

# Are the transient receptor potential melastatin (TRPM) channels important in magnesium homeostasis following traumatic brain injury?

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**Abstract.** Traumatic brain injury (TBI) confers a major burden to Western society and effective treatments are urgently required to improve the long-term deficits that inflict TBI survivors. Depletion of intracellular  $Mg^{2+}$  is a well-known phenomenon occurring after TBI and is associated with poor neurological outcome. However, despite success in pre-clinical experimental studies, therapies utilizing  $Mg^{2+}$  have not always proven to be clinically effective. Recent evidence implicates members of the transient receptor potential melastatin (TRPM) channel family in processes leading to neuronal cell death following ischemic injury, however, the exact mechanism by which this occurs is not completely understood. Specifically, TRPM7 and TRPM6 are two channels that have been identified as potentially playing a role in regulating  $Mg^{2+}$  homeostasis, although whether this role in magnesium regulation and neuronal injury is significant is controversial. The purpose of this review is to explore the relationship between TRPM family members and  $Mg^{2+}$  homeostasis, including their potential involvement in secondary injury processes leading to cell death following TBI.

**Key words:** neurotrauma, ischemia, magnesium transporters, free magnesium, calcium

## Traumatic brain injury

Traumatic injury to the central nervous system (CNS) is the leading cause of death and disability in people under 40 years of age [1]. Worldwide incidence rates of traumatic brain injuries (TBI) are estimated at 150-200 cases per 100,000 population per annum [2]. Motor vehicle accidents account for the majority of moderate and severe TBI cases, whereas falls and sporting accidents are responsible for most mild injuries [3]. Despite the major public health significance of TBI, there is currently no effective treatment regime and survivors are left with debilitating long-term motor, cognitive and behavioural deficits.

TBI is defined as craniocerebral trauma associated with decreased level of consciousness, amnesia, other neurological or neuropsychological

abnormalities, skull fracture, intracranial lesions or death [4]. The neurological dysfunction resulting from TBI is due to both direct, immediate mechanical damage to brain tissue (the primary injury) and indirect, delayed (secondary) injury mechanisms [5]. The primary event is irreversible and includes both focal (e.g. contusion, laceration) and diffuse (e.g. concussion, diffuse axonal injury) lesions [6]. In contrast, secondary injury is comprised of a series of complex biochemical changes that are triggered by the primary event and may continue for days to weeks after the insult [7]. These changes include disruption to the blood brain barrier (BBB), oedema, ischemia, hypertension, inflammation, excitotoxicity and oxidative stress, all of which can be deleterious to neuronal cells [6, 8, 9].

## **Magnesium decline following TBI**

Magnesium has been implicated as a crucial component of the secondary injury cascade that follows brain injury. Indeed, several lines of evidence suggest that magnesium deficit is associated with poor neurological outcome following TBI. A significant decline in serum ionised magnesium ( $Mg^{2+}$ ) levels has been measured in TBI patients, with the magnitude of  $Mg^{2+}$  decline being associated with the severity of TBI [10]. A decrease in brain intracellular free  $Mg^{2+}$  concentration has also been demonstrated after TBI in rats, and this is associated with the development of neurological deficits [11, 12]. Another study [13] examined the consequences of magnesium deficiency prior to TBI in rats, and found that the  $Mg^{2+}$ -deficient group had significantly greater cortical cell loss compared to the vehicle group, as well as cytoskeletal alterations in cortical and hippocampal neurons.

$Mg^{2+}$  deficiency in the absence of injury has been shown to evoke an inflammatory response in rats, resulting in leukocytosis, hyperplasia and increases in pro-inflammatory cytokines and substance P [14]. Low  $Mg^{2+}$  caused lipid peroxidation and activation of NF-KB in canine primary cerebral vascular smooth muscle cells [15]. This is relevant to TBI since lipid peroxidation can generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), thus activating multiple signalling pathways that may result in cell death. Finally, low  $Mg^{2+}$  diet in rats over generations led to a significant loss of dopaminergic neurons in the substantia nigra, suggesting a role of  $Mg^{2+}$  in the pathogenesis of Parkinson's disease [16].

## **Physiological role of magnesium**

$Mg^{2+}$  plays a vital role in a number of diverse biological processes and is an essential co-factor required for the function of numerous enzymes [17, 18].  $Mg^{2+}$  is necessary for all reactions that either consume or produce ATP, including glycolysis, oxidative phosphorylation and cellular respiration [19].  $Mg^{2+}$  also plays an important role in protein synthesis [20], the cell cycle and normal neuronal functioning [21] and is often referred to as a physiological calcium blocker [22]. Furthermore,  $Mg^{2+}$  maintains the stability, integrity and normal function of the cell membrane, and is essential for the activity of the membrane  $Na^+/K^+$ -ATPase [23].  $Mg^{2+}$  also modulates other ion transport pumps, carriers and channels, and therefore is

likely to be involved in the regulation of signal transduction and the intracellular concentrations of ions such as  $K^+$  and  $Ca^{2+}$  [24].

## **$Mg^{2+}$ neuroprotection in TBI**

Since magnesium is crucial to so many physiological processes, it is clear that a decline in  $Mg^{2+}$  concentration, such as that resulting from TBI, will adversely affect normal cellular functioning, the maintenance of membrane potential and the capacity for cells to undergo repair [25]. Indeed, several groups have investigated  $Mg^{2+}$  as a potential multifactorial therapy for TBI, since it is able to modulate many processes of the secondary injury cascade. For example, magnesium ions are able to regulate excitotoxic processes by blocking voltage- and ligand-gated  $Ca^{2+}$  channels, including N-methyl-D-aspartate (NMDA) receptors [26] and other voltage-gated calcium channels [27], thus acting as a  $Ca^{2+}$  antagonist.  $Mg^{2+}$  may also have protective effects on the BBB, thereby reducing the formation of vasogenic oedema [19]. It has also been demonstrated that  $Mg^{2+}$  inhibits the formation of reactive oxygen species [28] and potentiates presynaptic adenosine and relaxes vascular smooth muscle cells [29]. Adenosine stimulation has been reported to reduce neuronal damage and mortality in stroke [30]. Finally,  $Mg^{2+}$  preserves mitochondrial membrane potential and has been shown to improve oxidative phosphorylation and decrease lactic acid production when administered post-TBI [19]. Additional properties that render magnesium an attractive therapeutic agent are its low cost, ease of use, low risk of adverse effects and its ability to penetrate the BBB [27].

It is therefore not surprising that several groups have investigated whether  $Mg^{2+}$  administration reduces the mortality and morbidity associated with TBI. Post-TBI  $Mg^{2+}$  administration in rats significantly reduced cortical cell loss compared to the vehicle group [13] and was also shown to reduce apoptosis and the expression of the apoptosis-regulating proteins, p53 [31], Bax and Bcl-2 [32]. Another study [33] showed that magnesium chloride attenuated the neurological motor deficits in brain-injured rats. Research conducted in our own laboratory with magnesium salts [34] has also demonstrated a neuroprotective effect of  $Mg^{2+}$  following TBI.  $Mg^{2+}$  has been shown to improve neurological outcome when administered up to 24 hours after injury [35], however, the best results have been achieved when the therapeutic

window is restricted to 12 hours [25]. The study by Heath and Vink [11] found that the  $Mg^{2+}$  decline persisted for at least 4 days after injury. However, the concentration of  $Mg^{2+}$  in CNS injury has been shown never to fall below 0.2 mM [12]. Thus, it is likely that the length of time for which free  $Mg^{2+}$  concentration is reduced, rather than the magnitude of decline, is the parameter that influences neurological outcome [25]. It is clear that there exists substantial evidence that  $Mg^{2+}$  deficiency plays a role in the secondary injury cascade of TBI and that administration of magnesium salts is neuroprotective in experimental TBI.

Despite the positive results obtained using  $Mg^{2+}$  as a therapy for TBI in animal models, a recent phase III clinical trial [36] found that  $MgSO_4$  given to patients for 5 days after traumatic brain injury was not neuroprotective and possibly even had a negative effect on outcome. Given that all patients (including the control group) received magnesium to restore depleted serum levels to normal, it is unclear whether this restoration of serum magnesium level was sufficient to confer positive effects in all patients irrespective of the treatment group. This result was in marked contrast to the clinical trial reported by [37] which reported that acute Mg administration (less than 24 h) resulted in significant improvements in Glasgow outcome scores at 3 months, as well as in post-operative brain swelling and 1-month mortality. While differences in trial results have been ascribed to possible limitations in central magnesium transport [38], the potential role of the more recently described magnesium transporters in brain magnesium homeostasis and neuronal cell death has not been critically assessed.

### **Transient receptor potential melastatin (TRPM) channels**

Of all the potential eukaryotic magnesium transporters that have been identified to date, the recent discovery and description of the magnesium transporting transient receptor potential (TRP) channel family has generated the most interest. The TRP channel family is a diverse group of ion channels consisting of about 30 known members. The general properties of TRP channels are discussed in a number of excellent general reviews [39-43] and will not be discussed in detail here. Of interest to this review is the TRP melastatin (TRPM) subfamily that contains eight members, designated TRPM1-TRPM8. TRPM proteins are a heterogeneous group of ion channels with diverse expression patterns, permeabilities, activation mechanisms and physiological

functions [44-46]. The ubiquitously expressed TRPM7 is permeable to a wide range of divalent metal ions, including  $Zn^{2+}$ ,  $Ni^{2+}$ ,  $Ba^{2+}$ ,  $Co^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Sr^{2+}$ ,  $Cd^{2+}$  and  $Ca^{2+}$  [47], and is one of only a few identified mammalian  $Mg^{2+}$  transporters (see [48] for review). It consists of an ion channel fused to a protein kinase domain [49] and has been implicated in many cellular processes including synaptic transmission [50], the cell cycle [51], normal growth and development [52], regulation of vascular smooth muscle cells [53] and the proliferation of human retinoblastoma cells [54]. While deletion of TRPM7 has been shown to disrupt embryonic development without altering  $Mg^{2+}$  homeostasis [55], evidence from a number of other studies suggests that TRPM7 is necessary both for cell survival and potentially for magnesium homeostasis. Genetic deletion of TRPM7 in DT-40 chicken lymphocytes resulted in non-viable cells [49]. In another study [56], TRPM7-deficient cells were  $Mg^{2+}$ -deficient and growth arrested, but the viability and proliferation of these cells were rescued by supplementation of extracellular  $Mg^{2+}$ . The addition of high levels of other cations was ineffective in substituting for the loss of TRPM7, suggesting that TRPM7 regulates  $Mg^{2+}$  homeostasis in eukaryotic cells.

TRPM7 activity is inhibited by free intracellular  $Mg^{2+}$  and Mg. ATP complexes, and is strongly activated when intracellular Mg.ATP and  $Mg^{2+}$  concentrations are depleted [49, 57-60]. TRPM7 may also be regulated by G-protein-coupled receptors, either *via* the cyclic AMP (cAMP) and protein kinase A (PKA) pathway [61], or the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) [62]. Considering the vital role fulfilled by TRPM7 with regard to cell viability and  $Mg^{2+}$  homeostasis, mutations in TRPM7 could be expected to result in severe pathological consequences [63]. Indeed, the zebrafish *touchtone/nutria* phenotype, resulting from mutations in the TRPM7 gene, displayed growth retardation and serious alterations in skeletal development [52].

The pathogenesis of complex neurodegenerative disorders is believed to involve both genetic and environmental factors [64]. Two such disorders, amyotrophic lateral sclerosis and Parkinsonism-dementia complex of Guam (ALS-G and PD-G, respectively), have been found with a relatively high incidence on islands in the Western Pacific. Environmental factors such as deficiencies in dietary  $Ca^{2+}$  and  $Mg^{2+}$ , toxins from the cycad plant and high exposure to aluminium, manganese and other toxic metals may act as triggers for these

diseases [65]. With regard to genetic factors, Hemo-sura *et al.* [66] reported a missense mutation in the TRPM7 gene, Thr1482Ile, in a subgroup of ALS-G and PD-G patients but not in matched controls. The protein encoded by this variant displays a higher sensitivity to inhibition by levels of intracellular  $Mg^{2+}$ . Thr1482, which lies between the channel and kinase domains of TRPM7 is evolutionarily conserved between many species, and phosphorylation of Thr in this position is likely to be important for channel function. Isoleucine cannot be phosphorylated, therefore, the mutant allele found in ALS-G and PD-G patients could potentially confer a functional deficit. Since TRPM7 is important in maintaining homeostasis of  $Mg^{2+}$  and  $Ca^{2+}$ , the authors propose that the higher sensitivity to  $Mg^{2+}$  of the TRPM7 variant, combined with the  $Mg^{2+}$ - and  $Ca^{2+}$ -deficient environment, could result in severe cellular deficiencies of these metal ions, which may contribute to the aetiology of diseases such as ALS-G and PD-G [66]. The same group has recently reported a missense mutation of the TRPM2 gene (Pro1018Leu) in ALS-G and PD-G patients, which resulted in TRPM2 channels that were unable to sustain ion influx [65].

TRPM6 is the closest relative of TRPM7, with which it shares 50% homology at the amino acid level [67]. TRPM6 is able to form homomeric channels, as well as heteromeric channels with TRPM7, which are biophysically and pharmacologically distinguishable [68]. TRPM6 is mainly expressed in the kidney and small intestine [69], but has also been localised in the brain [70]. Mutations in the TRPM6 gene have been identified as the cause of hypomagnesaemia with secondary hypocalcaemia (HSH) [69, 71, 72], an autosomal recessive disorder characterised by low serum levels of  $Mg^{2+}$  and  $Ca^{2+}$  [73]. TRPM7 and TRPM6 contain serine/threonine protein kinase domains resembling elongation factor 2 (eEF-2) kinase and other atypical alpha-kinases [74]. The protein kinase domain of TRPM7 is able to phosphorylate serine and threonine residues of various substrates, as well as to undergo auto-phosphorylation [75]. Interestingly, TRPM6 is able to phosphorylate TRPM7, but not *vice versa* [76].

### **TRPM channels and ischemic cell death**

Over recent years, TRPM channels have gained attention as possible therapeutic targets for ischemia. TRPM7 and TRPM2 have been implicated in playing direct roles in  $Ca^{2+}$ -mediated neuronal death [77], although the exact mechanism by which this occurs requires further investigation. It

has been proposed that TRPM7 mediates cell death *via* a positive feedback loop whereby  $Ca^{2+}$  entry into cells as a result of injury causes the production of free radicals, which activate TRPM7, leading to further  $Ca^{2+}$  influx and additional free radical production [78]. Indeed, the activation of TRPM7 during ischemia is proposed to be a key factor contributing to excitotoxicity and other deleterious processes [79]. Consistent with this proposal, suppressing TRPM7 expression in CA1 hippocampal neurons has been shown to be protective against damage following ischemia [80].

TRPM2 is a non-selective cation channel, which is highly permeable to  $Ca^{2+}$ , and expressed in a wide range of human tissues, including immune cells and brain [81-83]. TRPM2 is activated in response to oxidative and nitrosative stress, and several groups have shown that activation of TRPM2 by reactive oxygen species, such as hydrogen peroxide, results in cell death *via* unregulated  $Ca^{2+}$  influx [84-86]. TRPM2 is also activated by ADP-ribose [87], levels of which are elevated in response to oxidative stress (for review, see [88]). TRPM2 has recently been shown to aggravate inflammation, specifically as a key participant in monocyte chemokine production induced by  $H_2O_2$  [89]. TRPM2 may form multimers with TRPM7, however this has yet to be demonstrated conclusively. Interestingly, silencing of TRPM7 also resulted in the silencing of TRPM2, suggesting that the expression of these proteins may be co-regulated [77, 78]. The role of TRPM2 and TRPM7 in ischemic neuronal cell death has been comprehensively reviewed in several articles [78, 90, 91]. The role of TRPM6 in these processes is currently unclear, however, given its localisation in the brain, its permeability to  $Mg^{2+}$ , ability to form functional heteromers with, and to phosphorylate, TRPM7, it too may be a mediator of cell death.

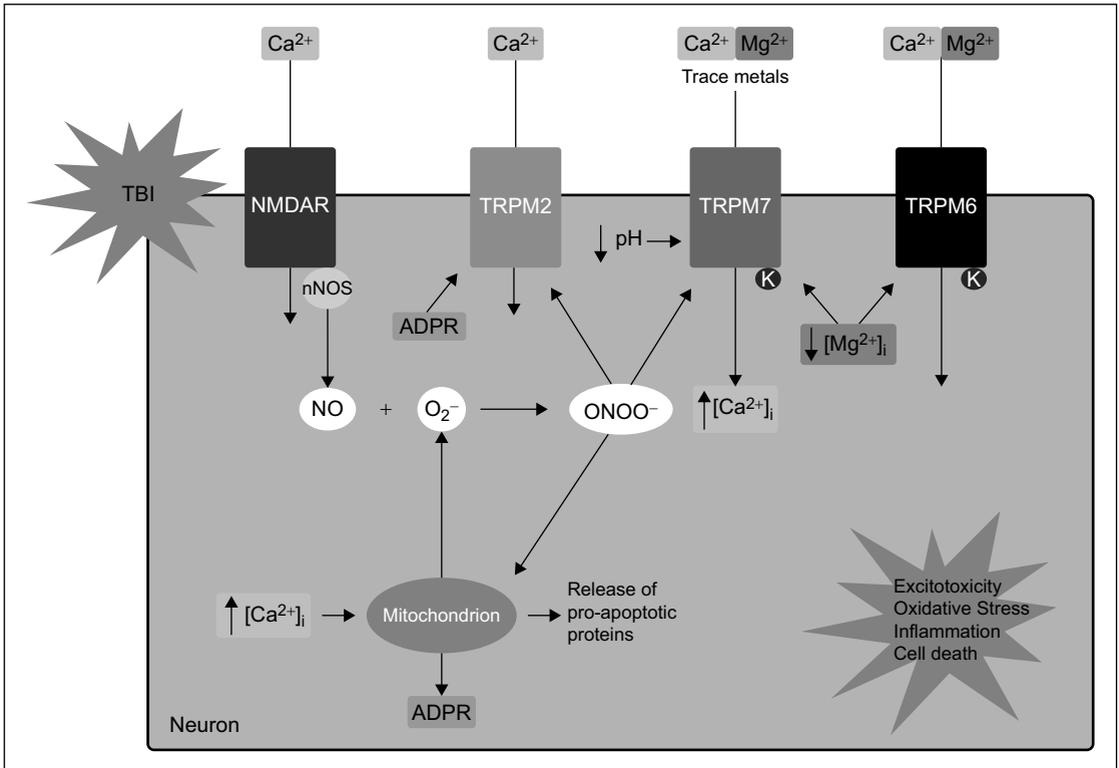
### **TRPM channels and TBI**

There are a number of secondary injury factors that contribute to neuronal cell death after TBI. Excitotoxicity resulting from  $Ca^{2+}$  influx through n-methyl-D-aspartate (NMDA) receptors is one mechanism of delayed cell death following CNS injury [79]. Several other secondary injury processes including oxidative stress, oedema formation, apoptosis and necrosis also require, to some extent, the influx of cations into neurons [92]. Therefore, non-selective cation channels, including TRPM channels, have gained attention as potential contributors to neuronal cell death.

There are several ways in which TRPM channels could contribute to cell death following TBI (figure 1). The role of TRPM2 and TRPM7 in ischemic neuronal death, as discussed in the previous section, is clearly established and is extremely relevant to TBI. Oxidative stress and the production of free radicals have been demonstrated to be key secondary injury factors in TBI [8, 9]. Since both TRPM2 and TRPM7 are activated by reactive oxygen and nitro-

gen species (ROS and RNS, respectively), oxidative stress could lead to the production of positive feedback loops, whereby unregulated  $\text{Ca}^{2+}$  influx *via* TRPM2 and TRPM7 channels stimulate secondary signalling pathways that further enhances oxidative stress, leading to tissue damage and cell death.

Deficits in  $\text{Mg}^{2+}$  concentration, as have been demonstrated following TBI, can also lead to the generation of ROS and RNS [15], further activating



**Figure 1.** Potential mechanisms of TRPM channel-mediated cell death in traumatic brain injury (TBI). Following TBI, the entry of  $\text{Ca}^{2+}$  into cells *via* the n-methyl-D-aspartate receptor (NMDAR) stimulates neuronal nitric oxide synthase (nNOS) leading to the production of nitric oxide (NO). Rises in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) promote  $\text{O}_2^-$  release from mitochondria, which reacts with NO to produce highly reactive peroxynitrite radicals (ONOO<sup>-</sup>). Free radicals damage cellular macromolecules and further activate TRPM2 and TRPM7 channels, allowing the influx of even more  $\text{Ca}^{2+}$ . Mitochondria also produce adenosine diphosphate ribose (ADPR), which stimulates TRPM2. TBI causes  $[\text{Mg}^{2+}]_i$  depletion and decreases in cellular pH, which activates TRPM7 and TRPM6 (and TRPM7/TRPM6 dimers). The kinase domains (K) of TRPM7 and TRPM6 may also influence signaling processes and inflammation. Disruption to the BBB allows water, proteins and metal ions to enter the brain (not shown); toxic trace metals (such as  $\text{Zn}^{2+}$ ) may enter cells through TRPM7, while inflammatory mediators (such as tumor necrosis factor- $\alpha$ ) further activate TRPM2. Prolonged depletion of  $\text{Mg}^{2+}$  and excess production of ROS and RNS could exacerbate this positive feedback loop and overactivate TRPM2 and TRPM7 (and potentially TRPM2/TRPM7 dimers). The resultant unregulated  $\text{Ca}^{2+}$  influx leads to excitotoxicity, enhances oxidative stress and inflammatory processes, and eventually activates pro-apoptotic signaling cascades that result in cell death.

TRPM7 and TRPM2, thereby enhancing inflammation, oxidative stress and cell death. ATP is also depleted as a result of severe brain injury [9]. As discussed, low intracellular  $Mg^{2+}$  and  $Mg$ .ATP levels strongly activate TRPM7 [47, 49, 93]. Given that  $Mg^{2+}$  levels remain suppressed for several days following injury [11], this is potentially a critical and persistent pathway leading to cell death after TBI. Furthermore, free radicals increase microvascular permeability, and have been shown to cause blood-brain barrier disruption and oedema following ischemia [94]. Indeed, oedema is one of the most important secondary injury factors in TBI with respect to patient outcome [95]. Changes in extracellular  $Ca^{2+}$  concentrations as a result of injury also activate TRPM7 [90] and TRPM2 [96]. All of these factors could potentiate the cell death process by the generation of free radicals and causing the sustained activation of TRPM2, TRPM7 and TRPM2/TRPM7 multimers. TRPM6 may also be involved in these processes, either alone or by association with TRPM7, however, this requires further investigation.

TBI results in massive influxes of  $Zn^{2+}$  ions to neurons, which is a major factor in neuronal cell toxicity and death [97, 98]. While voltage-gated calcium channels have been proposed to carry this current, zinc could also enter cells through TRPM7 channels, which is permeable to a wide range of divalent cations including  $Zn^{2+}$ . Although trace metal ions are necessary for the catalytic function of enzymes and normal cellular function, their accumulation above trace levels is highly toxic [47]. Precise regulation of ion channels such as TRPM7 is vital to maintain normal physiological conditions and their overactivation in pathological processes like TBI may lead to cell death. TRPM7 has a low, constitutive activity in resting cells that is likely to provide a constant flow of  $Mg^{2+}$  and  $Ca^{2+}$  into the cell [46]. TRPM7 channel activity is strongly activated when  $Mg^{2+}$  is decreased. Therefore, the depletion of  $Mg^{2+}$  following TBI combined with increases in ROS and RNS, which further stimulate TRPM7 in a positive feedback loop, may result in impaired inhibition of TRPM7 activity. The resultant overactivation of TRPM7 could therefore result in extensive entry of cations other than magnesium into the cell thus resulting in significant cell death.

TRPM7 has also been implicated in the pathological response to vessel wall injury, which may be relevant to both the primary and secondary injury processes of TBI. At the time of insult, shearing of nerve fibres results in massive ion fluxes across

cell membranes, loss of membrane potential and rapid release of neurotransmitters from damaged neurons. This results in excitotoxicity and evokes an inflammatory response, which stimulates further pathological processes, eventually leading to apoptosis and necrosis [99]. In response to shear stress, which results in an increase in fluid flow, a significant number of TRPM7 channels accumulated at the plasma membrane in less than 2 minutes, and an increase in TRPM7 current was detected in vascular smooth muscle cells [100]. This rapid response by TRPM7 most likely makes it one of the first molecules to react to shear stress. TRPM7 was also identified as the stretch-activated channel that is activated by osmotic swelling in epithelial cells, and is involved in cellular volume regulation by providing a  $Ca^{2+}$ -influx pathway [101], which may be relevant to cerebral oedema following TBI.

TRPM7 could also participate in cell death after TBI by responding to changes in extracellular pH [102, 103]. High concentrations of protons may be generated in pathological processes like TBI, leading to an acidic ( $pH < 6.0$ ) state and thus enhancing TRPM7 activity. Finally, the TRPM7 kinase domain is able to phosphorylate annexin I, a  $Ca^{2+}$ - and phospholipid-binding protein originally described as a mediator of the anti-inflammatory actions of glucocorticoids [104]. The biological function of annexin I phosphorylation by TRPM7 is currently unknown, but may have relevance to TBI since both TRPM7 and annexin I have been implicated in cell death.

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## Conclusion

The secondary injury cascade resulting from traumatic brain injury involves numerous pathological processes that can lead to cell death. Deficits in intracellular  $Mg^{2+}$  concentration occurring following TBI are associated with poor neurological outcome, but despite promising experimental studies,  $Mg^{2+}$  has not been proven clinically effective. It is unlikely that targeting a single factor will result in a significant improvement in outcome. Members of the TRPM channel family have been implicated in ischemic neuronal death and may also play a role in the injury processes occurring after TBI. In particular, TRPM7, which is crucial to  $Mg^{2+}$  homeostasis and cell survival, could be a critical mediator of cell death following TBI. However, their role in magnesium transport seems less important than their facilitation of other cation fluxes, as well as

the kinase role in inflammatory processes. TRPM channels may therefore represent novel therapeutic targets as part of a multifactorial treatment strategy for TBI, although not likely a major target for regulating brain magnesium homeostasis.

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