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Title: Synaptic Zn^{2+} and febrile seizure susceptibility

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Abstract:

Zn^{2+}, the second most prevalent trace element in the body, is essential for supporting a wide range of biological functions. While the majority of Zn^{2+} in the brain is protein-bound, a significant proportion of free Zn^{2+} is found co-localized with glutamate in synaptic vesicles and is released in an activity-dependent manner. Clinical studies have shown Zn^{2+} levels are significantly lower in blood and cerebrospinal fluid of children that suffer febrile seizures. Likewise, investigations in multiple animal models demonstrate that low levels of brain Zn^{2+} increase seizure susceptibility. Recent work provides human genetic evidence that disruption of brain Zn^{2+} homeostasis at the level of the synapse is associated with increased seizure susceptibility. In this review we will explore the clinical, functional and genetic data supporting the view that low synaptic Zn^{2+} increases cellular excitability and febrile seizure susceptibility. Finally, the review will focus on the potential of therapeutic Zn^{2+} supplementation for at risk patients.

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Abbreviations:

Febrile Seizures (FS)

Zn^{2+} transporter 3 (ZnT3)

Table of links

TARGETS
Transporters[a]
ZnT1 (Zinc transporter 1 / SLC30A1)
ZnT3 (Zinc transporter 3 / SLC30A3)
ZIP1 (Zinc transporter 1 / SLC39A1)
ZIP3 (Zinc transporter 3 / SLC39A3)
Ionotropic glutamate receptors[b]
GluN2B (GRIN2B)
GluN2A (GRIN2A)

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This Tables of Links list key protein targets in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b] (Alexander *et al.*, 2015a; Alexander *et al.*, 2015b))

Zn^{2+} is an essential element that supports a variety of biological functions throughout the body. It plays a critical role as a cofactor to stabilize proteins structurally and to catalyse a variety of key enzymatic reactions. Secondly, it is present as a free ion in glutamatergic vesicles and participates in synaptic transmission. Zn^{2+} is regionally enriched in the brain with high levels seen in the hippocampus, neocortex, amygdala and striatum (Frederickson, 1989; Frederickson *et al.*, 2005). There is a rich literature of the potential role of free Zn^{2+} in the pathogenesis of several neurological diseases including Parkinson's (Stelmashook *et al.*, 2014) and Alzheimer's disease (Baaj *et al.*, 2009) that will not be covered here. Instead we will focus on the impact of free Zn^{2+} in the context of seizures.

Zn^{2+} and Epilepsy

Epilepsy encompasses a broad range of seizure disorders differentiated by brain location, electroencephalogram signatures, clinical presentation and time of onset (Berg *et al.*, 2010). Several studies have implicated changes in Zn^{2+} homeostasis in the underlying pathology of epilepsy (Marger *et al.*, 2014). However, Zn^{2+} seems to play a variety of roles in setting seizure susceptibility with both pro- and anti-convulsant effects reported. For example, a recent meta-analysis concludes that serum Zn^{2+} concentration is significantly higher in non-medicated patients with epilepsy (Sagharadeh *et al.*, 2015). However, others suggest that Zn^{2+} levels are significantly lower in the serum of children with refractory epilepsy (Kheradmand *et al.*, 2014; Saad *et al.*, 2014). This mixed picture is likely to reflect the multiple biological roles of Zn^{2+}. The impact of altered Zn^{2+} levels will also depend on the brain region affected and developmental time windows during which seizures occur.

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In this review we will specifically explore the role of Zn^{2+} in febrile seizures (FS). For this seizure syndrome there is good evidence that Zn^{2+} is low in both the serum and cerebrospinal fluid of individuals with FS as compared to those with either fever or seizures alone (Amiri *et al.*, 2010; Burhanoglu *et al.*, 1996; Ganesh *et al.*, 2011; Lee *et al.*, 2012; Saghazadeh *et al.*, 2015). We present clinical, physiological and genetic data that demonstrate how disruption in Zn^{2+} homeostasis could contribute to FS susceptibility.

Febrile Seizures

FS is the most common seizure syndrome, affecting approximately 3% of all children. It is typically a self-limited disorder where a child under the age of 5 has seizures only in response to fever (Verity *et al.*, 1985). FS are a major health problem accounting for 1 in 200 paediatric emergency department admissions (Martindale *et al.*, 2011). Although often considered benign, FS can manifest in short-term physical, psychological, and behavioural problems in children and parents. Worse still, they can be the harbinger of various forms of epilepsy in later life with long-term studies showing that 7% of children with FS subsequently develop epilepsy (Annegers *et al.*, 1987). Despite their significant clinical burden, little progress in understanding the mechanistic causes of FS has been made over the last decade, making this area a key future research priority for the epilepsy field (Helbig *et al.*, 2013).

Zn^{2+} Levels and FS

Several clinical studies report low total Zn^{2+} levels in the blood of children who have been diagnosed with FS (Amiri *et al.*, 2010; Burhanoglu *et al.*, 1996; Ganesh *et al.*, 2011; Lee *et al.*, 2012). Meta-analyses confirm that the concentration of serum Zn^{2+} in FS patients is significantly lower than children with central nervous system infections, meningismus, encephalitis, febrile illness without seizure, afebrile seizure, or no illness (Saghazadeh *et al.*, 2015). Direct Zn^{2+} levels in serum may not always correlate with brain cerebrospinal fluid

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levels (Palm *et al.*, 1982), in part due to the fact that uptake across the blood brain barrier is mediated through active transport (Grabrucker et al., 2011). However, Burhanoglu and colleagues have measured cerebrospinal fluid Zn^{2+} levels in FS patients and found them to be significantly reduced, more specifically implicating brain Zn^{2+} levels (Burhanoglu *et al.*, 1996). Evidence from animal models further supports the idea that low brain Zn^{2+} can increase seizure susceptibility. First, manipulation of dietary Zn^{2+} intake alters seizure susceptibility in the *El* mouse model of epilepsy. *El* mice fed diets low in Zn^{2+} have reduced brain Zn^{2+} levels and increased seizure susceptibility (Fukahori *et al.*, 1990). Conversely, diets high in Zn^{2+} increase brain levels and protect these mice from seizures. Second, rats administered intraperitoneal injections of the Zn^{2+} chelator, sodium diethyldithiocarbamate, have an almost complete depletion of free Zn^{2+} in the hippocampus and develop spontaneous seizures (Blasco-Ibanez *et al.*, 2004). Collectively, this data implicates low brain Zn^{2+} levels as a cause of increased seizure susceptibility in FS.

Zn^{2+} homeostasis

Zn^{2+} levels in the brain can be influenced by dietary and other factors. The majority of clinical studies implicating low Zn^{2+} in increased FS susceptibility were conducted in developing countries where dietary Zn^{2+} deficiency is common (Amiri *et al.*, 2010; Burhanoglu *et al.*, 1996; Ganesh *et al.*, 2011; Saghazadeh *et al.*, 2015). Thus, in these settings low Zn^{2+} levels may be simply linked to nutritional deficiency. However, in countries where Zn^{2+} intake is adequate patients with FS still have 15-20% lower serum Zn^{2+} levels when compared to those with afebrile seizures (Lee *et al.*, 2012). Although it is still an open question as to why Zn^{2+} levels are lower in FS patients with normal diet intake there is an emerging and strengthening relationship between elevated serum markers of inflammation and Zn^{2+} deficiency (Ghashut *et al.*, 2016; Jung *et al.*, 2015; Murr *et al.*, 2012). Further, in a pig model of peritonitis, serum Zn^{2+} levels drop by half in 2 hours post-infection and over 75% by 6 hours, with free Zn^{2+} also falling (Hoeger *et al.*, 2015). Acute sepsis or severe malaria are also associated with low serum Zn^{2+}, and these levels correlate with changes in inflammatory markers (Ghashut *et al.*, 2016; Hoeger *et al.*, 2015). Zn^{2+} is essential to the

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execution and regulation of both innate and humoral immune responses, primarily through its capacity to act as a structural and/or catalytic protein cofactor (Rink *et al.*, 2007). Within hours of pathogen detection or trauma, inflammatory cytokines like TNF trigger the rapid sequestration of free Zn^{2+} from the serum to serve these functions (Cousins *et al.*, 1988). Intracellular Zn^{2+} quickly works as part of a negative feedback loop to extinguish the inflammatory response and restore Zn^{2+} homeostasis (Liu *et al.*, 2013), but in some cases inflammatory hypozincemia can persist for days or weeks, and is associated with worse prognosis in patients suffering severe forms of systemic inflammation like sepsis (Wong *et al.*, 2007).

It has been established that chronic dietary Zn^{2+} deficiency ultimately reduces the pool of Zn^{2+} available to neurons (Fukahori *et al.*, 1990), but it is not yet known if rapid inflammatory sequestration of serum Zn^{2+} can similarly deplete synaptic Zn^{2+} levels. Given their role in Zn^{2+} transporter modulation and Zn^{2+} sequestration by other cell types, it is also possible that early phase inflammatory cytokines can modulate synaptic Zn^{2+} levels directly. Both possibilities are areas of active investigation given FS, as its name suggests, is comorbid with infection and inflammation. We will now discuss the specific evidence linking *synaptic* Zn^{2+} levels and FS susceptibility.

Synaptic Zn^{2+} levels and FS

A significant proportion of free Zn^{2+} in the brain is found co-localized with glutamate in synaptic vesicles. Zn^{2+} is concentrated within glutamatergic vesicles exclusively by the solute carrier, Zn^{2+} transporter 3 (ZnT3) (Cole *et al.*, 1999). This Zn^{2+} is released in an activity-dependent manner resulting in concentrations as high as 100-300μM in the extracellular space (Pan *et al.*, 2011; Qian *et al.*, 2005; Vergnano *et al.*, 2014; Vogt *et al.*, 2000). Synaptic Zn^{2+} is present primarily in cortical and limbic structures and is enriched in the hippocampus (Frederickson *et al.*, 2005), a brain region frequently implicated in FS (Dube *et al.*, 2006; Wimmer *et al.*, 2010). A mouse ZnT3 gene knock-out strain exhibits

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increased susceptibility to pro-convulsant drug induced seizures (Cole *et al.*, 2000). Our own work demonstrated an increased susceptibility of ZnT3 null mice to FS (Hildebrand *et al.*, 2015), using environmental heat to increase core body temperature and trigger thermogenic seizures (Reid *et al.*, 2013; Richards *et al.*, 2013). Collectively, these data implicate low *synaptic* Zn^{2+} as a susceptibility factor in FS.

Genetic Susceptibility, Synaptic Zn^{2+} and FS

Genetic factors play an important role in FS susceptibility. About 25% of FS patients have a family history (Kjeldsen *et al.*, 2002) and monozygotic twins have a higher FS concordance than dizygotic twins (Eckhaus *et al.*, 2013). Based on the central role of synaptic Zn^{2+} in modulating excitability and clinical evidence implicating low cerebrospinal fluid and blood levels in FS we took a candidate gene approach and screened *SLC30A3*, that encodes ZnT3, in a FS population (Hildebrand *et al.*, 2015). The screen revealed a heterozygous variant (c.892C>T; p.R298C) that was enriched in FS populations from Australia and Europe but absent from population-matched controls. The variant is reported at over 10-fold lower frequency on the Exome Aggregation Database with a case-control analysis giving an odds ratio of about 11 (Hildebrand *et al.*, 2015). Given a lifetime prevalence of 2-3%, the 10 fold increase in risk would lead to an absolute risk of over 1 in 5 of developing FS for a patient harbouring the variant. Functional analysis of the variant demonstrated a loss-of-function due to a trafficking deficit (Hildebrand *et al.*, 2015). Taken together, the genetic, clinical and experimental animal data we have outlined here support the idea that a loss of synaptic Zn^{2+} is likely to increase the susceptibility of an individual to FS.

Low Synaptic Zn^{2+} and Neuronal Excitability

Multiple potential mechanisms may underlie increased neuronal excitability under conditions of low synaptic Zn^{2+} because extracellular Zn^{2+} interacts with a range of ion channels, receptors and transporters (Marger *et al.*, 2014). In particular, Zn^{2+} directly inhibits a number of ionotropic receptors commonly found at central synapses (Paoletti *et al.*, 2009). This

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includes $GABA_A$, AMPA and NMDA receptors. Zn^{2+} block of $GABA_A$ receptors has been implicated in the underlying pathogenesis of epilepsy (eg.(Buhl *et al.*, 1996)); here block of $GABA_A$ receptors are proposed to result in reduced neuronal network inhibition. Zn^{2+} has also been shown to inhibit astrocyte glutamate uptake potentially increasing the activation of both AMPA and NMDA receptors (Carver *et al.*, 2016; Suh *et al.*, 2007). However, as presented above, FS associates with reductions in Zn^{2+} levels which might be expected to lead to increased $GABA_A$-mediated transmission and increased glutamate uptake and therefore these proposed mechanisms seem unlikely contributors to increased excitability.

AMPA and NMDA receptors are localized in the postsynaptic density of glutamatergic synapses and are therefore well positioned to be modulated by synaptic Zn^{2+}. The role of AMPA receptors in driving excitability Zn^{2+} is unclear given the conflicting evidence as to whether endogenously released synaptic Zn^{2+} modulates these receptors (Kalappa *et al.*, 2015; Vergnano *et al.*, 2014). NMDA receptors stand out as a target for Zn^{2+} because of their high sensitivity, with levels as low as 10 nM causing significant inhibition (Chen *et al.*, 1997; Paoletti *et al.*, 1997; Traynelis *et al.*, 1998). Furthermore, Vergnano and colleagues demonstrate that activity-dependent release of Zn^{2+} at hippocampal synapses modulates NMDA-mediated excitability (Vergnano *et al.*, 2014). This impact on excitability is limited to higher stimulation frequencies, a situation likely to occur during neuronal hyperexcitability induced by high brain temperatures (Mizunuma *et al.*, 2009). Vergnano and colleagues also show that trains of evoked NMDA receptor-mediated synaptic events are significantly larger in ZnT3 null mice. As discussed above ZnT3 knockout mice are more sensitive to heat-induced seizures that model FS (Hildebrand *et al.*, 2015). A variety of mechanistic models could be proposed to explain excitability. However, a model where heat-mediated increases in hippocampal neuronal excitability cannot be constrained when synaptic Zn^{2+} levels are too low to modulate NMDA receptor activity provides a simple solution to explain increased FS susceptibility (Figure 1).

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Therapeutic Interventions

The possibility of recurrence of FS is high with approximately one-third of patients having a second occurrence (Annegers *et al.*, 1990). Reducing this risk in children would have significant clinical and economic implications since FS accounts for over 1 in 200 paediatric emergency department (ED) admissions (Martindale *et al.*, 2011). Moreover, there is some evidence that long febrile seizures may result in hippocampal damage and later epilepsy (Annegers *et al.*, 1987), so prevention of FS recurrence might reduce the frequency of later epilepsy. However, given the often self-limiting nature of FS it is imperative that any therapeutic intervention is safe and well tolerated. An obvious therapeutic option is supplementation of Zn^{2+} in FS patients who are shown to be deficient. Zn^{2+} can be given orally with Zn^{2+} acetate the most common administered and well tolerated form. Supporting this idea is the finding that Zn^{2+} supplementation can be protective in the hyperthermia-induced FS model in rats (Aydin *et al.*, 2016). However, one overriding concern for this approach is how effective it is in increasing brain levels of Zn^{2+} quickly. This is because the uptake of Zn^{2+} across the blood brain barrier is mediated through active transport and is highly regulated (Grabrucker *et al.*, 2011). This may limit the effectiveness of Zn^{2+} supplementation in FS since higher brain levels are likely to be required to be protective.

Alternative mechanisms of increasing Zn^{2+} brain levels rapidly need to be considered. One example is the development of Zn^{2+} loaded nanoparticles that may facilitate the delivery of Zn^{2+} to the brain (Chhabra *et al.*, 2015). Facilitating Zn^{2+} uptake by enhancing transport into the brain and individual neurons may also be possible. ZIP1, ZIP3 and ZnT1 are major transporters of Zn^{2+} in neurons (Qian *et al.*, 2011; Qin *et al.*, 2009) and are therefore good potential molecular targets. Further, given the proposed importance of synaptic Zn^{2+} in the underlying FS mechanism, agents that increase the activity of ZnT3 may be worth investigating. This could be either through direct activation or through secondary molecular targets. For example, Zn^{2+} transport activity by ZnT3 in neuronal cells can be potentiated by the over expression of the CLC-3 chloride channel (Salazar *et al.*, 2004).

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Future Directions

The evidence associating low Zn^{2+} serum and cerebrospinal fluid levels to increased risk of FS is compelling. Much of the clinical data collected to date has been in developing countries implicating dietary deficiency in Zn^{2+} as a potential risk factor in FS. However, prospective studies in which Zn^{2+} levels are measured in children and correlated with subsequent FS occurrence are required to better answer this question. Importantly, therapeutic intervention studies in which patients with FS are treated with Zn^{2+} supplementation will determine if recurrence can be reduced. As discussed, it is still unclear if chronic dietary Zn^{2+} deficiency or acute inflammatory Zn^{2+} depletion alone is sufficient to trigger the cascade of events leading to FS. Animal models of FS in which Zn^{2+} levels are manipulated will provide further mechanistic insight. Variation in ZnT3 leading to increased FS susceptibility is rare. However, deficiencies at other points in the Zn^{2+} transport pathway could have the same net effect. A candidate approach focusing on other known Zn^{2+} transporters is therefore worth considering. *SLC30A1* (ZnT1), *SLC39A1* (ZIP1) and *SLC39A3* (ZIP3) are good candidates given their role in controlling Zn^{2+} flux in neurons (Qian *et al.*, 2011; Qin *et al.*, 2009). Other gene candidates involved in Zn^{2+}-related mediation of downstream excitability include the GluN2B and GluN2A receptor subunits encoded by *GRIN2A* and *GRIN2B* respectively. Interestingly, genetic studies in epilepsy have identified mutations in NMDA receptors that alter Zn^{2+} sensitivity (Lesca *et al.*, 2013; Yuan *et al.*, 2014), although both decreases and increases have been observed (Lesca *et al.*, 2013; Serraz *et al.*, 2016; Yuan *et al.*, 2014).

Conclusions

FS has a significant clinical and economic impact with few proven preventative or therapeutic strategies currently available in the clinic. The evidence associating low Zn^{2+} serum and cerebrospinal fluid levels to increased risk of FS is strong (Saghazadeh *et al.*, 2015). The increased susceptibility of the ZnT3 null mouse to thermogenic seizures and the enrichment of a loss-of-function ZnT3 variants in patients directly link a reduction in

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synaptic Zn^{2+} to FS susceptibility (Hildebrand *et al.*, 2015). Data presented here support the idea that restoring synaptic Zn^{2+} concentrations is a potential therapeutic strategy that is simple and inexpensive, with future research required to test this idea both at the pre-clinical and clinical levels.

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Figure 1: A simplified cartoon of how FS and synaptic Zn^{2+} may be linked. Low synaptic Zn^{2+} through genetic or environmental factors results in the reduction of Zn^{2+} co-expressed with glutamate. During a fever the rate of hippocampal firing is increased. Under normal conditions the accumulation of Zn^{2+} in the synaptic cleft acts to block NMDA receptors and constrains excitatory synaptic input. When synaptic Zn^{2+} is low NMDA receptors contribute more to depolarising currents resulting in voltage changes that cross action potential firing threshold. This 'unconstrained' excitability is hypothesised to lead to enhanced FS susceptibility.

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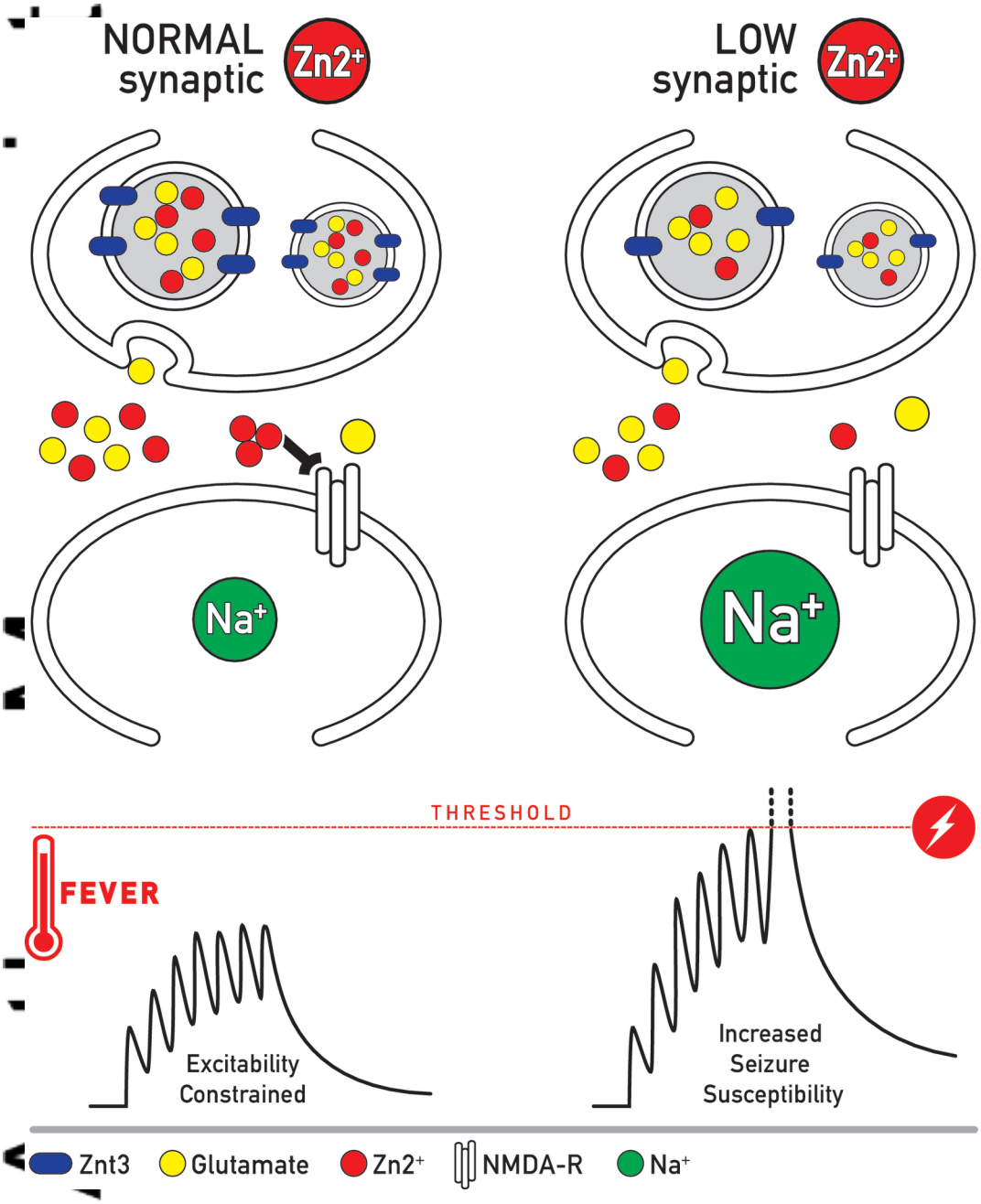
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