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SYMPOSIUM REVIEW

Role of TRPM7 in cerebral ischaemia and hypoxia

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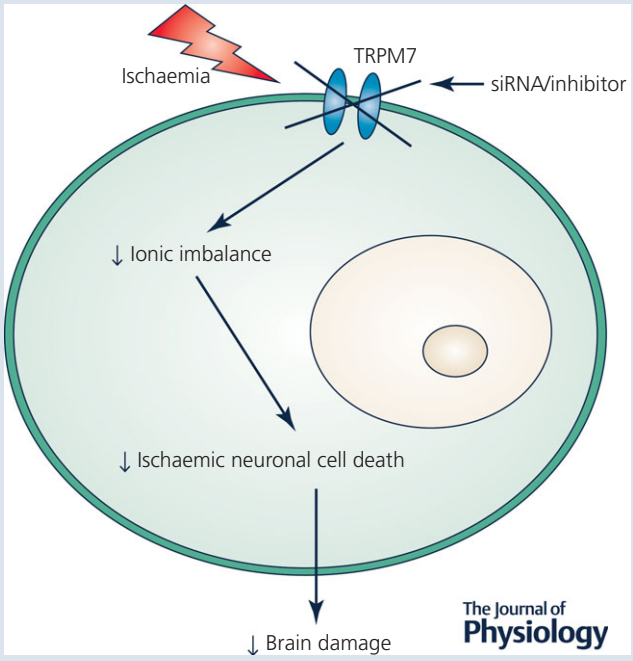

Abstract Transient receptor potential melastatin 7 (TRPM7) channel, a calcium-permeable non-selective divalent cation channel, is broadly expressed in various cells and tissues, including the brain. TRPM7 is thought to be coupled to the metabolic state and regulate calcium homeostasis in the cell. TRPM7 takes part in a wide range of cell biology processes that affect cell growth and proliferation, as well as in embryonic development and skeleton formation. TRPM7 plays a significant role in ischaemic and hypoxic brain injury and neuronal cell death. TRPM7, as a key non-glutamate mechanism of cerebral ischaemia, also triggers an intracellular ionic imbalance and neuronal cell death in ischaemia and hypoxia. We have reported that TRPM7 is expressed in neurons of the hippocampus and cortex and activation of TRPM7 induced ischaemic neuronal cell death; suppression of TRPM7 with virally mediated gene silencing using siRNA reduced

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ischaemic neuronal cell death and improved neurobehavioural outcomes *in vivo*. Recently, we also demonstrated that inhibition of TRPM7 using pharmacological means promoted neuronal outgrowth *in vitro* and provided neuroprotection against brain injury to hypoxia *in vivo*. Thus, we have shown the contributions of TRPM7 in many physiological and pathophysiological processes, including hypoxia and ischaemia.

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Abstract figure legend TRPM7, a calcium-permeable divalent cation channel, is an important player in the non-glutamate mechanism in stroke and mediates intracellular ionic imbalance and neuronal cell death. Inhibition of TRPM7 reduced neuronal cell death and brain damage in ischaemia and hypoxia.

Abbreviations AAV, adeno-associated virus; AET, anti-excitotoxic therapies; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASIC, acid-sensing ion channel; HIE, hypoxic–ischaemic encephalopathy; K_{ATP}, ATP-sensitive potassium channel; MCAO, middle cerebral artery occlusion; NCX, sodium–calcium exchanger; NMDA, *N*-methyl-D-aspartate; OGD, oxygen-glucose deprivation; STAIR, Stroke Therapy Academic Industry Roundtable; tPA, tissue plasminogen activator; TRP, transient receptor potential; TRPM7, transient receptor potential melastatin 7; VRAC, volume-regulated anion channel.

Introduction

Cerebral ischaemia and stroke (Dirnagl *et al.* 1999; Lipton, 1999) is a leading cause of mortality and a major cause of long-term immobility in the world based on statistics from the World Health Organization (WHO) (WHO, 2014; Mozaffarian *et al.* 2016). Stroke has a high mortality rate, and stroke prevalence is projected to increase. There is no effective treatment for stroke, except for the tissue plasminogen activator (tPA), which has a limited therapeutic window (Zivin, 2009). Stroke has already shown significant social and economic impacts worldwide (American Stroke Association, 2016). In addition, hypoxia could cause neonatal hypoxic–ischaemic brain injury and subsequent early-onset brain and behavioural disorders in children, termed hypoxic–ischaemic encephalopathy (HIE) (Vannucci, 2000; Nelson & Lynch, 2004). HIE is characterized by neurodevelopmental delay, motor and cognitive impairments, and epilepsy. Neonatal hypoxic–ischaemic brain injury and its related HIE have also caused noticeable burdens worldwide.

Stroke triggers intracellular calcium overload and ionic imbalance, eventually leading to neuronal cell death (Dirnagl *et al.* 1999; Lipton, 1999). The time course and sequence of events occurring in cerebral ischaemia includes (1) anoxic depolarization in seconds to minutes, (2) peri-infarct depolarization in minutes, (3) excitotoxicity in minutes, to later cause (4) apoptosis and (5) inflammation in days. During cerebral ischaemia, the excitatory neurotransmitter glutamate is released from the brain, acts on the glutamate receptor channels, and triggers a calcium overload and neuronal cell death (Dirnagl *et al.* 1999; Lipton, 1999). Excitotoxicity, mediated through NMDA and AMPA receptor channels (Besancon *et al.* 2008; Tymianski, 2011), has been the central focus of stroke research for decades. Preventing the calcium overload is theoretically considered to be neuroprotective. Blocking calcium-mediated glutamate receptor channels *in vitro* and *in vivo* inhibits intracellular calcium overload and prevents ischaemic brain damage. Even experimental studies have shown hopeful data; however, the subsequent clinical trials of anti-excitotoxic therapies (AET) could not support AET as a further therapeutic development (Davis *et al.* 2000). As a result, stroke researchers began searching for unconventional mechanism(s) outside the traditional glutamate mechanism. In addition to the traditional glutamate excitotoxicity mediated through NMDA and AMPA receptor channels (Besancon *et al.* 2008; Tymianski, 2011), new data indicates that a non-glutamate mechanism in cerebral ischaemia also causes intracellular ionic imbalance and neuronal cell death (Besancon *et al.* 2008; Tymianski, 2011). Ischaemic neuronal death is now accepted as a result of both glutamate-mediated excitotoxicity and the newly discovered non-glutamate mechanisms (Besancon *et al.* 2008; Tymianski, 2011). The newly accepted non-glutamate mechanism undeniably contributes to the disappointing results of the AET clinical trials. Thus, we may need to consider both glutamate and non-glutamate mechanisms in new drug development for stroke, as well as using multiple *in vivo* animal models of human disease based on the Stroke Therapy Academic Industry Roundtable (STAIR) Protocol (Stroke Therapy Academic Industry Roundtable

(STAIR), 1999), which emphasizes the necessity for testing potential stroke drugs using multiple animal stroke models in multiple species. The non-glutamate mechanism includes transient receptor potential (TRP) channels (Sun *et al.* 2009; Alim *et al.* 2013; Chen *et al.* 2015*a*), ATP-sensitive potassium (K_{ATP}) channels (Sun *et al.* 2006, 2007, 2015; Liu *et al.* 2016), acid-sensing ion channels (ASICs) (Xiong *et al.* 2004), hemichannels (Thompson *et al.* 2006), volume-regulated anion channels (VRACs) (Alibrahim *et al.* 2013), sodium–calcium exchangers (NCXs) (Pignataro *et al.* 2004), and other non-selective cation channels (Simard *et al.* 2006). Activation of these ion channels could be at the initial stages of cerebral ischaemia and/or later in the ischaemic events. For example, K_{ATP} channels are activated at the initiation of the ischaemic event during the anoxic depolarization; TRPM7 may be activated at the same time or shortly after the excitotoxicity; and TRPM2 may be activated in the later stages as it is involved in inflammation. Ion channels are the third largest target in drug development (Dabrowski *et al.* 2008). Thus, the non-glutamate mechanism is a target for neuroprotection.

Transient receptor potential channels

Ion channels play many fundamental roles in physiological and pathophysiological functions in the brain. We have been working on the non-glutamate mechanism in cerebral ischaemia and hypoxia, including TRP channels, K_{ATP} channels and VRAC channels. Here, the focus is on the role of TRP channels in cerebral ischaemia and hypoxia.

Transient receptor potential channels, also termed TRP channels, are a group of non-selective cation channels located on the cell membrane of various cell types (Clapham, 2003; Pedersen *et al.* 2005; Wu *et al.* 2010). TRP channels were originally discovered in fruit fly (*Drosophila*) (Minke *et al.* 1975) photoreceptors where they participated in phototransduction (Monteilh-Zoller *et al.* 2003). The channel received its name, transient receptor potential, because the *Drosophila* photoreceptors in the fly carrying the mutant *trp* gene initiated a transient response to light (Minke *et al.* 1975). The TRP channel superfamily now has approximately 30 mammalian TRP channels (Wu *et al.* 2010). The TRP channels, classified based on their homologous sequences, are divided into six sub-families: (1) TRPC (canonical), (2) TRPV (vanilloid), (3) TRPM (melastatin), (4) TRPA (ankyrin), (5) TRPML (mucolipin), and (6) TRPP (polycystin). TRP channels can be activated by different physical and chemical stimuli and play many physiological and pathological functions in various cells (Clapham, 2003; Pedersen *et al.* 2005; Wu *et al.* 2010).

The TRPM family has eight members, TRPM 1 to 8. TRPM7 (Clapham, 2003), the seventh member of the TRPM channel sub-family, is widely expressed in many tissues and cells including the brain and neurons. TRPM7 is a calcium-permeable non-selective divalent cation channel and is also permeable to other trace metal ions, i.e. $Zn^{2+} \approx Ni^{2+} >> Ba^{2+} > Co^{2+} > Mg^{2+} \geq Mn^{2+} \geq Sr^{2+} \geq Cd^{2+} \geq Ca^{2+}$ ions (Monteilh-Zoller *et al.* 2003).

TRPM7 plays an important role in a wide range of physiological and pathophysiological functions, and its channel activities can be modulated by diverse intracellular and extracellular factors (Penner & Fleig, 2007), such as Mg^{2+} and Mg^{2+}-complexed nucleotides (such as MgATP and MgGTP) (Takezawa *et al.* 2004; Demeuse *et al.* 2006), extracellular pH (Jiang *et al.* 2005; Li *et al.* 2007), shear stress (Oancea *et al.* 2006), etc. TRPM7 participates in a wide scope of cell biology processes ranging from cell proliferation, cell growth and cell adhesion (Nadler *et al.* 2001; Inoue & Xiong, 2009); TRPM7 overexpression reduces cell viability (Nadler *et al.* 2001; Su *et al.* 2006; Chen *et al.* 2010). TRPM7 also regulates embryonic development (Jin *et al.* 2008) and skeleton formation (Elizondo *et al.* 2005). Globally knocking out TRPM7 has been shown to be embryonically lethal in mice (Jin *et al.* 2008). Thus, TRPM7 is essential for development. TRPM7 channels are thought to be activated during ischaemia based on the metabolic state of the cell. Therefore, the favourable condition for TRPM7 activation during ischaemia would be low concentrations of Mg^{2+}-nucleotides (Demeuse *et al.* 2006) and acidic conditions (Rehncrona, 1985; Li *et al.* 2007).

In the brain, TRPM7 plays key roles both under physiological conditions, e.g. cell growth (Nadler *et al.* 2001; Inoue & Xiong, 2009; Turlova *et al.* 2016), and under pathophysiological conditions, e.g. hypoxia- and ischaemia-induced neuronal cell death (Aarts *et al.* 2003; Sun *et al.* 2009; Chen *et al.* 2015*a*) and survival of brain tumour cells (Chen *et al.* 2015*b*,*c*). In addition to showing the role of TRPM7 in ischaemia (Sun *et al.* 2009), we have recently also demonstrated the following: (1) inhibition of TRPM7 *in vitro* enhances neurite outgrowth and maturation in mouse culture hippocampal cells (Turlova *et al.* 2016); (2) TRPM7 plays a role in neonatal hypoxic–ischaemic brain injury in mice *in vivo* (Chen *et al.* 2015*a*); and (3) TRPM7 also plays an important role in cell survival in glioma cell lines *in vitro* (Chen *et al.* 2015*b*,*c*). Here, the role of TRPM7 in neuronal cell death and brain damage during ischaemia and hypoxia *in vivo* (Sun *et al.* 2009; Chen *et al.* 2015*a*), its pharmacology and its potential in drug development for stroke will be further discussed.

Molecular and pharmacological reagents are available for TRPM7; these include channel activators and blockers (Zierler *et al.* 2011; Chubanov *et al.* 2014; Chen *et al.* 2015*a*; Turlova *et al.* 2016), antibodies (Sun *et al.* 2009; Chen *et al.* 2015*a*,*b*; Turlova *et al.* 2016), and siRNA (Sun *et al.* 2009; Turlova *et al.* 2016). These will be beneficial

for studying TRPM7, and many studies have used various TRPM7 reagents.

TRPM7 plays a key role in anoxic cell death in cultured neurons (Aarts *et al.* 2003). We have demonstrated that TRPM7 plays a significant role in ischaemic brain damage and neuronal cell death *in vivo* (Sun *et al.* 2009). The study showed that virally mediated gene silencing of TRPM7 *in vivo* with siRNA increased neuronal cell survival and improved neurobehavioural outcomes after cerebral ischaemia (Sun *et al.* 2009). Later, we also showed that TRPM7 plays many important roles both *in vitro* and *in vivo*: inhibiting TRPM7 *in vitro* enhances hippocampal neuronal cell outgrowth (Turlova *et al.* 2016); blocking TRPM7 *in vivo* reduces brain damage in hypoxia (Chen *et al.* 2015*a*); and suppressing TRPM7 *in vitro* decreases glioma cell survival *in vitro* (Chen *et al.* 2015*b*,*c*).

TRPM7 in cerebral ischaemia and hypoxia

In the event of cerebral ischaemia and/or hypoxia, calcium overload and ionic imbalance inside the neuronal cells have been the accepted cellular and molecular mechanisms for ischaemic and/or hypoxic neuronal cell death and brain damage (Besancon *et al.* 2008; Tymianski, 2011). In addition to the traditional glutamate mechanism, which is mainly focused on glutamate receptor channel-mediated excitotoxicity, the non-glutamate mechanism also triggers intracellular ionic imbalance and initiates ischaemic and/or hypoxic neuronal cell death and brain damage in stroke (Besancon *et al.* 2008; Tymianski, 2011). The non-glutamate mechanism contains many other ion channels and newly described calcium-mediated non-selective cation channels, e.g. ATP-sensitive potassium channels (K_{ATP}) (Sun *et al.* 2006, 2007, 2015; Liu *et al.* 2016), transient receptor potential (TRP) channels (Sun *et al.* 2009; Alim *et al.* 2013; Chen *et al.* 2015*a*), volume-regulated anion channels (VRACs) (Alibrahim *et al.* 2013), hemichannels (Thompson *et al.* 2006), acid-sensing ion channels (ASICs) (Xiong *et al.* 2004), ion exchangers (Pignataro *et al.* 2004) and other non-selective cation channels (Simard *et al.* 2006). In this report, the focus is on the neuronal cell death and brain damage mediated by TRPM7 in stroke and hypoxia *in vivo*.

An early *in vitro* study reported that TRPM7 plays a key role in anoxic-induced neuronal cell death in culture (Aarts *et al.* 2003). This study originally described the so-called I_{OGD} currents in response to prolonged *in vitro* oxygen–glucose deprivation (OGD) challenge in primary culture cortical neurons, which eventually triggered a secondary neuronal cell death through a secondary increase in Ca^{2+} influx through the later identified TRPM7 (Aarts *et al.* 2003). The effects of the prolonged anoxic cell death were eventually unmasked with treatment of a cocktail of blockers aimed at blocking the glutamate NMDA and AMPA receptors and L-type calcium channels using MK-801, CNQX and nimodipine in the *in vitro* OGD experiment (Aarts *et al.* 2003). The study was facilitated by calcium imaging and electrophysiological approaches, as well as additional molecular biology verification. The *in vitro* study further used the TRPM7 siRNA directly against the TRPM7 in the mouse primary culture cortical neurons and showed that both the expression level of TRPM7 mRNA and the subsequent prolonged anoxia-mediated neuronal cell death were reduced. Thus, this was the first study that revealed the key role of TRPM7 in mediating Ca^{2+} influx and subsequent anoxic neuronal cell death *in vitro* during prolonged OGD. As OGD is a simplified *in vitro* anoxia model for studying neuronal cell death, it is not a good representation of 'ischaemia' because it lacks other factors *in vivo,* such as blood, neuronal circuitry, network connectivity and the involvement of other brain cells. An unrelated *in vivo* study using the middle cerebral artery occlusion (MCAO) model reported that TRPM7 expression levels in mRNA and protein were increased after MCAO (Jiang *et al.* 2008), indicating that TRPM7 may be implicated in cerebral ischaemia. As a result, we still need to confirm the study *in vivo* and use animal models to further investigate the pathophysiological role of TRPM7 in ischaemia and/or hypoxia.

We have later confirmed that TRPM7 also plays an important role in cerebral ischaemia *in vivo* (Sun *et al.* 2009). In the report, we showed that virally mediated gene silencing of TRPM7 in hippocampal CA1 neurons *in vivo* suppressed mRNA and protein TRPM7 expression levels, reduced CA1 neuronal death and preserved behavioural outcomes after cerebral ischaemia. TRPM7 pharmacology was not clear, and selective TRPM7 blockers were not available at that time; the study utilized a virally mediated gene silencing shRNA approach to suppress TRPM7 in adult rat hippocampal CA1 neurons. The adeno-associated viral vector (AAV) was used to package the TRPM7 shRNA, and a stereotaxic microinjection was used to deliver the AAV to the hippocampal CA1 area *in vivo*. We confirmed that the AAV infected the adult hippocampal CA1 neurons *in vivo*. We also showed suppression of TRPM7 in the infected hippocampal CA1 neurons at the mRNA level with RT-PCR, at the protein level with Western Blot and immunohistochemistry, and at the functional level with electrophysiology. We verified that transient suppression of TRPM7 in the adult hippocampal CA1 neurons showed no noticeable effects on neuronal cell survival, fine structures and electrophysiological properties. We then revealed that TRPM7 suppression significantly reduced hippocampal CA1 cell death *in vivo* and preserved behavioural outcomes using a global ischaemia model. We demonstrated that the surviving hippocampal CA1 neuronal cells were also healthy with intact morphology and fine cell structures. We also proved that the surviving hippocampal

CA1 neurons had well maintained electrophysiological properties. Lastly, we confirmed that suppressed TRPM7 *in vivo* not only reduced hippocampal cell death but also preserved the hippocampal-associated behavioural tasks, e.g. fear-associated and spatial-navigation memory (fear conditioning (Cheng *et al.* 2006; Sun *et al.* 2009) and Morris water maze (Morris *et al.* 1982; Sun *et al.* 2009)). This study was the first detailed *in vivo* study showing the important role of TRPM7 in cerebral ischaemia.

We also recently showed that TRPM7 plays a significant role in brain damage in hypoxic–ischaemic brain injury *in vivo* (Chen *et al.* 2015*a*). We reported that the non-selective TRPM7 inhibitor carvacrol significantly reduced the brain damage in a mouse hypoxic–ischaemic brain injury model (Chen *et al.* 2015*a*). The inhibition of TRPM7 also preserved behavioural outcomes after hypoxic–ischaemic brain injury (Chen *et al.* 2015*a*), and these included the geotaxic reflex (Ten *et al.* 2003; Sun *et al.* 2015; Chen *et al.* 2015*a*), which tests the vestibular and/or proprioceptive functions; the cliff avoidance reaction (Ten *et al.* 2003; Sun *et al.* 2015; Chen *et al.* 2015*a*), which assesses maladaptive impulsive behaviour; and the grip test (Liu *et al.* 2013; Sun *et al.* 2015; Chen *et al.* 2015*a*), which evaluates force and fatigability. Neuroprotective effects in reducing brain damage by TRPM7 inhibition in the hypoxic–ischaemic brain injury were arbitrated partly by promoting pro-survival signalling (e.g. Akt signalling) and inhibiting pro-apoptotic signalling (e.g. caspase-3 and Bcl/Bax signalling) (Chen *et al.* 2015*a*).

Conclusions and future direction

TRPM7, a calcium-mediated non-selective divalent cation channel, is one of the newly described non-glutamate mechanisms of neuronal cell death and brain damage in cerebral ischaemia and hypoxia. Both *in vitro* and *in vivo* studies have shown that TRPM7 plays a critical role in ischaemic and hypoxic neuronal cell death and brain damage. Thus, TRPM7 is a potential therapeutic target for drug development for stroke and hypoxic–ischaemic brain injury. With the new development of selective TRPM7 inhibitors (Zierler *et al.* 2011; Turlova *et al.* 2016), we could further study TRPM7 drug development in stroke and hypoxia following the Stroke STAIR protocol (Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

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Additional information

Competing interests

The author declares no conflicts of interest.

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