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Research Paper

Transient receptor potential melastatin 2 channels (TRPM2) mediate neonatal hypoxic-ischemic brain injury in mice

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ABSTRACT

Transient receptor potential melastatin 2 (TRPM2), a calcium-permeable non-selective cation channel, is reported to mediate brain damage following ischemic insults in adult mice. However, the role of TRPM2 channels in neonatal hypoxic-ischemic brain injury remains unknown. We hypothesize that *TRPM2*$^{+/-}$ and *TRPM2*$^{-/-}$ neonatal mice have reduced hypoxic-ischemic brain injury. To study the effect of TRPM2 on neonatal brain damage, we used 2,3,5-triphenyltetrazolium chloride (TTC) staining to assess the infarct volume and whole brain imaging to assess morphological changes in the brain. In addition, we also evaluated neurobehavioral outcomes for sensorimotor function 7 days following hypoxic-ischemic brain injury. We report that the infarct volumes were significantly smaller and behavioral outcomes were improved in both *TRPM2*$^{+/-}$ and *TRPM2*$^{-/-}$ mice compared to that of wildtype mice. Next, we found that TRPM2-null mice showed reduced dephosphorylation of GSK-3β following hypoxic ischemic injury unlike sham mice. *TRPM2*$^{+/-}$ and *TRPM2*$^{-/-}$ mice also had reduced activation of astrocytes and microglia in ipsilateral hemispheres, compared to wildtype mice. These findings suggest that TRPM2 channels play an essential role in mediating hypoxic-ischemic brain injury in neonatal mice. Genetically eliminating TRPM2 channels can provide neuroprotection against hypoxic-ischemic brain injury and this effect is elicited in part through regulation of GSK-3β.

1. Introduction

Hypoxic-ischemic brain injury is a common occurrence in newborn infants with an incidence of 2–6 neonates per 1000 live births in developed countries and as much as 10-fold higher in underdeveloped countries (Kurinczuk et al., 2010). Neonatal hypoxic-ischemic brain injury and its related condition hypoxic-ischemic encephalopathy (HIE), an early onset brain and behavioral disorder in children, represents a group of disabilities including cognitive and motor impairments, epilepsy and common complications in learning, vision and language. While hypothermia has been shown to be effective in reducing death and disability in term infants (Azzopardi et al., 2009; Gluckman et al., 2006; Shankaran et al., 2005), there are no effective treatments for preterm neonates suffering from hypoxic-ischemic brain injury.

The classical understanding of hypoxic-ischemic brain injury involves an initial primary energy failure causing excessive release of glutamate from synaptic terminals leading to the over-activation of *N*-methyl-D-aspartate (NMDA) receptors, calcium overload and ultimately cell death (Rothman and Olney, 1986). Indeed in the neonate, the excitotoxicity contributes to damage of brain regions with high metabolic activity, such as cerebral cortex, thalamus and putamen (Johnston et al., 2001; Johnston et al., 2002; Sie et al., 2000). While blocking NMDA and AMPA receptors reduced brain damage in the rat model of neonatal HI injury (Hagberg et al., 1994), efforts in blocking glutamate-mediated excitotoxicity pharmacologically did not achieve success in clinical trials, as NMDA receptors play an essential role in normal neurophysiological processes (Kalia et al., 2008; Lipton, 2004). However, it's been reported that glutamate's role in hypoxic-ischemic injury is not only a deleterious one, but also beneficial (Hoyte et al.,

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2004; Ikonomidou and Turski, 2002). Since then, research has also been directed towards ameliorate toxic calcium influx through non-glutamate targets of hypoxic-ischemic cell death (Bae and Sun, 2011).

One such non-glutamate target is the transient receptor potential melastatin 2 (TRPM2) channel which is a calcium-permeable non-selective cation channel. It forms a tetrameric structure with 24 transmembrane segments and is uniquely characterized by a NUDT9-H domain in the C-terminus (Faouzi and Penner, 2014; Perraud et al., 2003). TRPM2 is widely expressed throughout the central nervous system (CNS), liver, pancreas, lungs and heart (Fonfria et al., 2006b). At a cellular level, TRPM2 is found in neurons, microglia, neutrophils, macrophages, pancreatic β-cell and endothelial cells. TRPM2 channels were first discovered as a plasma membrane channel, but recent studies report that TRPM2 is also located in lysosomes. TRPM2 acts as a calcium-mediated channel found in pancreatic β cells that is activated by intracellular adenosine diphosphate ribose (ADPr) that contributes to calcium induced apoptotic signaling (Lange et al., 2009). Currently, the factors that determine the subcellular localization of TRPM2 remain unknown, but it is suggested that they are modulators of intracellular calcium levels.

Under normal conditions, the calcium-conducting activity of TRPM2 has been shown to be involved in several physiological processes including inflammation through cytokine release, synaptic transmission in the hippocampus, microglial activation and insulin secretion (Belrose et al., 2012; Hecquet et al., 2008; Kashio et al., 2012; Kraft et al., 2004; Lange et al., 2009; Olah et al., 2009). TRPM2 has also been shown to be up-regulated in adult rodents in transient middle cerebral artery occlusion (tMCAO) stroke model (Fonfria et al., 2006a). Following hypoxic-ischemic brain injury, there is a reported accumulation of H_2O_2 in the neonatal brain but not the adult brain (Lafemina et al., 2006). In primary rodent neurons, a brief exposure to H_2O_2 was shown to lead to a marked increase in intracellular Ca^{2+} through TRPM2, causing neuronal death (Kaneko et al., 2006). H_2O_2 can directly activate TRPM2 and also increase the production of other activators of TRPM2 including NAD^+, cADPr and ADPr leading to increased calcium influx (Chen et al., 2012; Fonfria et al., 2004; Hecquet et al., 2008; Kraft et al., 2004; Shimizu et al., 2014). ADPr is the major activator of TRPM2 and when bound by the NUDT9-H domain, it acts as a reserve pool for TRPM2 during channel activation (Kuhn and Luckhoff, 2004). Dysregulation of TRPM2 has been reported to be involved in triggering neuronal cell death leading to CNS diseases including neuropathic pain, bipolar disorder, amyotrophic lateral sclerosis, Alzheimer's disease and stroke (Naziroglu, 2011; Xie et al., 2010). These studies have used siRNAs and non-specific antagonists of TRPM2 to investigate the involvement of the TRPM2 channel in different disease conditions. However, with the recent development of a TRPM2-null mouse (Yamamoto et al., 2008), we can investigate the effect of TRPM2 channel in hypoxic-ischemic brain injury in neonatal mice. A recent study showed that genetic deletion of TRPM2 reduced ischemia-reperfusion brain injury in adult mice (Alim et al., 2013). However, the effect of TRPM2 in neonatal brain following hypoxic-ischemic brain injury remains unclear. Since there is an accumulation of H_2O_2 following hypoxic-ischemic injury in the neonatal brain but not the adult brain (Lafemina et al., 2006), we expect greater activation of TRPM2 in our neonatal hypoxic-ischemic brain injury model. Taken together, it is expected that TRPM2 is a potential novel therapeutic target for the neonatal hypoxic-ischemic brain injury. In this study, we used TRPM2-null mice as a tool to test the involvement of TRPM2 in neonatal hypoxic-ischemic brain injury. We report that genetic deletion of TRPM2 reduced brain damage and improved functional behavioral outcomes following the neonatal hypoxic-ischemic brain injury.

2. Materials and methods

2.1. Ethics approval

All protocols were performed in accordance to the Canadian Council on Animal Care (CCAC guidelines) and all animal treatments were approved by the local Animal Care and Use Program Committee (Office of Research Ethics at the University of Toronto). All of the experiments have been reported using the ARRIVE guidelines.

2.2. Animals and genotyping

The global *TRPM2*$^{-/-}$ was developed in C57B6J mouse from The Jackson Laboratories (Bar Harbor, ME, USA) by Dr. Yasuo Mori's lab at Kyoto University, Japan. The mouse was developed by replacing the third axon (S5–S6 linker in the pore domain) with a neomycin coding region. The knockout mice exhibit no differences in behavior or impairment in breeding, compared to wildtype C57BJ6 mice. For the breeding, males between 5 and 12 months of age and females between 3 and 6 months of age are used. The gestation period for transgenic and wildtype mice is 21 days. Postnatal day 7 (P7) littermates [wildtype (WT), *TPRM2*$^{+/-}$ and *TRPM2*$^{-/-}$, either sex, body weight ranging 5 to 5.5 g] were used for experiments. The genotypes of the mice were determined using polymerase chain reaction (PCR). Briefly, ear clippings were taken from the mice and placed in a Proteinase K and Tris-EDTA ear buffer (50 mM Tris-HCl/10 mM EDTA). Next, ear samples were mixed with phenol:chloroform and TE buffer and DNA was cleaned using 100% ethanol. Following DNA extraction, previously designed PCR primers (Yamamoto et al., 2008) were used in conjunction with the KAPA HotStart kit for PCR. The final PCR products were ran on a 1% agarose gel mixed with ethidium bromide for 60 min and DNA was visualized with ultraviolet light. Tissues for genotyping were taken at the endpoint of all experimental procedures; this also served as blinding measure for all the experiments.

2.3. Hypoxic-ischemic brain injury model

Hypoxic-ischemic (HI) brain injury was induced in (WT, *TPRM2*$^{+/-}$ and *TRPM2*$^{-/-}$) C57B6J postnatal day 7 (P7) pups using a previously described protocol with modifications (Chen et al., 2015; Sun et al., 2015; Xiao et al., 2015). Briefly, P7 pups of all genotypes were placed on the heating blanket and under anesthesia using a 3% isoflurane-oxygen mixture for induction and 2% for maintenance. Ischemia was achieved by isolating the right common carotid artery (separated from surrounding tissue and nerves) and ligating using a bipolar electrocoagulation device (Vetroson V-10 Bi-polar electrosurgical unit, Summit Hill Laboratories, Tinton Falls, NJ, USA). The remaining ligated artery was cut using microscissors. Following the surgery, pups were placed under a heat lamp until they regained consciousness and were returned to their mother for 90 min. Next, hypoxia damage was achieved by placing the pups in an airtight hypoxic chamber (A-Chamber A-15274 with ProOx 110 Oxygen Controller/E720 Sensor, Biospherix, NY, USA) fed with humidified 7.0% oxygen in mixture with 93% nitrogen gas for 120 min. The chamber temperature was kept at 37 °C using a homoeothermic blanket control unit (K-017484 Harvard Apparatus, Massachusetts, USA). The pups from the same litters underwent this part of the procedure simultaneously, and genotyping was performed after the data were collected. After the hypoxia, the pups were returned to their dams. Sham WT animals were anesthetized and their right common carotid artery was exposed and separated but no ligation or hypoxia took place.

2.4. Behavioral assessments

Behavioral outcomes were assessed 7 days following hypoxic-ischemic brain injury. Mice from the WT, *TPRM2*$^{+/-}$ and *TRPM2*$^{-/-}$ groups performed the geotaxis reflex and cliff avoidance reflex as previously described (Sun et al., 2015) and their scores were recorded. These reflex tests were chosen because they are strong indicators of sensorimotor function following brain damage. Moreover, these are reflexes that are present during early stages of development that are not strain or sex