{2+}]_i$, PKC and finally Zn^{2+} [128]. At low cytosolic concentrations, neuroprotective Zn^{2+}-effects have been related to Akt-phosphorylation and hsp70 upregulation [75,117,129] whereas the proapoptotic effect of Zn^{2+} is associated with caspase activation and release of proapoptotic proteins, such as cytochrome c or apoptosis-inducing factor (AIF) [2,130]. Interestingly, some of these factors are either $Ca_v2.3$ interaction partners, modulated by $Ca_v2.3$ or Zn^{2+}-related proteins, or upregulated in the KA seizure model, e.g. MAPK, hsp70, neurokinin1 and metallothioneins [74]. Summarising the above findings, they suggest a complex crosstalk among VGCCs, Ca^{2+}-, Zn^{2+}- and Cu^{2+}-ions, the exact nature of which, remains to be further understood. Given these findings, functional interactions among Zn^{2+}, Cu^{2+} and $Ca_v2.3$ R-type channels became the focus of recent research efforts. T-type Ca^{2+} channels are exceptionally sensitive to low concentrations of Ni^{2+}, Zn^{2+} and Cu^{2+} [123,131,132]. Nickel, a divalent cation has already been characterized as a selective blocker of $Ca_v2.3$ R-type, but also of $Ca_v3.2$ T-type Ca^{2+}-channels. Recently, a key structural motif for Ni^{2+} and Zn^{2+} binding to $Ca_v3.2$ 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 $Ca_v2.3$ Ca^{2+} channels as well. Following site-directed mutagenesis, electrophysiological studies revealed that both histidines are structural determinants of Ni^{2+} inhibition of the $Ca_v2.3$ R-type Ca^{2+} channel [133].

Recently, Sheglovitov *et al.* [134] carried out a series of exceptional experiments which may well revolutionize our knowledge on Zn^{2+} and Cu^{2+} effects on $Ca_v2.3$ Ca^{2+} channels. *In vitro* dose-concentration studies using HEK 293 heterologous expression systems and calibrated heavy metal ion concentrations revealed that $Ca_v2.3$ is a most sensitive target of Zn^{2+} and Cu^{2+} ions (IC_{50} = 1.3 ± 0.2 μM and IC_{50} = 18.2 ± 3.7 nM, respectively using voltage steps to -20 mV representative for effects on activation gating and IC_{50} = 8.1 ± 1.4 μM and IC_{50} = 269 ± 101 nM respectively representative for action on conductance with voltage steps to +20 mV), in contrast to other channels and receptors, e.g. NMDAR (IC_{50} = 270 nM) and $Ca_v3.2$ (IC_{50} = 900 nM) for copper [131,135].

Abolishing the effects on potential binding sites of divalent heavy metal ions by chelation or by substitution of key amino acid residues in the IS1–IS2 (H111) and IS3–IS4 (H179 and H183) loops substantially enhanced $Ca_v2.3$ mediated Ca^{2+} influx by shifting the voltage-dependence of activation toward more negative membrane potentials [136]. The authors further demonstrate that Cu^{2+} regulates the voltage dependence of $Ca_v2.3$ by affecting gating charge movements. The presence of Cu^{2+} 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 $Ca_v2.3$ Ca^{2+} 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 $Ca_v2.3$ channels at hyperpolarized potentials by shifting their voltage-dependent activation curve toward more negative voltages. Most importantly, the glutamate effect on $Ca_v2.3$ Ca^{2+} 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 $Ca_v2.3$ 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 $Ca_v2.3$ Ca^{2+} channels to glutamate is based on the presence of trace metals in the extracellular milieu. Notably, the concentration of free or loosely bound Zn^{2+} and

Cu^{2+} is elevated in the brain [142,143], and the release of these metals from synaptic vesicles in a voltage- and Ca^{2+}-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 $Ca_v2.3$ voltage-dependent gating. Based on the observed shifts in IV-curves and changes in current kinetics in the presence of various Zn^{2+} and Cu^{2+} concentrations, $Ca_v2.3$ VGCC turned out to be mid-voltage activated in a Zn^{2+} and Cu^{2+} low/free environment.

It has been estimated that an average HEPES–TEA solution contains 50 nM Cu^{2+}, which could be responsible for a 17 mV negative shift in the $Ca_v2.3$ activation curve. In addition, trace metal chelation also enhanced $Ca_v2.3$ current inactivation kinetics (Shcheglovitov *et al.,* 2012). These findings are likely to have an impact on our view on $Ca_v2.3$ VGCCs, require a thorough re-assessment of previously reported electrophysiological studies on $Ca_v2.3$ 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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