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Zinc signaling and epilepsy

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ABSTRACT

Evidence from both preclinical and clinical studies suggest the importance of zinc homeostasis in seizures/epilepsy. Undoubtedly, zinc, via modulation of a variety of targets, is necessary for maintaining the balance between neuronal excitation and inhibition, while an imbalance between excitation and inhibition underlies seizures. However, the relationship between zinc signaling and seizures/epilepsy is complex as both extracellular and intracellular zinc may produce either protective or detrimental effects. This review provides an overview of preclinical/behavioral, functional and molecular studies, as well as clinical data on the involvement of zinc in the pathophysiology and treatment of seizures/epilepsy. Furthermore, the potential of targeting elements associated with zinc signaling or homeostasis and zinc levels as a therapeutic strategy for epilepsy is discussed.

Contents

Abbreviations: 2-AG, 2-arachidonoylglycerol; AAS, atomic absorption spectrometry; AMPAR, α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; APV, D-(−)-2-amino-5-phosphonopentanoic acid; ASICs, acid-sensing ion channels; Ca-EDTA, calcium-ethylenediaminetetraacetic acid; CB1R, presynaptic cannabinoid type 1 receptor; CNS, central nervous system; DEDTC, diethyldithiocarbamate; DIOA, R-(+)-butylindazonedihydroindenyloxyalkanoic acid; EDTA, ethylenediaminetetraacetic acid; EEG, electroencephalography; EPSC, excitatory postsynaptic currents; GABA, γ-aminobutyric acid; $GABA_AR$, γ-aminobutyric acid A receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluN1–3, N-methyl-D-aspartate receptor subunits; GPCR, G protein-coupled receptor; GPR39, G protein-coupled receptor 39, zinc sensing receptor; ICP-MS, inductively coupled plasma mass spectrometry; KCC2, potassium-chloride cotransporter 2; KCNQ, potassium voltage-gated channel subfamily Q; LTP, Long-term potentiation; MRE, metal response element; MTF1, metal-regulatory transcription factor 1; mTOR, mammalian target of rapamycin; MTs, Metallothioneins; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt; NMDAR, N-methyl-D-aspartate receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RDA, Recommended Dietary Allowance; TFL-Zn, N-(6-methoxy-8-quinolyl)-p-carboxybenzoylsulfonamide; TPEN, N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine; TRPMs7, transient receptor potential melastin channels 7; TSQ, p-toluenesulfonamido-quinoline; VGCCs, voltage-gated calcium channels; ZIP, (Zrt-Irt-related proteins) zinc transporters (SLC39A); ZnA, zinc-adequate diet; ZnD, zinc-deficient diet; ZnPy, zinc pyrithione; ZnT, zinc transporters (SLC30A).

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1. Introduction

Epilepsy is one of the most prevalent neurological diseases, affecting more than 65 million people worldwide (Ngugi, Bottomley, Kleinschmidt, Sander, & Newton, 2010). In contrast to a seizure, which is a transient occurrence of signs and/or symptoms due to abnormal, excessive, or synchronous neuronal activity in the brain, and can be temporarily induced by brain trauma, injuries, drugs, temperature or hypoxia, epilepsy is a brain disease characterized by an enduring predisposition to generate spontaneous, unprovoked seizures (Fisher et al., 2014; Goldberg & Coulter, 2013). Despite substantial research, the process of epilepsy development, i.e., epileptogenesis, remains poorly understood. It is generally believed that following a precipitating event (e.g., brain trauma, febrile seizures, infection, stroke, status epilepticus or genetic malformation), molecular and cellular alterations occur that transform a physiological neuronal network into an epileptic state, promoting excitability, which results in chronic epilepsy (Goldberg & Coulter, 2013; Loscher, 2002b; Pitkanen, 2010).

The detailed mechanisms responsible for epilepsy development and progression are likely multifactorial and have not been elucidated thus far, which limits the search for novel drugs. Although epilepsy can be controlled pharmacologically, new drug treatments are needed because more than one third of patients remain treatment-resistant (Dalic & Cook, 2016). Moreover, the only available drugs suppress symptoms of epilepsy, i.e., either to stop or reduce the frequency or severity of seizures, independently of the underlying epileptic state or disease progression (Galanopoulou et al., 2012). There are currently no medications that modify the disease in people at risk. Such agents would modify the pathological changes underlying epileptogenesis or co-morbidities (Pitkanen, 2010). Thus, exploration of the pathophysiology of epilepsy is crucial to define novel drug targets and develop compounds with favorable criteria.

An imbalance between neuronal excitation and inhibition has been established as a mechanism underlying seizures (Staley, 2015), and the trace element zinc has been determined to interact with a variety of targets that mediate excitation or inhibition including excitatory (glutamatergic) and inhibitory (γ-aminobutyric acid (GABA)-ergic) systems. Thus, it is possible that zinc plays an important role in the pathophysiology of seizures and/or epilepsy. Moreover, zinc has been shown to be involved in neuropsychiatric disorders like depression (Sanacora, Treccani, & Popoli, 2012; Tokita, Yamaji, & Hashimoto, 2012) or autism (Rubenstein & Merzenich, 2003), which have also been characterized by a defective balance between excitation and inhibition (Foss-Feig et al., 2017; Javitt et al., 2011; Rubenstein & Merzenich, 2003; Sanacora et al., 2012; Tokita et al., 2012) and are common co-morbidities in epilepsy (Besag, 2015; Fiest et al., 2013), as has been described by our group and others (Doboszewska et al., 2017; Mlyniec, 2015; Nowak, 2015; Szewczyk, 2013; Szewczyk, Kubera, & Nowak, 2011; Vela et al., 2015).

The possible association between zinc and epilepsy is supported by the observation that the highest levels of zinc in the brain are in the hippocampus (Perez-Clausell & Danscher, 1985), a brain region critical for cognition (Kesner & Rolls, 2015; Smith & Bulkin, 2014; Zeidman & Maguire, 2016), mood (Fujii, Saito, Yanaka, Kosaka, & Okazawa, 2014; MacQueen & Frodl, 2011) and one of the most extensively studied regions of the brain with regard to epilepsy (Thom, 2014). Furthermore, structural changes in the hippocampus are frequently observed in patients with mesial temporal lobe epilepsy, the most common form of treatment resistant epilepsy (Dalic & Cook, 2016; Jobst & Cascino, 2015; Thom, 2014). Noteworthy is the fact that numerous rodent and human studies demonstrated that zinc is necessary for proper brain development and function (Levenson & Morris, 2011; Sandstead, 2012) such as learning and memory, social behavior or antidepressant-like behavior (Hagmeyer, Haderspeck, & Grabrucker, 2015). Given the role of zinc in these processes, it is plausible that this element is not only involved in the mechanisms underlying epilepsy, but also cognitive impairment or depressive symptoms that often occur in epileptic patients (Badawy, Johnson, Cook, & Harvey, 2012; Lenck-Santini & Scott, 2015).

The relationship between zinc and seizures/epilepsy is complex. Although a considerable amount of data has been obtained, results are often conflicting. Thereby, our goal was to provide an overview of both preclinical/behavioral, functional and molecular studies, as well as clinical data on the role of zinc in the pathophysiology of seizures/epilepsy. To accomplish such, we describe zinc homeostasis and zinc targets in the brain, and present approaches that have been utilized to estimate zinc levels in preclinical models of seizures/epilepsy and in epileptic patients, as well as following treatment with anti-seizure drugs. We attempt to draw a link between altered levels of zinc and targets influenced by extracellular/intracellular zinc, with a focus on both preclinical and clinical aspects of the relationship between zinc deficiency and seizures/epilepsy. Finally, we discuss the potential of targeting elements involved in zinc homeostasis or signaling as a therapeutic strategy for epilepsy.

2. Zinc physiology and homeostasis in the brain

Zinc is the second (after iron) most abundant trace metal in the human body. It is a redox-inactive metal and exists solely as a divalent cation. Zinc is required because of its catalytic function in more than 300 enzymes and because it stabilizes the folding of protein subdomains (Coleman, 1992). Zinc fingers, self-contained domains stabilized by a zinc ion bound to a pair of cysteines and a pair of histidines (Cys2-His2 zinc fingers), are widely prevalent families of proteins, comprising about 3% of the proteins encoded by the human genome (Klug, 2010). In addition to catalytic and structural function, zinc plays an important signaling function, both extracellularly and intracellularly (Kambe, Tsuji, Hashimoto, & Itsumura, 2015; Klug, 2010; Maret, 2017). In the central nervous system (CNS), extracellular zinc signaling involves neuromodulating functions and activating the G protein-coupled receptor (GPCR) 39 (GPR39) (Besser et al., 2009; Kay & Toth, 2008; Yasuda et al., 2007). Intracellular zinc signaling involves its second messenger function (Yamasaki et al., 2007) and modulating a variety of signaling pathways (Kambe et al., 2015).

Zinc homeostasis is maintained primarily via the gastrointestinal system by the processes of absorption of exogenous zinc in the distal duodenum or proximal jejunum, gastrointestinal secretion and mainly fecal excretion of endogenous zinc (Krebs, 2000). Following uptake into enterocytes and subsequent transport from enterocytes to the blood, zinc is distributed throughout tissues and organs. Blood plasma represents less than 0.1% of whole body zinc, while approximately 57% of the total zinc is found in skeletal muscle, 29% in bone, 6% in skin and 5% in liver (King, Shames, & Woodhouse, 2000). To enter the brain, which accounts for approximately 1.5% of the total body zinc (King et al., 2000), zinc crosses the blood-brain barrier, and, to lesser extent, the blood-cerebrospinal fluid barrier (Bobilya, Gauthier, Karki, Olley, & Thomas, 2008; Franklin, Pullen, & Hall, 1992).

In cells, zinc is distributed in the cytoplasm (50%), nucleus (30–40%), and membrane (10%) (Kambe et al., 2015). The vast majority of zinc is bound to proteins and is sequestered into organelles and vesicles (Eide, 2006). Attempts to measure labile or free ionic zinc levels have indicated a wide range of concentrations, with huge discrepancies for labile zinc measured in the mitochondria and the endoplasmic reticulum; cytosolic zinc concentrations have been reported to range between the pM and low nM range (Eide, 2006; Kambe et al., 2015; Sensi, Paoletti, Bush, & Sekler, 2009). Nevertheless, cytosolic labile zinc has been generally estimated at a very low concentration. In contrast, high concentrations of labile zinc accumulate in specialized secretory compartments, such as synaptic vesicles (Kambe et al., 2015; Sensi et al., 2009).

2.1. Zinc transporters and metallothioneins

At the cellular level, zinc homeostasis is maintained via the ZnT and ZIP transporters and metallothioneins (MTs) (Fig. 1). The ZnT

transporters are assigned to the Solute Carrier family 30A (SLC30A) and designated ZnT1-ZnT10. The ZIP (Zrt-, Irt-related protein) transporters, named after the original members iron-regulated transporter 1 (Irt1) and zinc-regulated transporter 1 (Zrt1), are assigned to the Solute Carrier family 39A (SLC39A) and comprise 14 members designated ZIP1-ZIP14 (Kambe et al., 2015; Liuzzi & Cousins, 2004; Schweigel-Rontgen, 2014). The ZnT and ZIP transporters appear to have opposite functions with the former decreasing and the latter increasing intracellular zinc (Liuzzi & Cousins, 2004).

ZnT3 is localized in the membranes of the synaptic vesicles (Wenzel, Cole, Born, Schwartzkroin, & Palmiter, 1997) and concentrates zinc into these vesicles (Palmiter, Cole, Quaife, & Findley, 1996), while ZnT1, which is localized in the plasma membrane, promotes efflux and has been suggested as a key player in reducing intracellular zinc levels (Palmiter, 2004; Palmiter & Findley, 1995). ZnT1 is expressed in brain regions such as the cerebral cortex or hippocampus (Sekler et al., 2002), and plays a direct role in zinc efflux from cortical neurons (Qin, Thomas, Fontaine, & Colvin, 2009). ZnT1 is concentrated at the postsynaptic density (PSD) (Boeckers, 2006) of hippocampal neurons (Mellone et al., 2015; Sindreu, Bayes, Altafaj, & Perez-Clausell, 2014) where it is colocalized with the postsynaptic glutamatergic marker PSD-95 (Mellone et al., 2015). Interestingly, a concerted action of zinc and the ProSAP/Shank PSD major scaffold proteins is essential for PSD integrity. Overexpression of zinc-sensitive ProSAP1/Shank2 or ProSAP2/Shank3 led to increased synaptic density, while depletion of synaptic zinc, along with the knockdown of zinc-insensitive Shank1, caused rapid disintegration of the PSD and the loss of several postsynaptic molecules including the N-methyl-D-aspartate receptor (NMDAR) (Grabrucker et al., 2011). Thus, ZnT1, which transports zinc from the intracellular to the extracellular space, may provide zinc concentrations necessary for maintaining the structural organization of the PSD. Further, ZnT1 binds directly to the cytoplasmic tail of the GluN2A subunit of the NMDAR at hippocampal synapses, constituting a novel NMDAR binding protein at the PSD (Mellone et al., 2015). Other ZnTs expressed in the brain include: ZnT4, ZnT5, ZnT6 (Rafalo et al., 2017; Rafalo-Ulinska et al., 2016). In addition, ZnT2 was present in choroidal epithelial cell culture, indicating the presence of this transporter in the blood-cerebrospinal fluid barrier (Fu, Zeng, Zheng, & Du, 2014).

In contrast to the ZnT transporters, the MTs are a family of cysteine-rich (33 mol %), low molecular weight (7 kDa), metal binding proteins (Thirumoorthy et al., 2011). The most well studied member of this family is the mammalian MT, which comprises two domains: a β-domain with 9 cysteine residues, which sequesters 3 cadmium or zinc or 6 copper ions, and an α-domain with 11 cysteine residues, which sequesters 4 cadmium or zinc or 6 copper ions (Sutherland & Stillman, 2011). Of the 4 major isoforms of MTs (I–IV), three (MTI, MTII, MTIII) have been localized in the CNS (Juarez-Rebollar, Rios, Nava-Ruiz, & Mendez-Armenta, 2017). Moreover, MTIII is found exclusively in the CNS and can be localized either intra- or extracellularly (Vasak & Meloni, 2017). Because the thiol side chain in cysteine undergoes redox reactions, MTs buffer zinc in a redox-sensitive manner and oxidative stress can mobilize zinc from MTs (Krezel, Hao, & Maret, 2007). Zinc mobilization from MTs upon oxidative stress is involved in intracellular zinc signaling (Pochwat, Nowak, & Szewczyk, 2015; Sensi et al., 2011). Recently, MT expression in leukocytes, which decreases during zinc deficiency and increases along with increased intake of zinc, has been

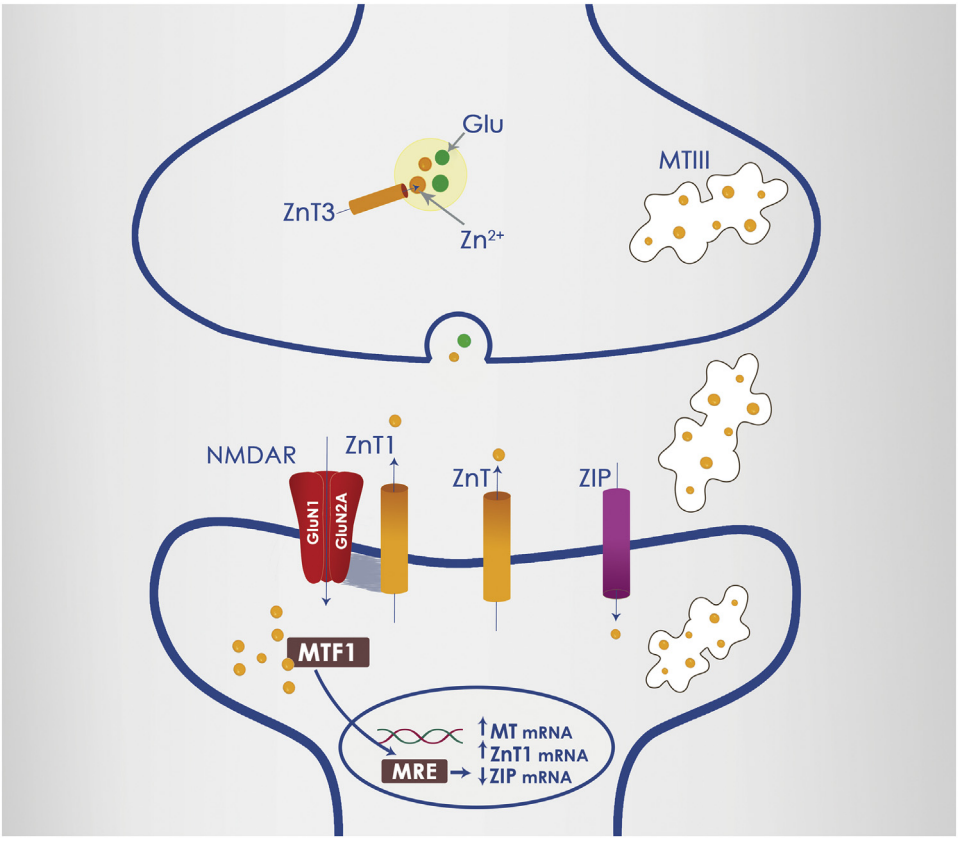

Fig. 1. Zinc homeostasis regulating proteins. ZnT transporters function to decrease intracellular zinc concentration either by promoting zinc efflux from cells or into intracellular vesicles, while ZIP transporters increase intracellular zinc concentration. ZnT3 packs zinc into the synaptic vesicle. ZnT1, which is localized in the plasma membrane, has been suggested as a key player reducing intracellular zinc levels. ZnT1 binds directly to the cytoplasmic tail of the GluN2A subunit of the NMDAR at hippocampal synapse, constituting novel NMDAR binding protein. MTs are a family of metal binding proteins. MTIII is found exclusively in the CNS and can be localized either intra- or extracellularly. MTF1 is activated by intracellular zinc and is transported to the nucleus where it recognizes promoters containing MRE, thus, increases transcription of genes, such as *Mt*, *Znt1* and represses expression of genes encoding *Zip*. ZnT, ZIP, zinc transporters; MTIII, metallothionein III; MTF1, metal-regulatory transcription factor 1; MRE, metal response element, NMDAR, N-methyl-D-aspartate receptor; Zn^{2+}, zinc ions; Glu, glutamate.

suggested as a more reliable marker for zinc status than plasma zinc concentration (Hennigar, Kelley, & McClung, 2016).

Metal-regulatory transcription factor 1 (MTF1), a Cys2-His2 zinc finger protein is the only currently known zinc-responsive transcription factor in mammals, and plays a key role in zinc homeostasis when intracellular zinc levels rise. MTF1 activated by zinc ions is transported to the nucleus where it recognizes promoters containing the metal response element (MRE), and increases the transcription of genes, such as *Mt*, *Znt1* and *Znt2* and represses expression of genes such as *Zip10* (Cousins, Liuzzi, & Lichten, 2006; Giedroc, Chen, & Apuy, 2001; Grzywacz et al., 2015; Kambe et al., 2015) (Fig. 1), thereby increasing extracellular levels of zinc.

3. Mechanisms of zinc action

As a result of ZnT3 activity, zinc is present inside synaptic vesicles of about 50% of glutamatergic neurons (Kay & Toth, 2008; Paoletti, Vergnano, Barbour, & Casado, 2009). In mice in which the *Znt3* gene is disrupted, Timm staining, a method to visualize zinc ions (free hydrated or loosely bound ions) (Represa, Le Gall La, & Ben-Ari, 1989; Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989), is undetectable (Cole, Wenzel, Kafer, Schwartzkroin, & Palmiter, 1999). Timm staining applied to different areas of the rat brain showed the presence of synaptic zinc in the following areas: olfactory bulb, septum, caudate-putamen, amygdaloid complex, neocortex, entorhinal cortex, hippocampal formation: subiculum, stratum radiatum, and oriens of both regio superior (CA1) and regio inferior hippocampi (CA3), the mossy fiber zone, the hilus fascia dentata and stratum moleculare in the dentate gyrus. Importantly, the highest levels of zinc were in the hippocampal mossy fiber boutons (Perez-Clausell & Danscher, 1985).

It was first demonstrated in 1984 that zinc is released into the extracellular space during excitation of hippocampal slices (Assaf & Chung, 1984) and is coreleased with glutamate (Qian & Noebels, 2005). The Monte Carlo simulation, also called probability stimulation, adapted to mimic the microenvironment of mossy fiber-CA3 and Schaffer collateral-CA1 synapses gave an indication of the zinc content in a single vesicle of approximately $1/20^{th}$ that of glutamate (Vergnano et al., 2014). However, there is no consensus on the concentration to which zinc rises in the synaptic cleft after release from presynaptic terminals. It has been estimated to range from 10 nM to over 100 μM (Paoletti et al., 2009). Recently, Vergnano et al. (2014) demonstrated that under resting conditions ambient synaptic zinc levels are extremely low (<10 nM) even at hippocampal mossy fiber synapses, while following short trains of synaptic stimuli, zinc rises to levels relevant for modulation of targets that contain nM zinc binding sites, but not μM zinc binding sites. In other words, this study questioned the physiological relevance of zinc-modulated targets which possess μM zinc binding sites. It should be noted that pathological conditions associated with intense neuronal activity, for example seizures, are likely to affect zinc concentrations in the synaptic cleft, thus leading to different effects in terms of modulating receptor activity. Unfortunately, the comprehensive study by Vergnano et al. (2014) did not asses the dynamics of zinc in the synaptic cleft following status epilepticus.

In spite of a lack of agreement whether it would be of physiological/pathophysiological relevance, exogenously applied zinc modulates a variety of ligand-gated, voltage-gated, and other ion channels including: glutamate NMDAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), GABA (γ-aminobutyric acid) A receptor ($GABA_AR$), voltage-gated calcium channels (VGCCs), potassium voltage-gated channel subfamily Q (KCNQ channels), transient receptor potential melastin channels 7 (TRPMs7), or acid-sensing ion channels (ASICs) (Marger, Schubert, & Bertrand, 2014; Noh et al., 2015; Toth, 2011). Moreover, some of these targets (NMDAR, AMPAR, VGCC, TRPMs7) serve as a route of entry of zinc into the postsynaptic neuron, while others (e.g., subtypes of VGCCs or KCNQ) may be influenced by both extracellular and intracellular zinc (Noh et al., 2015). Noteworthy is the fact that, while the neuromodulatory role of zinc has been known for several decades, research conducted over the last decade has suggested another important function of zinc, in terms of extracellular signaling – a neurotransmitter role, since the ion was proposed to serve as an agonist of the GPR39 receptor (Besser et al., 2009; Kay & Toth, 2008; Yasuda et al., 2007). All of the above-mentioned targets for zinc are summarized in Table 1 and will be discussed because of their importance/potential importance for seizures/epilepsy.

3.1. NMDAR

The NMDAR comprises four subunits surrounding a central cation-selective pore. Three major subtypes of NMDAR subunits have been identified: GluN1, GluN2A–D, and GluN3A–B (Cull-Candy, Brickley, & Farrant, 2001). The most widely expressed NMDAR is composed of two glycine binding GluN1 and two glutamate-binding GluN2 subunits (GluN2B or GluN2A or a mixture of the two). Two mechanisms of action of extracellular zinc on NMDAR have been characterized, viz., a voltage-independent, noncompetitive (allosteric) inhibition, responsible for reducing channel-opening frequency, and voltage-dependent inhibition, representing an open channel block (Christine & Choi, 1990; Paoletti et al., 2009). In contrast to the voltage-dependent inhibition, the voltage-independent inhibition is subunit-specific, with an affinity ranging from low nM for GluN1/GluN2A receptors to about 1 μM for GluN1/GluN2B receptors and ≥10 μM for GluN1/GluN2C and GluN1/GluN2D receptors (Paoletti et al., 2009).

It was long assumed that zinc tonically occupies the nM zinc sites of the NMDAR. However, a recent study using GluN2A-H128S knock-in mice, in which the high-affinity (nM) zinc inhibition of the NMDAR is specifically eliminated, indicated that under resting conditions, zinc levels are too low for tonic inhibition of GluN2A at hippocampal mossy fiber synapses (Vergnano et al., 2014). In this study, calcium-ethylenediaminetetraacetic acid (Ca-EDTA), which is more specific for zinc than ethylenediaminetetraacetic acid (EDTA) (Radford & Lippard, 2013) and another extracellular zinc chelator, tricine, which is a faster chelator than Ca-EDTA (Radford & Lippard, 2013), both potentiated NMDAR-mediated excitatory postsynaptic current (EPSC) recorded in low magnesium condition in wild-type, but not in GluN2A-H128S knock-in mice. However, under physiological magnesium levels, the effects of Ca-EDTA or tricine were indistinguishable between wild-type and GluN2A-H128S knock-in mice and no potentiation of NMDAR-EPSCs was observed, suggesting that tonic zinc levels present in low magnesium conditions are in the sub-μM range, while in normal magnesium conditions, extracellular zinc levels are insufficient for tonic inhibition of NMDAR. Therefore, the GluN2B subunit of the NMDAR is among the zinc targets in which physiological relevance of zinc modulation is questionable. Moreover, a single synaptic event was unable to produce zinc modulation of NMDAR-EPSC at mossy fiber synapses, while a short train of stimuli produced an increase in NMDAR-EPSC amplitude and even greater enhancement was observed in slices from GluN2A-H128S knock-in mice. In ZnT3 knockout mice, the amplitude of NMDAR-EPSCs increased continuously during trains of stimuli and resembled the pattern observed in GluN2A-H128S knock-in animals (Vergnano et al., 2014), supporting the inhibitory action of synaptically released zinc on the NMDAR.

In addition to modulation, NMDAR was found to be permeable to zinc, although the permeability to zinc is much lower than to calcium (Koh & Choi, 1994).

3.2. AMPAR

In addition to modulation of NDMARs, zinc modulates AMPARs, another subtype of ionotropic glutamate receptor. These receptors are composed of four types of subunits: GluA1, GluA2, GluA3, and GluA4 (Collingridge, Olsen, Peters, & Spedding, 2009). At lower concentrations (~30 μM), extracellular zinc potentiates AMPAR-induced currents, but

Table 1
Zinc targets relevant for seizures/epilepsy

	Target	Effects	Reference
Modulation by extracellular/intracellular zinc/permeability for zinc of:			
• ligand gated ion channels	NMDAR	voltage-dependent inhibition by extracellular zinc, voltage-independent subunit-specific inhibition by extracellular zinc: GluN1/GluN2A > GluN1/GluN2B > GluN1/GluN2C / GluN1/GluN2D zinc can enter the cell via NMDAR	Paoletti et al., 2009 Koh and Choi, 1994
	AMPAR	concentration-dependent potentiation/ inhibition by extracellular zinc zinc can enter the cell via AMPAR	Rassendren et al., 1990 Weiss and Sensi, 2000
	$GABA_AR$	subunit-specific inhibition by extracellular zinc: αβ or αβδ subunits are sensitive to zinc inhibition	Smart et al., 2004
• voltage-gated ion channels	VGCCs	subunit-specific inhibition by extracellular zinc zinc can enter the cell via VGCCs activation by intracellular zinc of nickel sensitive T-type calcium channel CaV3.2	Traboulsie et al., 2007; Huc et al., 2009; Sensi et al., 1999; van Loo et al., 2015
	KCNQ (Kv7, M-type)	activation by intracellular zinc	Gao et al., 2017.
	TRPMs	inhibition by extracellular zinc of TRPM1, TRPM2 and TRPM5 zinc can enter the cell via TRPMs7	Bouron et al., 2015 Inoue et al., 2010
• other ion channels	ASICs	subunit-specific inhibition by extracellular zinc: ASIC1a> ASIC2a / ASIC1a/2a / ASIC2a/3	Baron et al., 2001
Zinc as a neurotransmitter:	GPR39	activation by extracellular zinc	Kay and Toth, 2008; Besser et al., 2009; Yasuda et al., 2007

Zinc targets which are relevant for seizures/epilepsy. Extracellular or intracellular zinc was found to modulate a variety of ligand-gated ion channels including those binding glutamate: N-methyl-D-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR) or γ-aminobutyric acid: γ-aminobutyric acid A receptor ($GABA_AR$) as well as voltage-gated ion channels including: potassium voltage-gated channel subfamily Q (KCNQ channels), voltage-gated calcium channels (VGCCs), or transient receptor potential melastin channels 7 (TRPMs7) or acid-sensing ion channels (ASICs). Moreover, some of them (NDMAR, AMPAR, VGCC, TRPMs7) may serve as a route of entry of zinc into postsynaptic neuron. Furthermore, zinc was proposed to act as an agonist of the G-protein coupled receptor 39 (GPR39).

at higher (mM) concentrations, it inhibits them (Rassendren, Lory, Pin, & Nargeot, 1990). Zinc can also enter the postsynaptic neurons via AMPARs since GluA2-lacking-(calcium permeable)-AMPARs are also permeable for zinc (Inoue, O'Bryant, & Xiong, 2015; Kwak & Weiss, 2006; Weiss & Sensi, 2000). Of the several routes for zinc entry into postsynaptic neurons including VGCCs, NMDAR, or calcium–zinc permeable AMPA/kainate receptors, the latter present the highest permeability to exogenously applied zinc. Furthermore, zinc entry via calcium–zinc permeable AMPA/kainate receptors triggers reactive oxygen species generation and mitochondrial depolarization (Sensi, Yin, Carriedo, Rao, & Weiss, 1999).

3.3. $GABA_AR$

$GABA_ARs$ are ligand-gated channels permeable to chloride ions and 19 genes encoding $GABA_AR$ subunits (α1–α6, β1–β3, γ1–γ3, δ, ε, π, τ, ρ1–ρ3) have been identified. Depending on the postsynaptic or extrasynaptic localization of the $GABA_AR$, distinct forms of inhibitory neurotransmission ensue upon stimulation, i.e., fast, high-amplitude phasic currents upon quantal presynaptic GABA release or low-amplitude but persistent (tonic) currents activated by ambient GABA (Farrant & Nusser, 2005). Postsynaptic receptors contain mainly the α1, α2, and α3 subunits, β subunits, and the γ2 subunit, while extrasynaptic receptors contain the α4, α5, and α6 subunits, often along with the δ subunit, instead of the γ2 (Fritschy & Panzanelli, 2014). $GABA_ARs$ composed of αβ or αβδ subunits are sensitive to block by extracellular zinc, whereas the αβγ isoforms are much less affected. It has been suggested that extrasynaptic $GABA_ARs$ lacking γ subunits are a target for zinc, whereas, postsynaptic receptors are targets only when zinc concentrations rise to several hundred μM (Smart, Hosie, & Miller, 2004).

Molecular modeling approaches led to identification of three discrete sites that mediate zinc inhibition of $GABA_ARS$, one located within the ion channel, and the other two on the external amino (N)-terminal face of the receptor at the interfaces between α and β subunits. Low zinc sensitivity of $GABA_ARs$ containing the γ2 subunit results from disruption to two of the three sites after receptor subunit co-assembly (Hosie, Dunne, Harvey, & Smart, 2003).

3.4. GPR39

GPR39, a GPCR cloned in 1997 (McKee et al., 1997), is another important target for extracellular zinc. The application of zinc to hippocampal slices triggered intracellular calcium release mediated by a $G\alpha_q$ and IP3 pathway and induced phosphorylation of extracellular signal regulated kinase (ERK) and calcium-calmodulin-dependent protein kinase (CaMK). Moreover, the calcium response triggered by electrical stimulation of mossy fibers was inhibited in the presence of the extracellular zinc chelator Ca-EDTA and in ZnT3 knockout mice. In addition, knockdown of the expression of GPR39 attenuated zinc-triggered metabotropic responses in a neuronal cell line (Besser et al., 2009). Thus, it has been suggested that GPR39 mediates zinc-induced metabotropic signaling.

3.5. VGCCs

VGCCs are the primary mediators of calcium entry into neurons induced by membrane depolarization (Simms & Zamponi, 2014) and the entry point also for other ions including zinc. Zinc permeability depends on the specific subunit composition of the VGCC (Kerchner, Canzoniero, Yu, Ling, & Choi, 2000; Inoue et al., 2015). Extracellular zinc not only enters cells via VGCCs, but also blocks channel activity and calcium influx, especially of T-type channels (Busselberg, Michael, Evans, Carpenter, & Haas, 1992). Zinc inhibits the T-type calcium channel $Ca_V3.2$ with an IC_{50} in the sub μM range, and with lower IC_{50}s for the $Ca_V3.1$ and $Ca_V3.3$ isotypes. In addition, zinc can slow the deactivation kinetics of the $Ca_V3.3$/T-current, which in turn enhances calcium entry through $Ca_V3.3$ channels (Huc et al., 2009; Traboulsie et al., 2007). Moreover, intracellular zinc activates $Ca_V3.2$ (van Loo et al., 2015).

3.6. KCNQ

Members of the KCNQ channels, also referred to as M-type or Kv7 potassium channels, are activated by changes in transmembrane voltage, and are sensitive to plasma membrane phosphatidylinositol 4,5-

bisphosphate (PIP_2). The activity of these channels is inhibited through stimulation of muscarinic acetylcholine receptors, which decreases PIP_2. Five KCNQ channel subtypes, viz., KCNQ1–KCNQ5 have been identified with KCNQ2–KCNQ4 expressed in the CNS. The most abundant channel within the CNS is presumably the heteromeric KCNQ2/3 (Delmas & Brown, 2005; Robbins, 2001). KCNQ channels are activated by intracellular zinc, which reduces their dependence on the natural activator, PIP_2 (Gao et al., 2017).

3.7. TRPMs7

TRPMs7 are novel magnesium- and calcium- permeable cation channels. Their activation is involved in magnesium homeostasis as well as in calcium-mediated neuronal injury (Schmitz et al., 2003). Zinc inhibits several subtypes of TRPMs, and permeates TRPM7s more efficiently than magnesium or calcium (Inoue, Branigan, & Xiong, 2010).

3.8. ASICs

ASICs are proton-gated channels widely expressed in neuronal tissues, including the hippocampus, the amygdala, the cerebral cortex, the olfactory bulb and the cerebellum (Cristofori-Armstrong & Rash, 2017). They are voltage insensitive sodium channels with the ASIC1a channel also exhibiting some permeability to calcium. The six subunits of the ASICs are encoded by four genes, viz., ASIC1a and 2a, ASIC2a and 2b, ASIC3, and ASIC4, and the activity of these channels can be modulated by divalent metal ions such as zinc. At nM concentrations, extracellular zinc inhibits ASIC1a channels, while at μM concentrations, zinc can potentiate other ASIC channels such as the homomeric ASIC2a and heteromeric ASIC1a/2a and ASIC2a/3 channels (Baron, Schaefer, Lingueglia, Champigny, & Lazdunski, 2001).

4. Zinc levels following seizures

Several methods have been used to estimate zinc levels in preclinical models of seizures/epilepsy including atomic absorption spectrometry (AAS), which allows quantification of one metal at a time, inductively coupled plasma mass spectrometry (ICP-MS) and total reflection X-ray fluorescence (TXRF) (Cerchiaro, Manieri, & Bertuchi, 2013), which enable measurements of the total content of multiple metals at once, and imaging techniques including laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), synchrotron X-ray fluorescence (SXRF) microscopy, X-ray fluorescence microscopy, which enable measurements in a spatially resolved manner, thus assessing distribution of metals in cells and tissues (Qin, Caruso, Lai, Matusch, & Becker, 2011). Changes in zinc in animal seizure/epilepsy models are summarized in Table 2 and discussed below.

4.1. Total zinc levels

Total zinc levels in plasma were measured using AAS in Wistar Audiogenic Rats (Garcia-Cairasco, Umeoka, & Cortes de Oliveira, 2017) and Wistar non-epileptic rats, both in the basal state and after the induction of seizures by audiogenic or electroshock stimuli, respectively (Doretto, Simoes, Paiva, & Osorio-Neto, 2002). The audiogenic rats had lower plasma zinc levels than the non-epileptic rats both at baseline and following seizures. Although no differences were observed in zinc levels in whole brain in mice 24 h after administration of a dose of pentylenetetrazole that induced seizures compared to mice who received vehicle, decreased zinc concentrations were observed in the striatum and CA1/CA2 regions of the hippocampus (Takeda, Itoh, Hirate, & Oku, 2006a). Similarly, 24 h after the last of 3 injections of kainate to mice who exhibited status epilepticus, zinc concentrations in the hippocampus, amygdala and cerebral cortex decreased as measured by AAS (Takeda, Hirate, Tamano, & Oku, 2003b). Using X-ray fluorescence microscopy, decreased zinc levels were demonstrated in the CA3 region of the hippocampus and in the dentate gyrus of rats subjected to pilocarpine-induced status epilepticus (Chwiej et al., 2008). However, electrical kindling led to increased zinc levels in the hilus of the dentate gyrus of rats exhibiting tonic seizures, and zinc levels in this region were positively correlated with the cumulative intensity and duration of tonic seizures and with the total duration of clonic seizures (Chwiej et al., 2014). This finding suggests that an increased level of zinc is not a consequence of electrical stimulation, but rather an effect of subsequent seizure activity. Because this study did not include an unkindled group, this conclusion has to be taken with caution.

4.2. Labile zinc levels

The presence of labile zinc in the brain was first demonstrated in 1955 by Maske using intraperitoneal dithizone injection (Maske, 1955). This development was followed by the use of autometallography, a histological method based on the silver-amplified detection of sulfide nanocrystals formed with metal ions, originally proposed by Timm in 1958 and further optimized by Danscher. The first fluorescent stain for zinc, 8-hydoxyquinoline, was developed later, and currently, fluorescent sensors from the Zinpyr family represent one of the largest families of fluorescein-based sensors used widely to study the role of labile zinc (Carter, Young, & Palmer, 2014).

4.3. Timm staining and epilepsy

In 1955, Maske reported that injection of dithizone to rats resulted in the staining of the mossy fiber region of the hippocampus (Maske, 1955). Further, in the hippocampus of patients with temporal lobe epilepsy, the mossy fibers often branch out of the dentate hilus and abnormally innervate the dentate inner molecular layer (Schmeiser et al., 2017), a phenomenon termed mossy fiber sprouting. Hippocampal mossy fiber sprouting is one of the most prominent features of temporal lobe epilepsy, which is present in humans and in animal models (Sierra, Grohn, & Pitkanen, 2015).

Mossy fiber sprouting has been demonstrated by Timm staining in many animal models of epilepsy including: the intrahippocampal administration of kainic acid (Davenport, Brown, & Babb, 1990) and pilocarpine (Mello et al., 1993); activation of the synchronous perforant pathway and kindling of limbic pathways (Sutula, He, Cavazos, & Scott, 1988), amygdala or entorhinal cortex (Represa et al., 1989); and kindling induced by the repeated administration of subconvulsant doses of pentylenetetrazole (Golarai, Cavazos, & Sutula, 1992). Mossy fiber sprouting has also been demonstrated in seizure models including: flurothyl-induced recurrent seizures (Tian, Ni, & Sun, 2015); tetanus toxin seizures (Mitchell, Gatherer, & Sundstrom, 1996); and the epileptic Scn2a(Q54) mouse model (Anderson et al., 2014). It has also been shown to occur in epileptic human hippocampus (Babb, Kupfer, Pretorius, Crandall, & Levesque, 1991; Mathern, Babb, Micevych, Blanco, & Pretorius, 1997; Sutula et al., 1989).

In the electrical kindling model induced by perforant path stimulation, the mean Timm score for rats who experienced five afterdischarges was higher than in the control group and correlated significantly with the increasing number of afterdischarges and class V seizures evoked by perforant path, amygdala, or olfactory bulb stimulation. Mossy fiber synaptic terminals developed in the supragranular region of the dentate gyrus 4 days after initiation of kindling in a time course compatible with axon sprouting. The induced alterations in the terminal projections of the mossy fiber pathway progressed with the evolution of behavioral kindled seizures, became permanent in parallel with the development of long-lasting susceptibility to evoked seizures, and were observed as long as 8 months after the last evoked kindled seizure (Cavazos, Golarai, & Sutula, 1991).

One hour of pilocarpine-induced status epilepticus was sufficient to develop chronic epilepsy in mice, and was associated with robust hippocampal mossy fiber sprouting (Chen, Feng, Mao, Ye, & Zeng, 2013).

Table 2
Epilepsy/seizures and zinc levels.

Species/stimulus	Method	Zinc pool measured	Outcome	Reference
Wistar Audiogenic Rat	AAS	Total	Decreased zinc in plasma	Doretto et al. (2002)
Mice/pentylenetetrazole (40 mg/kg)	AAS	Total	Decreased zinc in CA1/CA2 regions of the hippocampus and striatum	Takeda, Itoh, Hirate, and Oku (2006a)
Mice/kainate (12 mg/kg 3×)	AAS	Total	Decreased zinc in the hippocampus, amygdala and cerebral cortex	Takeda, Hirate, Tamano, and Oku (2003b)
Rats/pilocarpine-status epilepticus	X-ray fluorescence	Total	Decreased zinc in CA3 region of the hippocampus and dentate gyrus	Chwiej et al. (2008)
Rats/electrical kindling	X-ray fluorescence	Total	Increased zinc in hilus of dentate gyrus	Chwiej et al. (2014)
Rats/kainic acid	TSQ staining	Free zinc	Reduced TSQ fluorescence in the neuropil/increased TSQ fluorescence in pericaria and proximal dendrites (blocked by administration of diazepam prior to kainic acid)	Frederickson et al. (1989)
Rats/pilocarpine-status epilepticus	TSQ staining	Free zinc	Reduced TSQ fluorescence in the neuropil/increased TSQ fluorescence of neuronal somata	Suh et al. (2001)
Mice/kainate (40 mg/kg)	TFL-Zn staining	Free zinc	Increased TFL-staining in the CA1 region of the hippocampus in ZnT3 KO and WT mice following kainate injection	Lee et al. (2000)
Rats/pilocarpine-status epilepticus	TFL-Zn staining	Free zinc	Increased TFL-staining in the CA1 region 1 day after status epilepticus, declined 2–4 days after the status	van Loo et al. (2015)

AAS - atomic absorption spectrometry; TFL-Zn - N-(6-methoxy-8-quinolyl)-p-carboxybenzoylsulfonamide; TSQ - p-toluenesulfonamido-quinoline; KO - knockout; WT - wild type.

In the penicillin-induced recurrent developmental seizures model, sprouting of mossy fibers paralleled decreased levels of ZnT1 in the hippocampus (Ni, Jiang, Tao, Cen, & Wu, 2009). ZnT1 expression is induced in the presence of increased cytoplasmic zinc, thereby preventing accumulation of toxic zinc levels (Langmade, Ravindra, Daniels, & Andrews, 2000). Thus, decreased ZnT1 mRNA levels may suggest either decreased intracellular zinc in this model or conversely, increased intracellular zinc, possibly as a result of decreased function of ZnT1.

In contrast to increased mossy fiber sprouting and Timm staining, a selective loss of Timm staining in the mossy fiber pathway was observed following stimulation of the perforant path for 24 h, which evokes hippocampal granule cell spikes and epileptiform discharges throughout the stimulation period (Sloviter, 1985). Similarly, attenuated Timm staining was observed in the hippocampal mossy fiber, stratum radiatum and stratum oriens 24 h after treatment with a dose of pentylenetetrazole that led to tonic and clonic seizures in mice (Takeda, Itoh, Hirate, & Oku, 2006a). Timm staining was also attenuated in the mossy fiber, stratum radiatum and stratum oriens of mice who displayed status epilepticus at 24 h after the last injection of kainate (Takeda, Hirate, Tamano, & Oku, 2003b).

Interestingly, in the intrahippocampal kainate model of mesial temporal lobe epilepsy, Timm staining in the hippocampus increased during the first 2 weeks post-kainate administration, followed by a progressive decline with a complete loss of staining at 56 days post-kainate administration. This effect was associated with increased VGluT1-, Synapsin-1-, and ZnT3-immunoreactivity in the sprouted mossy fiber boutons (Mitsuya, Nitta, & Suzuki, 2009). VGluT1 is one of the key transporters of glutamate into synaptic vesicles, and its expression is thought to correlate with the amount of glutamate loaded into and released from vesicles, thereby regulating the efficacy of glutamatergic neurotransmission (Wojcik et al., 2004). Synapsin-1 colocalizes with VGluT1 in the hippocampus (Bogen et al., 2006). Because zinc is coreleased with glutamate from synaptic vesicles (Paoletti et al., 2009), these results may suggest increased release of zinc from the vesicles. Thus, it has been argued that the progressive reduction and disappearance of Timm staining at 14 days after kainate administration may be a result of reduced vesicular zinc in the mossy fiber terminals, not a result of the loss of mossy fibers (Mitsuya et al., 2009). Two months after the induction of status epilepticus in rats by pilocarpine administration, the intensity of neo-Timm staining was lower in the nucleus reuniens and the rhomboid nucleus (Hamani, Paulo, & Mello, 2005), parts of midline thalamus, a region which is also involved in seizures (Bertram, Mangan, Zhang, Scott, & Williamson, 2001).

The distribution of the mossy fiber synaptic terminals was examined using the Timm method in surgically excised hippocampus and dentate gyrus from patients who underwent lobectomy of the anterior part of the temporal lobe for refractory partial complex epilepsy. The dentate gyrus of epileptic patients demonstrated intense Timm granules and abundant mossy fiber synaptic terminals in the supragranular region and the inner molecular layer (Sutula et al., 1989). It has been proposed that the severity of mossy fiber sprouting correlates with the severity of temporal lobe epilepsy and with the frequency of spontaneous recurrent seizures (Chang & Lowenstein, 2003). However, in electrically-induced (via stimulation of the lateral nucleus of the amygdala) self-sustained status epilepticus model of temporal lobe epilepsy, the density of mossy fiber sprouting did not correlate with the severity or duration of status epilepticus or delay from status epilepticus to occurrence of first or second spontaneous seizure. Mossy fiber sprouting was present in all animals with spontaneous seizures and in two animals in which spontaneous seizures did not develop (Nissinen, Lukasiuk, & Pitkanen, 2001). Thus, despite substantial research conducted over many years using Timm staining and mossy fiber sprouting, data on the association between fiber sprouting, seizures/epilepsy, and zinc homeostasis regulating proteins and/or elements of zinc signaling pathways are rare.

To clarify the role of synaptic zinc in seizures, the actions of diethyldithiocarbamate (DEDTC), a cell membrane permeable metal chelator, which reversibly blocks Timm staining (Danscher, Haug, & Fredens, 1973) were investigated. Injections of DEDTC decreased the duration of both behavioral seizures and electrical afterdischarges, and decreased the EEG spike frequency, without changing the progression of behavioral seizure severity in the amygdala rapid kindling model (Foresti, Arisi, Fernandes, Tilelli, & Garcia-Cairasco, 2008), which could suggest that chelatable zinc plays a facilitatory role in epileptogenesis. On the other hand, it has been reported that DEDTC exerts a proconvulsant action in the model of electrical stimulation of the perforant pathway (Mitchell & Barnes, 1993) and kainic acid model (Dominguez et al., 2006), which could suggest the opposite. DEDTC is the main metabolite of disulfiram, a drug indicated in alcoholism, which has been reported to induce *de novo* seizures in the absence of ethanol (McConchie, Panitz, Sauber, & Shapiro, 1983). Noteworthy is that the intraperitoneal injection of DEDTC alone induced convulsive behavior (Blasco-Ibanez et al., 2004), which could suggest that in the normal hippocampus, synaptic zinc may play a role in avoiding over excitation. Moreover, metal chelating compounds such as DEDTC may act as ionophores, which bind a metal, transport it across a lipid bilayer and release it, causing an intracellular rise of metal ions (Ding & Lind, 2009).

Thus, a proconvulsant action of DEDTC may be associated with increased levels of intracellular zinc. Furthermore, DEDTC is known to form complexes with other metals (Viola-Rhenals et al., 2018), and thus, this nonselective action might have influenced the outcome of the above experiments.

4.4. Synaptic and intracellular zinc in seizures/epilepsy

p-toluenesulfonamido-quinoline (TSQ) staining for zinc in axonal boutons was used to study the effects of kainic acid-induced seizures upon zinc in the boutons of hippocampal mossy fibers. Compared to untreated rats, rats given kainic acid who exhibited sustained seizures displayed a marked loss of zinc fluorescence in the mossy fiber regions. The reduced fluorescence was detectable within 3 h of kainic acid administration, was most pronounced at 12–24 h, and was still detected 48 h later. These findings suggested that zinc is released rapidly from mossy fiber boutons during seizures (Frederickson, Hernandez, Goik, Morton, & McGinty, 1988).

The initial study by Sloviter using Timm staining following perforant path stimulation for 24 h demonstrated a selective loss of Timm stain in the mossy fiber pathway (Sloviter, 1985). Further, studies combining Timm staining and in vivo microdialysis in mice exhibiting status epilepticus 24 h after the last of 3 injections of kainate, indicated decreased total zinc concentrations in the hippocampus, amygdala and cerebral cortex, attenuated Timm staining in mossy fibers, stratum radiatum and stratum oriens, and increased extracellular zinc concentrations in the hippocampal fluid (Takeda, Hirate, Tamano, & Oku, 2003b), supporting the release of zinc from the synaptic vesicles into the synaptic cleft.

Study by Frederickson, Hernandez, and McGinty (1989) using TSQ also demonstrated that seizures not only caused a loss of zinc staining from presynaptic boutons in many limbic and cerebrocortical regions, but simultaneously led to the development of intense fluorescence for zinc in postsynaptic neurons. In contrast to samples from untreated animals, in which fluorescence was vivid in discrete regions of the hippocampus and amygdala where the staining reflected dense plexuses of zinc-containing boutons, after 12 h of seizures induced by kainate, changes in the staining pattern were observed. Fluorescence in the neuropil was reduced while scattered individual neurons developed intense fluorescence throughout their pericaria and proximal dendrites (Frederickson et al., 1989). Also, in the pilocarpine-status epilepticus model, marked reduction in TSQ fluorescence in the neuropil paralleled the appearance of neuronal somata fluorescent for zinc (Suh, Thompson, & Frederickson, 2001). Thus, it has been proposed that status epilepticus causes translocation of zinc from synaptic boutons into postsynaptic neurons, leading to increased intracellular zinc.

Several sites of origin could contribute to the increased intracellular zinc. Following seizures, a combined increased zinc release from synaptic vesicles into the extracellular space and zinc mobilization from intracellularly located MTs could be involved. Because MTIII is localized not only intracellularly, but also extracellularly (Vasak & Meloni, 2017), zinc released from extracellularly located MTs could increase extracellular zinc, which could then enter the postsynaptic neuron via NMDAR, AMPAR, VGCC or TRPM7.

Indeed, staining of hippocampal sections with a zinc-specific fluorescent dye, N-(6-methoxy-8-quinolyl)-p-carboxybenzoylsulfonamide (TFL-Zn) revealed that zinc accumulated in CA1 and CA3 neurons after kainate induced seizures both in wild-type mice and in *Znt3*-null mice, suggesting that increased intracellular zinc observed in TFL-Zn staining originated from sources other than synaptic vesicles. Further, injection of Ca-EDTA into the cerebral ventricle almost completely blocked zinc accumulation in *Znt3*-null mice, suggesting that increases in extracellular zinc may be a critical event for zinc accumulation (Lee, Cole, Palmiter, & Koh, 2000).

However, this latter observation does not exclude the possibility that intracellularly mobilized zinc could be pumped out of cells by zinc outward transporters, leading to increased extracellular zinc levels, considering that zinc mobilization from MTs is involved in intracellular zinc increases (Sensi et al., 2011). After kainate-induced seizures, cytoplasmic zinc accumulation and neuronal death in the hippocampal CA1 region and the thalamus were substantially lower in *Mt3*-null mice than in wild-type mice, suggesting that MTIII can function as a source of increased intracellular zinc levels (Lee, Kim, Palmiter, & Koh, 2003).

It should be noted that studies have provided an estimation of intracellular zinc concentrations associated with extracellular zinc exposure. In a series of experiments, which exclude the possibility that changes in cell permeable mag-fura-5 AM fluorescence results from changes in magnesium concentration, it was shown that the application of KCl and 300 μM zinc to cortical neurons caused an increase in somatic intracellular zinc to approximately 35–45 nM, which was terminated by the application of N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane permeable zinc chelator. The response to KCl could be blocked completely by concurrent application of the nonselective VGCCs blockers gadolinium and verapamil, suggesting that VGCCs serve as a route of entry of zinc into neuronal somata.

Moreover, application of NMDA in the presence of 300 μM extracellular zinc produced a quick increase in neuronal intracellular zinc in an NMDA concentration-dependent manner that was sensitive to the competitive NMDAR antagonist D-APV. A maximal intracellular zinc concentration of approximately 32 nM was induced by 300 μM NMDA. Furthermore, application of kainate in the presence of 300 μM extracellular zinc also produced a quick increase in neuronal intracellular zinc in a concentration-dependent manner, which was sensitive to NBQX, an antagonist of the AMPAR. To sum up, an estimate of approximately 30 nM of neuronal somata intracellular zinc was provided following exposure to extracellular zinc via the following routes of entry: VGCC, NMDAR or AMPAR (Sensi et al., 1997).

Moreover, widespread neuronal death was observed over 24 h following 5–10 min exposure of cortical cell cultures to 300 μM zinc in the presence of kainate or elevated extracellular potassium. Exposure to zinc under depolarizing conditions caused an increase in intracellular zinc to several hundred nM as measured using Newport Green diacetate, lasting 20–40 min, despite termination of zinc exposure. The concentration of extracellular zinc between 30 μM and 1 mm was suggested to determine both the elevated level of intracellular zinc and zinc-dependent widespread neuronal death (Canzoniero, Turetsky, & Choi, 1999). However, recent studies by van Loo et al. (2015) and by Lee et al. (2000), which utilized TFL-Zn staining following pilocarpine-induced status epilepticus or kainate-induced seizures, respectively, do not provide the estimation of intracellular zinc after the status, but the count of TFL-Zn positive neurons.

Importantly, the above-mentioned studies estimating extracellular/intracellular zinc levels are based on fluorescence, and thus, face the difficulty of relating fluorescence intensity to the analyte concentration. Indeed, most fluorescent probes (such as TSQ, TFL-Zn, Zinquin, Newport Green, ZnAF-1, 2) are suited for displaying the presence of zinc, not for quantifying it (Frederickson, 2003; Thompson et al., 2002). Nevertheless, these tools are valuable as they represent a semi-quantitative approach, while the ratiometric fluorescent probes being developed (Bourassa et al., 2018; Iniya, Jeyanthi, Krishnaveni, Mahesh, & Chellappa, 2014) may help to resolve difficulties in measuring zinc concentrations in the future. Recently, chromis-1, a membrane-permeant, zinc selective ratiometric fluorescent probe, optimized for two-photon microscopy, was used to estimate changes in labile zinc levels during maturation of oligodendrocytes (Bourassa et al., 2018).

To sum up, although up-to-date studies neither provided direct extracellular nor intracellular zinc concentrations associated with seizures/epilepsy (as is in the case of zinc concentrations under physiological conditions), the action of zinc on numerous targets was shown to be associated with seizures/epilepsy (Fig. 2) and will be discussed in detail in subsequent sections of this review.

5. Possible mechanisms mediating a role for zinc in seizures/epilepsy

5.1. Zinc, NMDARs and seizures/epilepsy

Genetic studies have indicated that zinc actions at NMDARs are altered in epilepsy. Mutations in genes encoding NMDAR subunits have been identified in individuals with epilepsy (Endele et al., 2010). A *de novo* missense mutation (L812M) in a gene encoding NMDAR GluN2A (*GRIN2A*), identified in a child with intractable infantile-onset treatment-resistant epilepsy, epileptic encephalopathy, and profound developmental delay, enhances agonist potency, decreases sensitivity to negative modulators including zinc, prolongs the synaptic response time course, and increases single-channel open probability (Yuan et al., 2014). Based on clinical reports, 9 GluN2A missense mutations associated with various epilepsy-cognitive phenotypes were generated, and the resulting GluN2A-containing receptors were studied. Two of these mutations resulted in marked alterations in zinc sensitivity, one leading to an increase (GluN2A-R370W) and the other to a decrease (GluN2AP79R) of zinc sensitivity (Serraz, Grand, & Paoletti, 2016).

A link between decreased availability of zinc, NMDAR function and seizures, is supported by studies using metal ion chelators including citrate or EDTA, which attenuated the inhibitory action of zinc on NMDAR-mediated transmitter release from neurons and membrane currents in the oocytes (Westergaard, Banke, Wahl, Sonnewald, & Schousboe, 1995). Citrate is released from astrocytes, not stored in neurons and serves as an extracellular chelator (Westergaard et al., 1994). Intrathecal injection of fluorocitrate in mice resulted in seizures after an average latency of 15 s, while intracerebroventricular injection produced seizures after 36.5 min (Hornfeldt & Larson, 1990). Thus, it was proposed that extracellular zinc chelation by citrate leading to potentiation of NMDAR function may be a mechanism underlying the convulsive effects of citrate (Bhutia, Kopel, Lawrence, Neugebauer, & Ganapathy, 2017). However, the signals of zinc-Newport Green dichlorofluorescein, a membrane permeant probe, were reduced by the addition of citrate or isocitrate, suggesting that endogenous citrate may act as an intracellular zinc chelator (Sul et al., 2016).

Injection of DEDTC, which blocks Timm staining (Danscher et al., 1973), during injection of a nonconvulsive dose of kainate, neither of which led to pathological alterations in the hippocampus, produced damage to the hippocampal CA3 region that was dependent on NMDARs, whereas damage to CA1 was dependent on AMPAR and NMDARs (Dominguez et al., 2006). These results support a protective role of synaptic zinc against hippocampal damage. However, because DEDTC may act as an ionophore causing intracellular rise of zinc ions (Ding & Lind, 2009), the observed damage may be associated with increased intracellular zinc levels. Thus, although DEDTC and citrate influence both extracellular and intracellular zinc levels, and support a link between zinc, NMDAR and seizure occurrence, studies do not indicate which pool of zinc is of relevance.

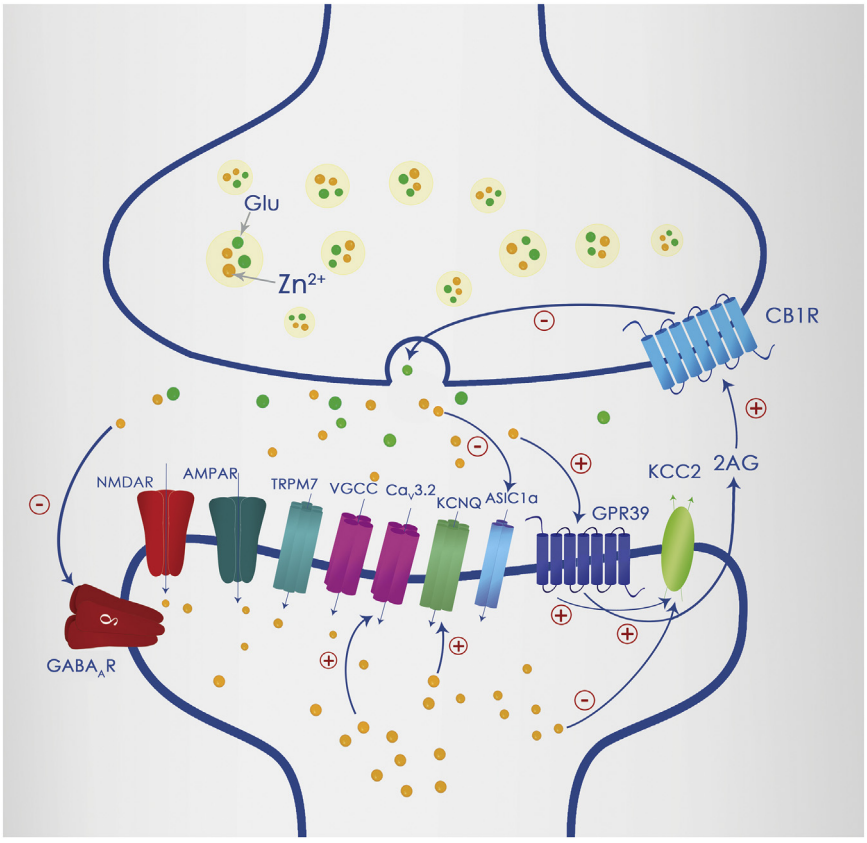

Fig. 2. The possible pro- and anti-seizure/epileptogenic effects of extracellular and intracellular zinc. Extracellular zinc activates the GPR39 receptor leading to enhanced activity of KCC2, which is crucial for GABA-ergic inhibition. Moreover, activation of GPR39 inhibits glutamate release by promoting 2-AG synthesis and its action on presynaptic CB1R. Further, extracellular zinc seems to be neuroprotective due to the inhibited activity of the ASIC1a channel. On the other hand, extracellular zinc inhibits extrasynaptic δ-$GABA_A$Rs and thereby prevents neurosteroid activation of tonic inhibition and neurosteroid-dependent protection from seizures. Extracellular zinc can enter the postsynaptic neuron via NMDARs, GluA2-lacking-(calcium permeable)-AMPARs, VGCCs and TRPMs7. Intracellular zinc may produce detrimental effects. In contrast to the effects of extracellular zinc on KCC2, increase in intracellular zinc inhibits KCC2, thus may lead to seizures/epilepsy. Moreover, intracellular zinc may induce seizures/epilepsy via activation of T-type calcium channels Cav3.2. However, intracellular zinc may also produce positive effects via activation of KCNQ channels, which may help to survive cell excitotoxicity associated with seizures. 2AG, 2-arachidonoylglycerol; AMPAR, α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; ASIC1a, subtype of acid-sensing ion channels; Cav3.2, subtype of T-type calcium channels; CB1R, presynaptic cannabinoid type 1 receptors; Glu, glutamate; $GABA_A$R, γ-aminobutyric acid type A receptor; GPR39, G protein-coupled receptor 39; KCC2, potassium-chloride cotransporter 2; KCNQ (M-type, Kv7), potassium voltage-gated channel subfamily Q; NMDAR, N-methyl-D-aspartate receptor; VGCC, voltage-gated calcium channels; TRPMs7, transient receptor potential melastin channels 7; Zn^{2+}, zinc ions; +, activation; -, inhibition.

5.2. Zinc, $GABA_AR$ and seizures/epilepsy

Substantial data support a relationship between zinc action at $GABA_AR$ and seizures/epilepsy. Changes in $GABA_AR$ sensitivity to extracellular zinc were observed in models of epilepsy with most of the data suggesting that zinc-mediated increased $GABA_AR$ inhibition is associated with seizures.

Repetitive mossy fiber stimulation to release synaptic zinc did not affect $GABA_AR$-mediated currents evoked by photorelease of GABA, suggesting that zinc released from the recurrent mossy fiber pathway did not reach a concentration at postsynaptic $GABA_AR$ sufficient to inhibit agonist-evoked activation (Molnar & Nadler, 2001). However, in epileptic hippocampus, increased $GABA_AR$ sensitivity to zinc was observed (Cohen, Lin, Quirk, & Coulter, 2003; Coulter, 2000). Zinc enhanced the rundown of $GABA_AR$ currents of $GABA_ARs$ microtransplanted to *Xenopus* oocytes from surgically resected brain tissues of patients afflicted with drug-resistant mesial temporal lobe epilepsy. Zinc-induced increase of $GABA_AR$ current rundown was concentration-dependent and reached a plateau at a concentration slightly above 40 μM. Moreover, $GABA_ARs$ microtransplanted from the cerebral cortex of adult rats subjected to pilocarpine-induced post-status epilepticus model of temporal lobe epilepsy, showed greater rundown than control tissue, which was enhanced by zinc at a concentration of 40 μM (Palma et al., 2007). Furthermore, in the pilocarpine-induced post-status epilepticus model, regionally distinct modifications in hippocampal GABAergic function were observed – the maximal sensitivity of GABA-evoked currents to blockade by zinc increased in the dentate gyrus of epileptic animals but not in CA1 (Gibbs III, Shumate, & Coulter, 1997).

Changes in $GABA_AR$ sensitivity to zinc were also observed in the amygdala kindling model. Although reduced inhibition has been generally associated with epilepsy, persistent enhancement of $GABA_AR$-mediated inhibition and a doubling of the number of activated functional postsynaptic $GABA_ARs$ in granule cells were found in the amygdala kindling model (Otis, De, & Mody, 1994). In brain slices obtained from amygdala kindled rats, the excitatory drive onto inhibitory interneurons was increased and was paralleled by a reduction in the presynaptic autoinhibition of GABA release. This increased inhibition was sensitive to zinc. The preserved benzodiazepine sensitivity after kindling excluded the possibility that kindling produced loss of γ subunits, which are crucial for benzodiazepine sensitivity. Therefore, it was suggested that molecular reorganization of $GABA_AR$ other than loss of γ subunits may account for such an effect (Buhl, Otis, & Mody, 1996).

Increase in zinc inhibition of $GABA_AR$ was also observed in a model induced by inhibition of brain cholesterol synthesis at a young age, which induces a permanent absence-like epileptic condition (Smith & Bierkamper, 1990). Increase of zinc inhibition of the $GABA_AR$ responses of nucleus reticularis of thalamus neurons of rats injected with the cholesterol synthesis inhibitor were observed together with a loss of ability of benzodiazepines to enhance such responses (Wu et al., 2004), suggesting that in this model, decreased γ2 subunit expression may be present.

In contrast, a decrease in sensitivity of GABA currents to zinc was noted in the cortex from pediatric epilepsy surgery patients with type II cortical dysplasia (Andre et al., 2010), a malformation of cortical development that is a prevalent cause of intractable epilepsy in children (Marin-Valencia, Guerrini, & Gleeson, 2014). Also, $GABA_AR$ currents from rats undergoing status epilepticus induced by the administration of lithium followed by pilocarpine were less sensitive to zinc (Kapur & Macdonald, 1997).

Endogenous neurosteroids, such as allopregnanolone, are positive allosteric modulators of synaptic and extrasynaptic $GABA_ARs$, and, similar to zinc, have preferential affinity for extrasynaptic δ subunit containing receptors (Carver & Reddy, 2013). Evidence from both animal models and clinical cases support the involvement of neurosteroids in epileptogenesis, and allopregnanolone has been shown to counteract seizures (Biagini et al., 2013). Zinc displayed a concentration-dependent, reversible, noncompetitive blockade of allopregnanolone-sensitive tonic current in dentate gyrus granule cells with an IC50 of 16 μM, while zinc inhibition of tonic currents was lacking in the dentate gyrus granule cells from δ subunit knockout mice. Furthermore, intrahippocampal infusion of zinc elicited rapid epileptiform activity and significantly blocked the antiseizure activity of allopregnanolone in the hippocampal kindling model of epilepsy (Carver, Chuang, & Reddy, 2016). Thus, this study provided a novel mechanism whereby extracellular zinc selectively inhibits extrasynaptic δ subunit-containing $GABA_ARs$ and thereby prevents neurosteroid activation of tonic inhibition and neurosteroid-dependent protection from seizures.

In addition, zinc may affect $GABA_AR$ function via modulation of the endogenous kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH mediates the phosphorylation of $GABA_AR$, preventing rundown of GABAergic responses on acutely dissociated pyramidal neurons from the rat cortex (Laschet et al., 2004). Endogenous phosphorylation has been shown to be deficient in human epileptogenic cortex obtained during surgery of patients with partial seizures (Laschet et al., 2007). Membrane-bound phosphatases, whose inhibition favors the phosphorylated state of the receptor and contributes to the maintenance of its function, were identified. The effects of zinc on these phosphatases were described and were variable depending on the phosphatases, i.e., zinc activated the catalytic site of protein phosphatases 1 class 1, considered as a metalloenzyme, whereas zinc inhibited the protein phosphatases class 2C by competing with Mg^{2+}/Mn^{2+} at the metal binding site of the catalytic domain (SidAhmed-Mezi et al., 2014).

5.3. Zinc, GPR39, KCC2 and seizures/epilepsy

Application of zinc to hippocampal slices, as well as electrical stimulation of mossy fibers, up regulated potassium-chloride cotransporter 2 (KCC2) activity (Chorin et al., 2011). Importantly, mossy fiber stimulation-dependent up regulation of KCC2 activity was not observed in slices from GPR39 knockout animals (Chorin et al., 2011).

KCC2 is a neuron-specific chloride extruder that uses the potassium gradient for maintaining low intracellular chloride. Outwardly-directed electrochemical chloride gradient across the neuronal membrane has been long established to be fundamental for proper function of postsynaptic $GABA_AR$ function. It was found that deletion of KCC2 by decreasing expression in cultured hippocampal neurons, increased intracellular chloride concentrations and produced deficits in GABAergic inhibition (Kelley et al., 2018). Therefore, the study by Chorin et al. (2011) suggests the importance of extracellular zinc signaling via GPR39 in inhibitory neurotransmission (Sensi et al., 2011). Moreover, changes in KCC2 expression or function have been associated with chronic epilepsy (Di Cristo, Awad, Hamidi, & Avoli, 2018). Local ablation of KCC2 in the hippocampus resulted in the development of spontaneous, recurrent seizures and hippocampal sclerosis. Hence, deficits in KCC2 function resembles mesial temporal lobe epilepsy (Kelley et al., 2018), which suggest the role of GPR39 in epilepsy development and progression.

Furthermore, adult GPR39 knockout mice displayed enhanced susceptibility to seizures triggered by a single intraperitoneal injection of kainate, when compared to wild-type littermates. The maximal seizure severity score was higher in GPR39 knockout mice and significantly higher seizure scores were observed from 40 min following the injection of kainate. In all, 82% of GPR39 knockout mice exhibited at least stage 5 seizures (loss of posture or status epilepticus) compared to 27% of the wild-type mice. Kainate also substantially enhanced seizure-associated gamma oscillatory activity in juvenile GPR39 knockout hippocampal slices, a phenomenon that was reproduced in wild-type tissue by extracellular zinc chelation. Importantly, kainate-induced synaptic zinc release enhanced surface expression and transport activity of KCC2 in wild-type, but not in GPR39 knockout hippocampal neurons (Gilad et al., 2015; Khan, 2016). Therefore, it was proposed that

GPR39-dependent up regulation of KCC2 activity provides homeostatic adaptation to an excitotoxic stimulus by increasing inhibition and that GPR39 may be a target for dampening seizures (Gilad et al., 2015; Khan, 2016).

Synaptic zinc and GPR39 are also necessary for triggering the synthesis of the endocannabinoid, 2-arachidonoylglycerol (2-AG) in the dorsal cochlear nucleus. The postsynaptic production of 2-AG, one of the best-described endocannabinoids, inhibits presynaptic probability of neurotransmitter release via activation of presynaptic cannabinoid type 1 receptors (CB1R) on inhibitory and excitatory neurons (Lu & Mackie, 2016). An important fact is that inhibition of monoacylglycerol lipase, the 2-AG degrading enzyme, delayed kindling progression in the amygdala kindling model of temporal lobe epilepsy (von Ruden, Bogdanovic, Wotjak, & Potschka, 2015), suggesting 2-AG as a target for antiepileptogenic intervention. Zinc-induced inhibition of transmitter release was absent in mice lacking either ZnT3 or GPR39. Moreover, measurement of 2-AG levels revealed that zinc-mediated initiation of 2-AG synthesis was absent in mice lacking GPR39 (Perez-Rosello et al., 2013). Hence, a mechanism has been proposed whereby extracellular zinc acting on GPR39 inhibits glutamate release by promoting 2-AG synthesis.

In contrast to the effects of extracellular zinc on KCC2, a rise in intracellular zinc may inhibit KCC2 activity (Hershfinkel et al., 2009). Zinc-mediated KCC2 inhibition produced a depolarizing shift of $GABA_AR$ reversal potentials in rat cortical neurons (Hershfinkel et al., 2009). A role for glia is supporting by findings that seizures occur in up to 90% of glioma patients (Pace et al., 2017), and coculturing neurons with patient-derived glioma cells resulted in depolarization of the reversal potential of $GABA_AR$-evoked currents. Using selective antagonists of NMDAR and AMPARs, it was found that activation of both receptors is necessary to observe a $GABA_AR$ reversal potential shift. With the aid of a specific blocker of KCC2, R-(+)-butylindazonedihydroindenyloxyalkanoic acid (DIOA), it was demonstrated that the glioma-induced $GABA_AR$ reversal potential depolarization is due to reduced function and not expression of KCC2. Moreover, the membrane permeant zinc chelator, TPEN, recovered $GABA_AR$ reversal potential depolarization, while TPEN application had no effect when KCC2 was blocked by DIOA, suggesting that rises in zinc levels cause KCC2 inhibition. Furthermore, the fast, extracellular chelator of zinc, tricine, did not abolish the effects of coculture on E_{GABA} depolarization, indicating that extracellular zinc in not necessary for GABA reversal potential depolarization. Altogether, these experiments pointed to chloride disequilibrium in cocultures of neurons with patient-derived glioma cells due to intracellular zinc-dependent KCC2 impairment (Di Angelantonio et al., 2014).

Thus, extra- and intracellular zinc may produce opposite effects on KCC2 activity. Synaptic (extracellular) zinc may activate the GPR39 receptor, leading to enhanced activity of KCC2 and presumably enhanced neuronal inhibition, while increase in intracellular zinc may trigger the opposite effect, i.e., a decrease in KCC2 function leading to decreased inhibition.

5.4. Zinc, $Ca_V3.2$ and seizures/epilepsy

To unravel the cascade following increase in intracellular zinc in postsynaptic neurons, van Loo et al. (2015) incubated neural NG108-15 cells with a solution containing 200 μM zinc, a concentration expected to increase intracellular zinc (Canzoniero et al., 1999) and potassium. They found increased mRNA expression and increased protein levels of a nickel-sensitive T-type calcium channel $Ca_V3.2$, but not other VGCCs sensitive to nickel. Whole-cell patch-clamp recordings of calcium currents showed an increase in functional Cav3.2 channels. Moreover, it was demonstrated that zinc is necessary for activation of the $Ca_V3.2$ gene promoter.

While delineating the region responsible for zinc-induced activation of the $Ca_V3.2$ promoter by analyzing $Ca_V3.2$ promoter deletion luciferase reporter constructs, it was found that $Ca_V3.2$ gene regulation is under the control of a zinc-responsive transcription factor (van Loo et al., 2015). The only currently known such transcription factor in mammals is MTF1 (Cousins et al., 2006; Giedroc et al., 2001; Grzywacz et al., 2015). Incubation of cells, in which MTF1 was able to bind the MRE, but did not possess the ability to activate transcription, with zinc and potassium resulted in a repression of zinc-induced $Ca_V3.2$ promoter activation. Furthermore, levels of MTF1 expression were increased 6 and 12 h after pilocarpine-induced status epilepticus and returned to basal values thereafter. TFL-staining of hippocampal slices from pilocarpine-treated rats for zinc showed increased staining in the CA1 region, which appeared 1 day after status epilepticus and declined 2–4 days after. In addition, analysis of hippocampal $Ca_V3.2$ and MTF1 expression levels in patients with treatment resistant temporal lobe epilepsy and hippocampal sclerosis demonstrated a positive correlation between MTF1 and $Ca_V3.2$ levels.

Thus, this comprehensive study revealed a novel mechanism whereby a rise in intracellular zinc activates MTF1, which binds to MRE in the $Ca_V3.2$ gene promoter and increases transcription of this gene. The increase in $Ca_V3.2$ mRNA leads to enhanced expression of $Ca_V3.2$ channels and larger nickel-sensitive-T-type calcium currents (van Loo et al., 2015). Importantly, in CA1 pyramidal cells, increase in these currents causes regular firing cells to convert to burst firing, thereby enhancing the excitability of the hippocampal network (Yaari, Yue, & Su, 2007).

5.5. Zinc, KCNQ channels and seizures/epilepsy

While increased intracellular zinc seems to be associated with hyperexcitability in terms of its action on KCC2 or Cav3.2, protection from seizures/epilepsy has been suggested through the effects of intracellular zinc on KCNQ (M-type) channels. Most importantly, mutations in KCNQ channels account for most of the mutations of potassium channels linked with human diseases (Robbins, 2001). Mutations of KCNQ2 and KCNQ3 resulting in decreased activity are associated with familial neonatal convulsions (Cooper, Harrington, Jan, & Jan, 2001), a dominantly inherited form of epilepsy.

Zinc pyrithione (ZnPy), a zinc ionophore that delivers free zinc ions across the plasma membrane and increases intracellular zinc ion concentrations (Krezel & Maret, 2016), potentiates the activity of KCNQ subtypes, except for KCNQ3, and treatment with ZnPy rescues mutant KCNQ2 channels that cause benign familial neonatal convulsions (Xiong, Sun, & Li, 2007). While it has been proposed that ZnPy binds to the KCNQ channel's pore from the extracellular site (Xiong et al., 2007), Gao et al. (2017) reported that intracellular zinc directly and reversibly increases the activity of recombinant and native KCNQ channels by reducing or virtually abolishing the channel requirement for PIP_2, thereby permitting a PIP_2-gated ion channel to operate independently of this important signaling molecule. Moreover, other zinc ionophores such as Zn-DEDTC were shown to open KCNQ channels (Gao et al., 2017). Thus, it has been proposed that activation of KCNQ channels by intracellular zinc may represent a strategy to survive cell excitotoxicity associated with seizures.

An interplay between T-type calcium and M-type potassium channels, with regard to zinc action on these channels, has been demonstrated by Huang et al. (2016). These authors determined that M-channel enhancement and T-channel inhibition contribute to the redox-mediated anti-nociceptive effect of neuropeptide substance P in peripheral nerves. They also demonstrated that substance P is able to modulate T-type channel activity in nociceptors by turning off channel sensitivity to zinc by a redox-dependent mechanism (Huang et al., 2016). Another example of a possible interplay between these channels was suggested by Martinello et al. (2015), who reported that acetylcholine activation of muscarinic M1 receptors in adult hippocampal granule cells increased axonal T-type calcium channel function, which in turn elevated axonal

basal calcium levels, leading to inhibition of the axonal K_v7/M current, which decreased the spike threshold in granule cells (Martinello et al., 2015).

5.6. Zinc, TRPM7 and seizures/epilepsy

Knockdown or inhibition of the function of TRPM7 in mouse cortical neurons reduces both intracellular zinc concentrations and zinc-mediated cell injury (Inoue et al., 2010). Other studies have shown that the local anaesthetic lidocaine reduces neuronal injury through inhibition of the TRPM7-mediated intracellular zinc increase (Leng et al., 2015). As yet, however, there is no specific TRPM7 inhibitor. Some compounds inhibiting TRPMs have poor selectivity, preventing a complete understanding of the role of TRPM7 in disorders related to brain damage. Carvacrol, a naturally occurring monoterpenic phenol, which (among other mechanisms) acts on TRPM7, was active in the perforant path stimulation kindling model, probably through its action on TRPM7 channels. Carvacrol inhibited recurrent status epilepticus and early seizures in vivo, and had a protective effect against status epilepticus-induced cell death both in CA1 and hilus (Khalil, Kovac, Morris, & Walker, 2017). Thus, the positive effects of this compound in this model may be associated with decreasing intracellular zinc levels through inhibition of TRPM7.

5.7. Zinc, ASIC channels and seizures/epilepsy

Activation of ASIC channels depends on pH, and desensitization is observed after slow acidification. Acidosis, which is the result of an accumulation of lactic acid, has been found in epileptic seizures (Xiong, Pignataro, Li, Chang, & Simon, 2008). The decrease of pH in the brain mainly activates calcium-permeable ASIC1a channels, which results in calcium accumulation and acidosis-mediated brain injury (Hey, Chu, Seeds, Simon, & Xiong, 2007). The increase in extracellular zinc seems to be neuroprotective due to the inhibited activity of the ASIC1a channels and the acid-induced increase in intracellular calcium (Hey et al., 2007)

6. Zinc levels in epilepsy – clinical data

Observations from clinical studies corroborate the link between zinc and epilepsy. A recent meta-analysis, which included 60 articles, 40 pertaining to epilepsy and 20 pertaining to febrile seizures, published between January 1970 and August 2013, investigated a relationship between zinc levels in serum, hair, or CSF and epilepsy or febrile seizures. Significantly increased serum zinc concentrations were noted in 285 non-treated patients with epilepsy, compared to 335 healthy control subjects. In contrast, serum zinc concentration were lower in 361 patients with febrile seizures than in 176 healthy controls (Saghazadeh et al., 2015). Although febrile seizures increase the risk of developing epilepsy, they are recognized as a distinct syndrome separate from epilepsy (Patel et al., 2015) and are not a subject of this review (for review on febrile seizures and zinc see: Reid et al., 2017).

However, lower serum zinc levels were demonstrated in untreated children with epilepsy compared to healthy controls (Wojciak, Mojs, Stanislawska-Kubiak, & Samborski, 2013). Decreased serum zinc concentrations were also demonstrated in children with generalized intractable epilepsy compared to age-matched healthy children (Saad, Hammad, Hassan, & Badry, 2014; Seven, Basaran, Cengiz, Unal, & Yuksel, 2013). Furthermore, lower serum zinc levels in children and adolescents with intractable epilepsy in comparison with a controlled epilepsy group was shown (Kheradmand et al., 2014) (Table 3).

7. The effects of anti-seizure treatment on zinc levels

7.1. Preclinical data

Many preclinical studies assessed Timm staining after administration of current/novel treatments for seizures. Vigabatrin did not prevent mossy fiber sprouting in the kainic acid-induced post status epilepticus model regardless of when the treatment was started. Sprouting increased in the septal end of the hippocampus when vigabatrin treatment began 1 h after the onset of status epilepticus (Pitkanen, Nissinen, Jolkkonen, Tuunanen, & Halonen, 1999). Lamotrigine treatment, started 2 h after the beginning of electrically induced status epilepticus and continued for 11 weeks, did not affect mossy fiber sprouting (Nissinen, Large, Stratton, & Pitkanen, 2004). In contrast, eslicarbazepine acetate treatment resulted in a significant decrease in mossy fiber sprouting into the inner molecular layer of pilocarpine-injected mice, as detected by Timm staining (Doeser et al., 2015).

Inhibition of mossy fiber sprouting has been proposed as an antiepileptogenic strategy although data is conflicting (Buckmaster, 2014). Mossy fiber sprouting was suppressed by systemic treatment with rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), which possesses anti-seizure and possibly antiepileptogenic properties, in pilocarpine- and kainic acid-induced status epilepticus models (Citraro, Leo, Constanti, Russo, & De, 2016; Meng, Yu, Song, Chi, & Tan, 2013). It was demonstrated that pilocarpine-induced status epilepticus longer than 1 h resulted in the development of spontaneous motor seizures, which are associated with robust mossy fiber sprouting (Chen et al., 2013). A 1-month focal, continuous, unilateral infusion of rapamycin in rats subjected to pilocarpine-induced status epilepticus,

Table 3
Summary of clinical data.

	Outcome	Reference
Zinc levels in epilepsy	Increased serum zinc concentration in 285 nontreated patients with epilepsy vs. 335 healthy controls	Saghazadeh et al. (2015) (a meta-analysis study)
	Decreased serum zinc concentration in untreated children with epilepsy vs. healthy controls	Wojciak et al. (2013)
	Decreased serum zinc concentration in children with generalized intractable epilepsy vs. healthy controls	Saad et al. (2014); Seven et al. (2013)
Anti-seizure treatment and zinc levels	Decreased serum zinc concentration in 138 epileptic patients on valproate monotherapy vs. 145 untreated epileptic patients	Saghazadeh et al. (2015) (a meta-analysis study)
	Decreased levels of zinc in the hair of 68 epileptic patients on valproate monotherapy vs. 73 untreated epileptic patients	
	Decreased serum zinc concentration in children and adolescent with intractable epilepsy vs. controlled epilepsy group	Kheradmand et al. (2014)
Zinc administration in epilepsy	A significant improvement (> 50% reduction in seizure frequency) in 31% of children with intractable epilepsy who received oral zinc (1 mg/kg/day) in addition to valproate and levetiracetam, for 6 months, vs. 4.5% of patients who received placebo, valproate and levetiracetam	Saad et al. (2015)
Zinc intake in epilepsy	Zinc intake in children with intractable epilepsy: 5.7 ± 0.7 mg in children 1–3.9 years of age; 8.6 ± 0.9 in children 4–8.9 years of age (lower than in healthy children but not below the RDA)	Volpe et al. (2007)
	Zinc intake in children (mean age 9.06 ± 3.17) with intractable epilepsy and severe physical and developmental disabilities: 4.1 mg/day (below the RDA)	Bertoli et al. (2006)

RDA - Recommended Dietary Allowance.

which began several hours after status epilepticus, reduced aberrant Timm staining in the granule cell layer and molecular layer, while infusion for 2 months inhibited mossy fiber sprouting more pronouncedly. However, after rapamycin infusion ceased, aberrant Timm staining approached untreated levels. When the onset of infusion began after mossy fiber sprouting had developed for 2 months, rapamycin did not reverse aberrant Timm staining. These findings show that inhibition of the mTOR signaling pathway suppressed development of mossy fiber sprouting but suppression required continual treatment, and rapamycin did not reverse already established axon reorganization (Buckmaster, Ingram, & Wen, 2009), thus did not fulfill criteria for an antiepileptogenic treatment.

Moreover, in the pilocarpine-induced status-epilepticus model, treatment with a high dose of rapamycin (10 mg/kg/day), which began 24 h after status epilepticus and was continued for 2 months, blocked mossy fiber sprouting, but did not reduce seizure frequency (Heng, Haney, & Buckmaster, 2013), suggesting that inhibition of mossy fiber sprouting may not be associated with antiseizure or antiepileptogenic effect in this model. Furthermore, animals in which mTOR was deleted from 44% of the astrocyte population, exhibited a lower seizure frequency compared to controls; however, down regulation of mTOR did not rescue mossy fiber sprouting (Wang, Sha, Sun, Shen, & Xu, 2017). These results argue against the involvement of hippocampal mossy fiber sprouting in the activity of rapamycin.

In contrast, sodium butyrate, a histone deacetylase inhibitor, displayed antiepileptogenic effects in the hippocampus kindling model, which was associated with powerful reduction in mossy fiber sprouting (Reddy, Clossen, & Reddy, 2018). These results suggest that histone deacetylation is a critical epigenetic mechanism in epileptogenesis, and support the reduction of mossy fiber sprouting as an antiepileptogenic mechanism.

In the intrahippocampal kainate model of mesial temporal lobe epilepsy, following an initial increase during the first 2 weeks of post-kainic acid administration, Timm staining progressively decreased in the hippocampus, and disappeared completely at 56 days post-kainic acid administration in the dentate gyrus. However, Timm staining was visible when midazolam was administered continuously for 24 h. Midazolam also completely inhibited the seizure activity in this model (Mitsuya et al., 2009).

Regardless of mossy fiber sprouting being involved in antiepileptogenic effect or not, it is commonly assessed while searching for novel treatment possibilities, however, concurrent data on zinc homeostasis regulating proteins or elements of zinc signaling pathways, which would clarify the role of zinc in these processes, have not been found by us while preparing this review.

7.2. Clinical data

A recent meta-analysis examined not only the levels of zinc in non-treated epileptic patients, but also the effects of anti-seizure treatment on zinc levels (Saghazadeh et al., 2015). Significantly lower serum levels of zinc were present in 138 epileptic patients who received valproate monotherapy than in 145 epileptic patients without anticonvulsant therapy, as well as in 131 epileptic patients on carbamazepine monotherapy compared to 161 controls without epilepsy. A decrease in serum zinc concentrations was observed in 458 treated epileptic patients who received anticonvulsant monotherapy, compared to 570 control subjects without epilepsy. In addition, zinc concentrations in hair were significantly lower in 68 epileptic patients on valproate monotherapy than in 73 untreated epileptic subjects, as well as in 67 treated epileptic patients relative to 121 control subjects.

Serum zinc levels in epileptic children under drug treatment (sodium valproate, carbamazepine, phenytoin, clonazepam, and levetiracetam) were lower compared to healthy children (Armutcu et al., 2004; Talat, Ahmed, & Mohammed, 2015). Also, a 2-year monotherapy with valproic acid in children decreased serum and hair zinc concentrations compared to children who were intended to be put on this drug after epilepsy diagnosis and compared to children admitted to the hospital because of upper respiratory tract disorders (Altunbasak et al., 1997) (Table 3). However, not altered serum zinc levels in children or adolescents with valproic acid treatment were also observed (Kaji et al., 1992; Verrotti et al., 2002).

8. The effects of zinc administration in epilepsy

8.1. Preclinical data

Given a variety of mechanisms induced by zinc which may be of relevance for seizures/epilepsy, data on the effects of zinc administration in experimental animals could be helpful to clarify the net effects. Administration of zinc in preclinical models of seizures/epilepsy was, however, found to exert either a pro- or anti-seizure effect, depending on the dose and form of zinc, its route of administration, and the applied model.

A 4-week continuous infusion of zinc chloride solution at a concentration of 1000 μM, but not at 10 μM (both solutions were administered at a rate of 0.25 μl/h) into the hippocampal hilus, delayed the development of behavioral seizures in a kindling model induced by daily awake commissural stimulation at 60 Hz. In this study, after discharges were recorded from a dentate gyrus electrode and development of behavioral seizures was assessed according to Racine score. Both doses, i.e., 10 μM and 1000 μM, inhibited progression of afterdischarge duration (Elsas, Hazany, Gregory, & Mody, 2009).

In contrast, intrahippocampal injection of zinc sulfate alone (600 μg/kg) to rabbits has been considered as a chronic model of experimental epilepsy. In this model, animals showed evidence of complex partial seizures, which may secondarily generalize. The electrohippocampalogram and electrocorticogram discharges changed correspondingly during both types of seizures, and lasted for weeks (Pei & Koyama, 1986). Thus, zinc is a metal (in addition to aluminum, cobalt, and iron) that can be used to induce seizures at preclinical level (De Deyn, D'Hooge, Marescau, & Pei, 1992).

The effects of subcutaneous injections of zinc chloride on seizures induced by the intraperitoneal administration of kainic acid (10 mg/kg) in rats and by noise (80–120 dB) in the DBA/2J mouse were also studied. Zinc salt (20–200 mg/kg) substantially reduced the frequency of noise-induced running fits, clonic and tonic seizures, and deaths in mice, but had no significant effect on the incidence or severity of kainic acid-induced seizures in rats (Morton, Howell, & Frederickson, 1990). Another study demonstrated that zinc chloride administered subcutaneously (35 mg/kg) 15 min prior to kainic acid administration (12 mg/kg, i.p.) induced wet dog shakes and convulsions similar to pretreatment with saline. Further, a single dose of zinc chloride did not affect or had a slight protective effect on kainate-induced lesions, while 2 doses of zinc administered prior to kainate led to more pronounced lesions (Nave & Connor, 1993).

The chronic (3-week) intraperitoneal administration of zinc sulfate at a dose of 60 mg/kg increased the severity of pilocarpine-induced seizures, while a dose of 3 mg/kg reduced the severity of pilocarpine-induced seizures and increased the latency to attain the forelimb clonus. Moreover, following administration of a combination of 3 mg/kg zinc and effective (100 mg/kg) or subeffective (45 mg/kg) doses of valproic acid, a reduction in the severity of limbic seizures and no forelimb clonus were observed (Baraka, Hassab El, & El, 2012), similar to the effect observed following administration of valproic acid alone.

Chronic treatment with zinc at doses of 2, 20, and 200 mg/kg, administered as zinc sulfate orally for 2 weeks did not affect maximal electroshock seizures, but 2 mg/kg exerted a protective effect on pentylenetetrazole-(60 mg/kg) induced seizures. Moreover, zinc at 200 mg/kg, decreased the number of kindled animals from 66.7% to 14.3% and reduced the seizure severity score in the pentylenetetrazole kindling model (Kumar, Katyal, & Gupta, 2015).

8.2. Clinical data

A systematic review published in 2013, which focused on possible treatments for seizures associated with the autism spectrum disorder in children, provided a recommendation D (limited, inconsistent, or inconclusive evidence) for the use of zinc in seizures and justified this rating by the fact that the evidence for the use of zinc in seizures was based only on basic research and no clinical trials at that time (Frye et al., 2013).

Since then, a double-blind, placebo-controlled trial of the efficacy of zinc supplementation of anti-seizure therapy in children with intractable epilepsy has been published (Saad et al., 2015). In all, 45 children (24 boys and 21 girls) aged between 3 and 12 years, from Egypt, were enrolled in the study. They were eligible for inclusion when they presented idiopathic generalized intractable epilepsy defined as one or more seizures per month and a failure of adequate trials of at least 2 tolerated and appropriately chosen and used anti-seizure drugs schedules. The patients were randomly allocated to the group that received oral zinc at a dose of 1 mg/kg/day or placebo in addition to currently used anti-seizure drugs, valproate and levetiracetam, for 6 months. It was found that 31% of the participants who received zinc showed a significant improvement, defined as > 50% reduction in seizure frequency compared with 4.5% of patients who received placebo (Saad et al., 2015) (Table 3).

It should be noted that a recently published meta-analysis, which found significantly increased serum zinc concentrations in non-treated patients with epilepsy compared with healthy volunteers (Saghazadeh et al., 2015), did not differentiate between children and the adult population. Importantly, a previous work demonstrated significantly decreased serum zinc concentration in children with generalized intractable epilepsy compared to age-matched healthy children (Saad et al., 2014). According to Ghasemi, Zahediasl, Hosseini-Esfahani, Syedmoradi, and Azizi (2012), pediatric serum zinc reference values are 9.7–31.5, 9.2–30.9, and 9.3–31.1 μmol/L in Iranian boys, girls, and total population respectively. The values of zinc levels found in the study by Saad et al. (2014) were 95.8 ± 29 μg/dL (14.7 ± 5 μmol/L) in children with intractable epilepsy and 145 ± 37 μg/dL (22 ± 6 μmol/L) in healthy children, i.e., the serum zinc concentrations in epileptic children were within the range of normal values. The study by Saad et al. (2015), which demonstrated the potential role of zinc as an adjunct therapy in children with intractable epilepsy, did not provide data on pre- or post-intervention serum zinc levels. However, zinc deficiency is more prevalent in developing countries like Egypt, in which the study of Saad et al. (2015) was conducted, than in developed countries. Therefore, it is possible that beneficial effects of zinc in terms of enhancing anti-seizure therapy occur in populations in which pre-intervention serum zinc concentration could have been already reduced.

9. Zinc-deficient diet and epilepsy

9.1. Preclinical data

Experiments assessing the effects of zinc deficient (ZnD) and zinc adequate (ZnA) diets on seizures/epilepsy may be another step in delineating the role of zinc in epilepsy. We reported altered expression of NMDAR and AMPAR subunits at the protein level in the hippocampi of rats fed the ZnD diet, which was associated with depressive-like behavior (Doboszewska et al., 2015a; Doboszewska et al., 2015b). These results are suggestive of altered function of NDMAR and AMPAR, and thus of imbalance between excitation and inhibition, in ZnD rats. Moreover, ZnD mice display features of the autism spectrum disorder (Grabrucker et al., 2018), which is comorbid with epilepsy.

The results of experiments examining directly the relationship between the ZnD diet and seizures/epilepsy in rats and mice of different ages have been summarized in Table 4. Chronic (4–8 weeks) ZnD diet increased susceptibility to seizures induced by postural stimulation in a genetically epileptic mouse (El) mouse model of idiopathic generalized epilepsy with complex partial seizures (Fukahori & Itoh, 1990), by kainate injection in ddY mice and Wistar rats (Takeda, Hirate, Tamano, Nisibaba, & Oku, 2003a), and by NMDA injection in ddY mice (Takeda, Itoh, Nagayoshi, & Oku, 2009), but not to pentylenetetrazole in ddY mice (Takeda, Itoh, Hirate, & Oku, 2006a). These observations indicate that the occurrence of behavioral seizures in the ZnD animals may depend on the stimuli that was used to induce seizures.

Furthermore, while a 2-week ZnD diet administered in to 4-week old rats did not produce differences in the latency of clonic convulsions or in maximum seizure score in response to kainate injection compared to the ZnA group, a 4-week ZnD diet produced shorter latencies of clonic convulsions and higher seizure scores (Takeda, Itoh, Tamano, & Oku, 2006b). Therefore, the duration of the ZnD diet may be critical. Similarly, the duration of the diet may explain differences observed by Fukahori and Itoh (1990) and Nagatomo et al. (1998). Fukahori and Itoh (1990) reported an increased susceptibility to seizures in El mice fed a ZnD diet, whereas Nagatomo et al. (1998) reported no differences in seizure scores between El mice fed the ZnD and ZnA diet, and an increased effectiveness of zonisamide in ZnD mice, suggesting protective effect of lowering dietary zinc amount in terms of treatment with this drug. However, Fukahori and Itoh (1990) utilized an 8-week ZnD diet while Nagatomo et al. (1998) used a 4-week diet. Hence, the duration of the diet, in addition to the mechanism of action of the chemical agent used for induction of seizures, is critical for seizures occurrence in the ZnD animals.

The effects of ZnD diet on neurotransmitter and zinc release in the hippocampus were studied in rats using in vivo microdialysis (Takeda, Hirate, Tamano, Nisibaba, & Oku, 2003a; Takeda, Tamano, & Oku, 2005). A 2-week ZnD diet given to 4-week old rats did not change basal extracellular zinc levels in the hippocampus, compared to ZnA rats. However, stimulation by 100 mM KCl, increased extracellular zinc levels in the hippocampus of the ZnD rats compared to basal levels, and this increase was more pronounced in ZnD rats than in the ZnA rats (Takeda, Itoh, Tamano, & Oku, 2006b).

After 4 weeks on the ZnD diet, the basal zinc concentration in the hippocampal extracellular fluid of the ZnD rats was less than 50% of that of the ZnA rats. The basal concentrations of glutamate, GABA and glycine in the perfusate of the ZnD rats were not significantly different from those of the ZnA rats. Zinc concentrations increased in the perfusate of ZnD rats after the administration of kainate, but were lower than the basal levels in the ZnA rats. Glutamate concentrations in the perfusate of the ZnA rats were negligibly increased after the administration of kainate, whereas they were markedly increased in ZnD rats, while GABA concentrations were significantly increased in the ZnA rats but unchanged in the ZnD rats. Glycine concentrations were significantly increased in both the ZnA and ZnD groups after the administration of kainate, suggesting enhanced release of glutamate and a decrease in GABA concentrations as a possible mechanism for the increased seizure susceptibility in ZnD young rats (Takeda, Hirate, Tamano, Nisibaba, & Oku, 2003a). We have observed decreased evoked zinc release in the prefrontal cortex of the rats after 6 weeks of ZnD diet administration, while the evoked glutamate release was increased in the ZnD rats, compared to the ZnA rats (Doboszewska et al., 2016). These results further support increased excitability in the ZnD animals.

In 8-week old rats fed the ZnD diet for 4 weeks, basal extracellular glutamate and zinc levels in the hippocampus were higher in ZnD rats than in ZnA rats. After the administration of kainate, the extracellular concentrations of glutamate and aspartate in the hippocampus were significantly higher in ZnD rats compared to ZnA rats. The extracellular zinc concentrations were significantly decreased in ZnD rats after kainate, compared to basal levels. In ZnA rats, extracellular glutamate concentrations were unchanged after the administration of kainate. Extracellular GABA concentrations in the hippocampus significantly increased in both groups after stimulation with 100 mM KCl, but no significant difference was observed between the ZnA and ZnD rats (Takeda et al., 2005). The latter observations suggest that differential biochemical changes may occur depending on the age of animals and the length of ZnD diet.

Table 4
The effects of the zinc deficient diet (ZnD), zinc adequate diet (ZnA) or zinc supplemented diet (ZnS) on seizure susceptibility and the levels of neurotransmitters and/or zinc.

Species	Diet	Stimulus	Outcome	Reference
Male Wistar rats, 4 weeks old	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 2 weeks	Kainate (5 mg/kg, i.p.)	Shorter latency in wet dog shakes and myoclonic jerks in the ZnD rats; no difference in the latency in clonic convulsions/maximum seizure score between the groups; no difference in Timm staining in the hippocampus; no difference in basal extracellular zinc level in the hippocampus; increased extracellular zinc level in the hippocampus of the ZnD rats after stimulation with 100 mM KCl, compared to basal extracellular zinc level in the ZnD rats; more pronounced increase in extracellular zinc after stimulation with 100 mM KCl in the ZnD rats than in the ZnA rats (in vivo microdialysis)	Takeda, Itoh, Tamano, and Oku (2006b)
Male ddY mice and Wistar rats, both 3 weeks old, feeding the diet begun at 4 weeks of age	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 4 weeks	In mice: kainate (12 mg/kg, every 60 min, 3×, i.p.)	Higher maximum seizure scores of the ZnD mice; all the ZnD mice exhibited status epilepticus vs. 30% of the ZnA mice; shorter latency in myoclonic jerks of the ZnD mice; no differences in the latency in clonic and tonic convulsions and in status epilepticus; decreased Timm staining in the hippocampal mossy fibres, stratum radiatum and stratum oriens in the ZnD mice.	Takeda, Hirate, Tamano, Nisibaba, and Oku (2003a)
		In rats: kainate (5–10 mg/kg, i.p.)	Kainate 10 mg/kg: clonic convulsion ca. 2 h after the injection in the ZnA group; all the ZnD rats exhibited status epilepticus and died; kainate 5 mg/kg: wet-dog shakes, e.g. head bobbing and twitching in the ZnA rats, the convulsion became severer with time, clonic convulsion immediately after the injection in the ZnD rats; increased glutamate release in the hippocampus of the ZnD rats; no increase in GABA release in the ZnD rats after treatment with kainate, compared to basal level in ZnD rats (in vivo microdialysis)	
Male mice, 3 weeks old, feeding of the diet begun at 4 weeks of age	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 4 weeks	Kainate (12 mg/kg, every 60 min, 3x, i.p.)	Increased neuronal loss in the CA1, CA2 and CA3 pyramidal cell layers of the ZnD mice; decreased extracellular zinc concentration in the CA3 region of the hippocampus of the ZnD mice (measured using ZnAF-2)	Takeda et al. (2005a)
Male Wistar rats, 8 weeks old	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 4 weeks	Kainate (5 mg/kg, i.p.)	Increased basal extracellular zinc and glutamate levels in the hippocampus of the ZnD rats; decreased extracellular zinc and no increase in extracellular glutamate level in the hippocampus of the ZnD rats after kainate injection, compared to basal level in ZnD rats; increased extracellular GABA in the hippocampus of the ZnD rats after stimulation with 100 mM KCl, compared to basal level in ZnD rats (in vivo microdialysis)	Takeda et al. (2005b)
Male ddY mice, 4 weeks old	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 4 weeks	PTZ (40 mg/kg, 50 mg/kg and 50 mg/kg, once a day for 2 days, i.p.)	No differences in the latency in each seizure; no differences in maximum seizure score; (more elevated maximum seizure score in the ZnD mice along with the increase of the dose of PTZ but no significant difference); no significant difference in the latency in each seizure between the groups after challenge with PTZ (50 mg/kg×2 times)	Takeda, Itoh, Hirate, and Oku (2006a)
Male ddY mice, 3 weeks old, feeding the diet begun at 4 weeks of age	ZnA 44 mg Zn/kg, ZnD 2.7 mg Zn/kg, 4 weeks	NMDA (150 mg/kg, i.p.)	Significantly higher maximum seizure scores of the ZnD mice; 40% of the ZnD mice exhibited status epilepticus and died within 2 h vs. none of the ZnA mice	Takeda et al. (2009)
EL genetic mouse model of idiopathic generalized epilepsy with complex partial seizures	ZnA 34.5 mg Zn/kg, ZnD 0.4 mg Zn/kg, ZnS 246 mg Zn/kg, 4 weeks	Postural stimulation once a week, begun at 4 weeks of age	No differences in the seizure scores; the seizure scores of the ZnD mice treated with zonisamide (75 mg/kg) lower than of the zonisamide in ZnA or ZnS mice	Nagatomo et al. (1998)
EL genetic mouse model of idiopathic generalized epilepsy with complex partial seizures	ZnA 38.9 mg Zn/kg, ZnD 1.1 mg Zn/kg, ZnS 248 mg Zn/kg, 8 weeks	Postural stimulation once a week, begun at 4 weeks of age, generalized tonic-clonic seizures induced at 7–8 weeks of age	Increased seizure susceptibility in the ZnD mice; decreased seizure susceptibility in the ZnS mice; higher Zn levels in the CA1 and CA3 regions of the hippocampus in mice with low behavioral scores of the ZnS group vs. mice with high behavioral scores of the ZnD group and vs. mice with average behavioral scores of the ZnA group (measured by a flameless AAS)	Fukahori and Itoh (1990)

i.p. – intraperitoneal; PTZ – pentylenetetrazole; NMDA – N-methyl-D-aspartate.

9.2. Clinical data

Recently, knowledge of the prevalence of zinc deficiency and its impact on health has grown (Jurowski, Szewczyk, Nowak, & Piekoszewski, 2014). It is estimated that approximately 17% of the world's population is at risk of inadequate zinc intake. The regional estimated prevalence of inadequate intake of zinc ranges from 7.5% in high-income regions to 30% in South Asia. Within regions, individual countries have a fairly

consistent estimated prevalence of inadequate zinc intake, with specific countries in South and Southeast Asia, Sub-Saharan Africa, and Central America having the greatest risk of inadequate intake of zinc. The aforementioned estimations were based on the absorbable zinc content of the national food supply and estimated physiological requirements for absorbed zinc. Country-specific estimated prevalence of inadequate zinc intake was negatively correlated with the total energy and zinc contents of the national food supply as well as the percentage of zinc obtained from foods of animal source, and positively correlated with the phytate: zinc molar ratio of the food supply.

Moreover, the prevalence of inadequate intake of zinc was correlated with the prevalence of stunting, i.e., low height-for-age, in children under 5 years of age (Wessells & Brown, 2012). An important fact is that zinc deficiency, which largely arises from inadequate intake of zinc or its poor absorption, has been ranked among the leading risks for both mortality and the burden of disease (WHO, 2009). For children under 5 years, zinc deficiency is estimated to be responsible for 13% of lower respiratory tract infections (mainly pneumonia and influenza), 10% of malaria episodes, and 8% of diarrhea episodes, worldwide (WHO, 2009).

A cross-sectional study compared the nutrient intake of 43 children, 1 to 8 years of age with intractable epilepsy, having one or more seizures every 28 days, for whom at least three appropriate anti-seizure drugs failed, to nutrient intake of healthy children of the same age from the National Health and Nutrition Examination Survey 2001 to 2002, conducted in the United States, and to the Recommended Dietary Allowance (RDA). The children were divided into two age categories: 1–3.9 years of age and 4–8.9 years of age (in order to correspond to RDA). It was found that 49% of children with intractable epilepsy, in both age groups, were below the percentage of the estimated energy requirement for sedentary children, and 70% were below the percentage of the estimated energy requirement for low active children. In both age groups decreased intake of fiber, niacin, folate, phosphorus, and selenium was observed. Moreover, the younger (1–3.9 years of age) group with intractable epilepsy had lower intakes of vitamin C, riboflavin, vitamins B_6 and B_{12}, calcium, magnesium, zinc, and copper, than healthy children (Volpe, Schall, Gallagher, Stallings, & Bergqvist, 2007).

The RDA for zinc for male and female children 1–3 years of age is 3 mg and for children 4–8 years is 5 mg (Anon, 2001). The mean intake of zinc in children with intractable epilepsy in the study of Volpe et al. (2007) was 5.7 ± 0.7 mg in children 1–3.9 years of age and 8.6 ± 0.9 in children 4–8.9 years of age. Thus, the mean zinc intake in children enrolled in the above-mentioned study was not below the RDA, although it was lower than in healthy children. However, as Loewe et al demonstrated, there is significant heterogeneity in the RDA for zinc (Lowe et al., 2013). Moreover, there is still need for a reliable biomarker for zinc status. Furthermore, tools for measuring the effects of dietary inhibitors of zinc absorption and their impact on population dietary zinc requirements are necessary (Lowe et al., 2013).

Bertoli et al. (2006) assessed the nutritional status of children with intractable epilepsy and severe physical and developmental disabilities in 17 subjects (13 boys and 4 girls), 3–16 years of age (mean age 9.06 ± 3.17), from Italy. 40% of subjects were malnourished, while 24% showed wasting (low weight-for-height). The mean zinc intake in those children was 4.1 mg/day (The RDA for zinc in children 1–3 years: 2 mg; 4–8 years: 5 mg; 9–13 years: 8 mg; 14–18 years: 11 mg for boys and 9 mg for girls (Anon., 2001) and adequacy index [nutrient daily intake/RDA × 100] was < 60% (Bertoli et al., 2006). Thus, in the study of Bertoli et al. (2006) the mean zinc intake was below the RDA (Table 3).

These data, together with data from preclinical studies on the effects of the ZnD diet administration on seizures, suggest that intake of zinc should be monitored in epileptic patients, particularly in children.

10. Zinc homeostasis regulating proteins and epilepsy

A recent study showed increased levels of MTI/II in the hippocampi of patients with pharmacoresistant mesial temporal lobe epilepsy and temporal lobe epilepsy associated with tumor or dysplasia, suggesting the involvement of MTs in the pathophysiology underlying temporal lobe epilepsy (Peixoto-Santos et al., 2012). MTI/II levels did not correlate with any clinical variables, but mesial temporal lobe epilepsy patients with secondary generalized seizures had less MTI/II than mesial temporal lobe epilepsy patients without secondary generalized seizures. Changes in the expression of MTs and ZnTs at both protein and mRNA levels were also found in the flurothyl-induced model of recurrent neonatal seizures (Tian et al., 2015) (Table 5).

Generation of animals lacking genes encoding zinc homeostasis regulating proteins could be helpful to clarify the role of these proteins in susceptibility to seizures, thus delineating the role of different pools of zinc in this process. The mouse lacking the gene encoding ZnT3 has been the most widely studied, but animals lacking MTIII, ZnT3 and MTIII as well as Zip1 and Zip3 have been also generated and their susceptibility to seizures has been evaluated (Table 6).

The initial research with the aid of *Znt3* knockout animals revealed few abnormalities in behavior (Cole, Martyanova, & Palmiter, 2001; McAllister & Dyck, 2017). The absence of spontaneous seizures or acoustic- or handling-induced seizures was observed in *Znt3* −/− mice, however the incidence of myoclonic jerks and tonic-clonic seizures was higher in the *Znt3* −/− mice than in *Znt3* +/+ mice in response to kainic acid and the ED50 for tonic-clonic seizures was significantly lower. Also the maximum seizure severity observed during the 2-h period following kainic acid administration was higher in *Znt3* −/− mice. No abnormalities in EEG patterns were observed in *Znt3* −/− mice during the 10 min observation period prior to kainic acid injection, but *Znt3* −/− mice had more severe electrographic seizures in response to kainic acid than in *Znt3* +/+ littermates. While *Znt3* +/+ mice spent approximately 18 % of the time in electrographic seizure, *Znt3* −/− mice spent about 65 % of the observation time in electrical epileptiform discharge. Moreover, electrical seizures in the *Znt3* −/− mice were longer in duration than in *Znt3* +/+ with shorter interictal periods (Cole et al., 2001; McAllister & Dyck, 2017). Thus, *Znt3* −/− mice are characterized by higher susceptibility to seizures elicited by kainic acid injection.

Interestingly, *Znt3* −/− mice displayed longer latency to myclonic jerks induced by bicuculline and no differences in seizure susceptibility in response to pentylenetetrazole or flurothyl (Cole et al., 2001; McAllister & Dyck, 2017). Taken together, these observations suggest that while lack of synaptic zinc *per se* does not produce seizures, it does influence susceptibility to seizures induced by chemical agents depending on the mechanism of action of the agent used for seizure induction. Bicuculline is a competitive $GABA_AR$ antagonist, while pentylenetetrazole is $GABA_AR$ ionophore-blocking convulsant ligand (Kalueff, 2007). Also flurothyl belongs to a group of drugs with prevailing or suspected effects on $GABA_AR$ (Velisek, 2006). In turn, kainic acid is an agonist of kainic acid receptors, ionotropic receptors binding glutamate (Levesque & Avoli, 2013).

Administration of the ZnD diet was also found to increase susceptibility to seizures induced by kainate injection (Takeda, Hirate, Tamano, Nisibaba, & Oku, 2003a), and by NMDA injection (Takeda et al., 2009), but not by pentylenetetrazole (Takeda, Itoh, Hirate, & Oku, 2006a) (Table 4). In addition, *Gpr39* knockout animals display higher susceptibility to seizures induced by injection of kainic acid than wild-type littermates (Gilad et al., 2015) (Table 6). Therefore, it seems that both animals lacking *Znt3* and animals fed the ZnD diet are more susceptibile to convulsant agents targeting the glutamatergic system than to agents targeting the GABAergic system.

It was found that mice lacking *Znt3* had lower levels of the GABA metabolizing enzyme, 4-aminobutyrate aminotransferase (GABA transaminase) within the barrel cortices, and deceases in the relative expression of the *Dlg4* (encoding postsynaptic density protein 95 (PSD-95), *Grin2a* (encoding GluN2A subunit of the NMDAR), and *Mt3* (encoding MTIII) genes (Nakashima, Butt, & Dyck, 2011). Decreased levels of the GABA transaminase protein could suggest increased levels of GABA in *Znt3* null mice, which may account for lesser susceptibility of these mice to

Table 5
The effects of epilepsy or seizures on the expression of zinc homeostasis regulating proteins.

Patients/model	Results	Reference
Patients with temporal lobe epilepsy (TLE)	Increased level of MTI/II in the hippocampi of patients with pharmacoresistant mesial temporal lobe epilepsy (MTLE) and TLE associated with tumor or dysplasia; no correlation between MTI/II levels and clinical variables; lowered level of MTI/II in MTLE patients with secondary generalized seizures vs. MTLE patients without secondary generalized seizures	Peixoto-Santos et al. (2012)
Flurothyl-induced model for recurrent neonatal seizures	Increased protein levels of ZnT3 and MTIII in the hippocampus; ketogenic diet decreased expression of ZnT3 and MTIII proteins	Tian et al. (2015)
Flurothyl-induced model for recurrent neonatal seizures	Increased levels of ZnT4 mRNA 6 h after the last seizures in the cerebral cortex	Ni et al. (2011b)
Flurothyl-induced model for recurrent neonatal seizures	Increased levels of ZnT1 (except at 6 and 24 h), and ZnT2 (except at 6 h) mRNA in the hippocampus from 1.5 to 24 h after the last seizures; decreased ZnT3 mRNA level at 24 h	Ni et al. (2011a)
Flurothyl-induced model for recurrent neonatal seizures	Decreased ZnT1 and ZnT3 mRNA levels at 12 h and 48 h after the last seizures in the cerebral cortex; increased ZnT3 and decreased ZnT1 mRNA levels in the hippocampus at 14 days after the last seizure; decreased ZnT3 mRNA level in the cerebral cortex at 14 days after the last seizure	Ni et al. (2010)
Flurothyl-induced model for recurrent neonatal seizures	Decreased ZnT1 mRNA level in the hippocampus of single seizure and recurrent seizure rats; no differences in ZnT3 mRNA level at postnatal day 90	Ni et al. (2009)

Table 6
Studies in knockout (KO) animals of genes associated with zinc homeostasis or signaling – the effects on seizures.

Model	Results	Reference
ZnT3 KO	Higher maximal seizure severity scores in ZnT3 KO compared to WT in response to kainic acid (12, 15, 18 mg/kg, i.p.); lower susceptibility to seizures elicited by bicuculline (only longer latency to myclonic jerks); no differences in seizure susceptibility in response to pentylenetetrazole or flurothyl; typical seizure-related neuronal damage in the hippocampus in response to kainic acid	Cole et al. (2000)
MTIII KO	Higher susceptibility to seizures elicited by kainic acid	
ZnT3 and MTIII double-KO	The same response to kainic acid as ZnT3 KO	
ZnT3 KO	Similar maximal seizure scores in ZnT3 KO and WT mice following kainate (40 mg/kg, i.p.) (seizures were terminated with phenytoin 2 h later)	Lee et al. (2000)
Zip1 and Zip3 double-KO	More severe seizure activity following kainic acid (15 mg/kg, i.p.) (EEG monitoring); attenuated CA1 cell damage following kainic acid	Qian et al. (2011)
ZnT3 KO	More severe seizure activity following kainic acid (15 mg/kg, i.p.) (EEG monitoring); less hippocampal cell damage following kainic acid injection	
GPR39 KO	Lack of mossy fiber stimulation-dependent upregulation of KCC2	Chorin et al. (2011)
ZnT3 KO	Lack of mossy fiber stimulation-dependent upregulation of KCC2	
GPR39 KO	Enhanced susceptibility to seizures induced by kainic acid (10 mg/kg, i.p.); lack of kainate-induced synaptic zinc release-dependent upregulation of KCC2	Gilad et al. (2015)

i.p. - intraperitoneal, KCC2 - potassium chloride cotransporter 2, WT - wild type.

agents whose action is mediated via $GABA_A$Rs. On the other hand, slices from *Znt3* knockout mice exhibited a greater attenuation of $GABA_A$-mediated inhibitory postsynaptic potentials during tetanic stimulation compared with slices from wild-type animals, suggesting that under conditions of intensive activation, lack of synaptic zinc results in reduction of inhibition (Lopantsev, Wenzel, Cole, Palmiter, & Schwartzkroin, 2003). Further, the susceptibility of mice lacking *Znt3* to seizures may be influenced not only by the mode of action of the agent used for induction of seizures but also by its dose (Lee et al., 2000).

In the study by Qian et al. (2011) the same dose of kainic acid, which usually evoked a full body convulsion in either *Zip-1,3* −/− or +/+ group within the first 30 min, caused forelimb tremors in *Znt3* −/− mice within the first hour of the injection. However, EEG monitoring of *Znt3* −/− mice after the first injection of kainic acid showed more seizure activity than the +/+ group even though their behavioral seizure activity scores were low. Although their average latency for the initial onset of high frequency EEG spiking was not significantly different from mice in the *Znt3* +/+ group, *Znt3* −/− mice displayed longer, on average, seizure episodes of high frequency EEG spiking. After the second dose of 15 mg/kg kainic acid injection 1 h later, *Znt3* −/− mice started to develop full body convulsions. Hence, this study confirms that increased susceptibility to electrographic seizures following injection of kainic acid is associated with the lack of *Znt3*.

Sacrificed 24 h later, the majority of *Znt3* −/− mice did not show any cell damage in the CA1 area (Qian et al., 2011). In contrast, Cole, Robbins, Wenzel, Schwartzkroin, and Palmiter (2000) observed typical seizure-related neuronal damage in the hippocampus in response to kainic acid (Cole et al., 2000). However, Cole et al. (2000) assessed the hippocampal damage 3 days after stage 5 behavioral seizures.

Qian et al. (2011) also explored the effects of double knockout of *Zip1* and *Zip3* transporters on electrographic seizures and hippocampal neurodegeneration. An important observation is that *Zip1,3* −/− double knockout mice exhibited reduction of zinc uptake into CA1 neurons, as demonstrated with the aid of FluoZin-3 dye and membrane impermeable fluorescent tracer Alexa 568. *Zip-1,3* null mutants displayed normal patterns of EEG activity during routine monitoring, but revealed a latent hyperexcitability upon administration of kainic acid (15 mg/kg or 30 mg/kg in two divided doses one h apart).

Zip-1,3 null mutants exhibited more synchronized high frequency spike bursting than wild-type mice, and 3/8 mutant mice showed essentially constant EEG seizure activity, lasting beyond the 4 hour experimental monitoring window. Among the 6 +/+ mice recorded for EEG activity, none displayed seizures beyond 4 h, and the EEG seizure discharges were discontinuous. The average latency for the initial onset of high frequency EEG spiking was 11 ± 9 min in the −/− group, which was less than one half of that in the +/+ group. Moreover, 17% of the tested mutants died during a prolonged seizure, compared to only 4% in the +/+ group after injection of 15 mg/kg of kainic acid.

Thus, not only *Znt3* but also *Zip-1,3* null mutants are characterized by higher susceptibility to seizures elicited by kainic acid injection. In addition, in *Zip-1,3* null mutants, attenuated CA1 cell damage following injection of kainic acid was observed. Hence, Qian et al. (2011) concluded that reduction of Zn uptake into neurons by knockout of *Zip-1,3* transporters or abolishment of synaptic Zn by knockout of *Znt3*, can protect neurons from neurodegeneration associated with seizures.

The observation that decrease in intracellular zinc via *Zip-1,3* knockout may be associated with increased seizure susceptibility is not surprising in view of the fact that intracellular zinc is necessary for the activation of KCNQ channels, and activation of KCNQ may protect from seizures/epilepsy.

11. Summary and conclusions

Clinical studies, which demonstrated changes in serum zinc in patients with epilepsy as well as during treatment with common anti-seizure drugs (Saghazadeh et al., 2015), support a relationship between epilepsy and zinc. Recent positive data on the effects of zinc supplementation in children from Egypt with intractable epilepsy (Saad et al., 2015) suggests that zinc administration may be helpful as an optimizing strategy for epilepsy, at least in those with decreased basal zinc supply/absorption.

Although there is no consensus on the relevance of zinc modulation of targets that are affected by exogenously applied zinc as a consequence of no agreement on physiological/pathophysiological concentrations of extracellular zinc, several well-known targets for zinc (for example NMDAR and GABAR) have been shown to mediate the link between zinc and seizures/epilepsy. Despite the fact many limitations are present when attempting to measure zinc levels, indirect evidence, e.g., increased expression of MTF1 (which is induced by intracellular zinc) after status epilepticus (van Loo et al., 2015), suggests that increase in intracellular zinc is associated with seizures. Because MTF1 mediates intracellular zinc increases secondary to status epilepticus, which often precedes chronic epilepsy, it seems to be an attractive future drug target in epilepsy. However, a study using *Zip-1,3* −/− double knockout mice, in which decreased zinc uptake into CA1 neurons parallels increased seizure susceptibility (Qian et al., 2011), questions decreasing intracellular zinc level as a treatment strategy for dampening seizures.

In fact, both extracellular and intracellular zinc may produce either protective or detrimental effects in terms of seizures via modulation of a variety of targets, which are summarized in Fig. 2. For example, extracellular zinc may inhibit extrasynaptic δ-$GABA_A$Rs and thereby prevent neurosteroid activation of tonic inhibition and decrease neurosteroid-dependent protection from seizures. On the other hand, extracellular zinc may activate the GPR39 receptor leading to enhanced activity of KCC2, thus increasing GABAergic inhibition. In contrast, intracellular zinc may inhibit KCC2, thus leading to seizures/epilepsy. Also activation of Cav3.2 channels by intracellular zinc may lead to seizures/epilepsy, but activation of KCNQ channels by intracellular zinc may result in protection from seizures/epilepsy.

Knowledge about absolute zinc concentrations would help to understand how changes in extracellular/intracellular zinc levels influence its molecular targets and which of the above effects will be prevailing. The ratiometric fluorescent probes, which are being increasingly developed, may help resolve these issues in the near future. Undoubtedly, zinc is necessary for the balance between excitation and inhibition and either excess or deficiency of any zinc pool may produce harmful effects.

Zinc homeostasis regulating proteins as well as recently described as the zinc-sensing - GPR39 receptor seem also to represent interesting drug targets in epilepsy. Our preliminary data obtained using TC-G 1008, a potent GPR39 agonist (Peukert et al., 2014), which was tested by us in the maximal electroshock seizure threshold test and the pentylenetetrazole kindling model, argue, however, against GPR39 activation as a therapeutic strategy for alleviating seizures/epilepsy (unpublished data). These data highlights the complexity of zinc signaling with regard to seizures/epilepsy. Therefore, further studies, with the aid of ligands selectively acting on targets associated with zinc homeostasis and/or signaling, are needed to demonstrate whether targeting these proteins will produce anti-seizure effect. Furthermore, these ligands should be tested in models of epilepsy, such as the amygdala kindling model, which has a good predictive validity for temporal lobe epilepsy (Loscher, 2002a), in order to demonstrate whether targeting these proteins would produce an antiepileptogenic effect.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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