EMERGING ROLES OF TRP CHANNELS IN BRAIN PATHOLOGY

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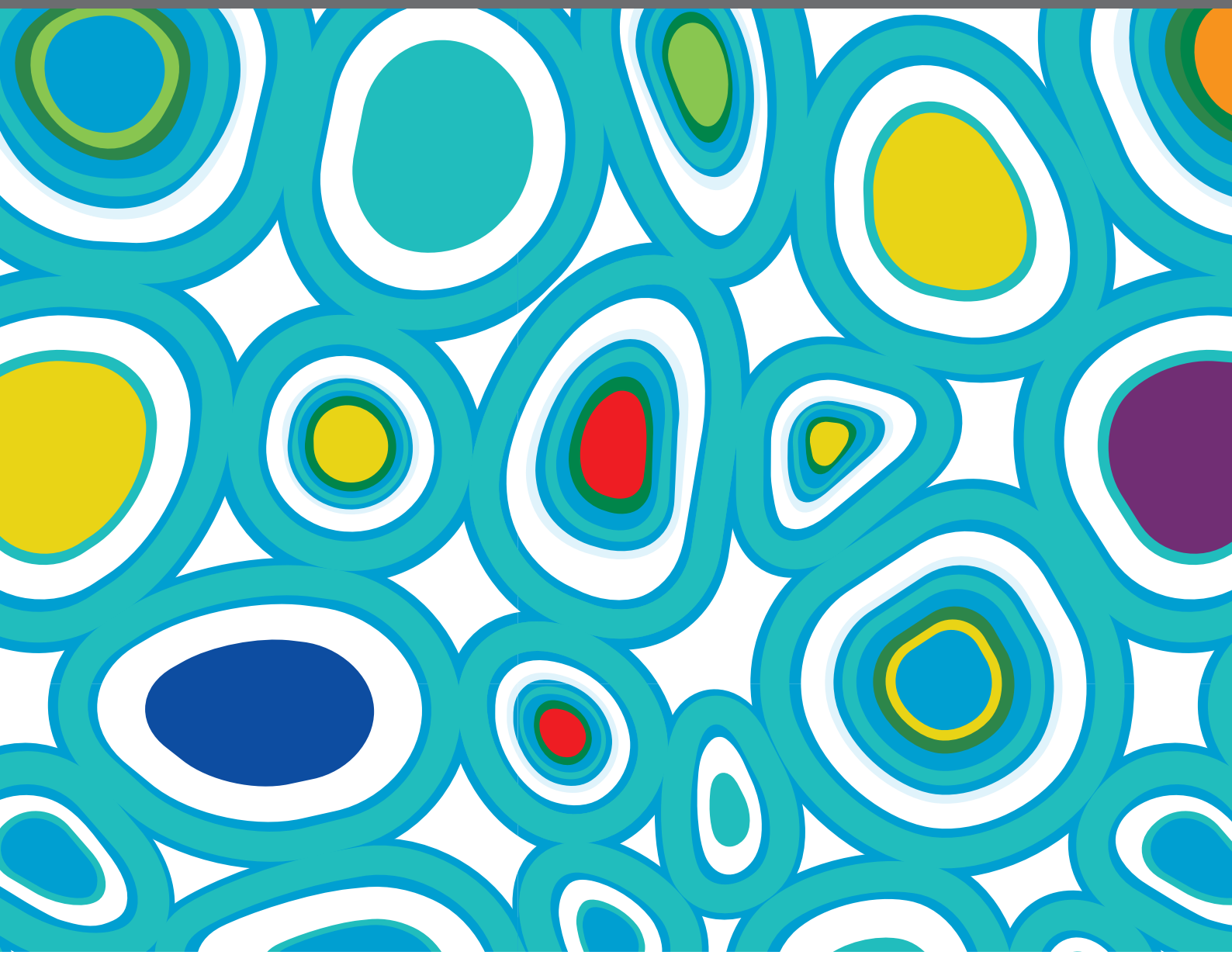

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EMERGING ROLES OF TRP CHANNELS IN BRAIN PATHOLOGY

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Editorial: Emerging Roles of TRP Channels in Brain Pathology

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Keywords: TRP channels, ischemic stroke, neurodegenerative diseases, psychiatric disorders, glioma, Ca^{2+} signalling

Editorial on the Research Topic

Emerging Roles of TRP Channels in Brain Pathology

The mammalian transient receptor potential (TRP) ion channel superfamily comprises six subfamilies, TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) (Ramsey et al., 2006; Venkatachalam and Montell, 2007). TRP channels are tetrameric and each subunit contains intracellular N- and C-termini and six membrane-spanning segments, with the fifth and sixth segments and the re-entrant loop between them forming the ion-conducting pore (Cao, 2020). They function as non-selective cation channels, with prominent Ca^{2+} permeability for most of them, and are activated by diverse physical, chemical and biological stimuli. Their Ca^{2+} permeability, poly-modal activation and wide expression place these channels in a vital position mediating Ca^{2+} signalling in a range of physiological processes. Not surprisingly, accumulating evidence supports an important role for the TRP channels in the pathogenesis of numerous diseases (Nilius et al., 2007). Many TRP channels are expressed in the brain. This Research Topic, including 14 review and original research articles, offers critical and new insights into the role of TRP channels, particularly the Ca^{2+}-permeable ones, in multiple brain pathologies.

TRPC IN ISCHEMIC BRAIN DAMAGE

Brain is highly vulnerable to damage by ischemia, if severe or lasting, and reperfusion after transient ischemia. Jeon et al. have critically evaluated the literature and also presented their recent study regarding the role of TRPC channels in ischemic brain damage. A complex role for the TRPC channels is emerging from studies, using middle carotid artery occlusion followed by reperfusion (MCAO/R), an *in vivo* model of ischemic stroke, and neuronal death induced by oxygen and glucose deprivation followed by reoxygenation (OGD/R), an *in vitro* model of ischemia/reperfusion, in combination with using transgenic knockout (KO) mice and neurons or brain slices derived from KO mice or using pharmacological interventions. TRPC channels, including TRPC3, TRPC4, TRPC6, and TRPC7, play a significant role in mediating ischemic brain damage. TRPC1 was proposed to act as a protective mechanism against ischemic brain damage via inhibiting generation of reactive oxygen species (ROS). However, Jeon et al. showed that TRPC1-KO attenuated OGD/R-induced neuronal death.

Neuroinflammation is another mechanism for ischemic brain damage and many other brain pathologies. Liu et al. showed that inhibition or depletion of TRPC6 suppressed OGD/R-induced Ca^{2+} response, production of interleukin (IL)-1β and IL-6, two neurotoxic proinflammatory cytokines, caspase-3 activation and apoptosis in astrocytes. Inhibition of TRPC6 also attenuated

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MCAO/R-induced caspase-3 activation, elevated level of IL-1β and IL-6 in the peri-infarct areas, and infarction in mice. These results support a critical role for TRPC6 in astrocytes in mediating neuroinflammation and ischemic brain damage.

TRP IN NEURODEGENERATIVE AND NEUROLOGICAL DISEASES

Alzheimer's disease (AD), Parkinson's diseases (PD), Huntington's disease, amyotrophic lateral sclerosis and epilepsy represent the most common neurodegenerative and neurological diseases. Extensive research efforts have been devoted to exploring the TRP channels in the pathogenesis of these conditions. Lee et al. have provided a concise overview of the potential involvement of the TRP channels in AD (TRPC1, TRPC6, TRPV, TRPM2, TRPM7, and TRPML1), PD (TRPC1, TRPC4, TRPC5, TRPM7, and TRPML1), Huntington's disease (TRPC1 and TRPC5), amyotrophic lateral sclerosis (TRPC4, TRPM2, TRPM3, TRPM7, and TRPML1) and epilepsy (TRPC4, TRPC5, TRPV4, TRPM7, and TRPA1). They further elaborated diverse TRP-mediated Ca^{2+}-dependent downstream signalling pathways to the associated pathologies. Vaidya and Sharma have drawn their attention to the TRP channels expressed in substantial nigra pars compacta and other brain areas affected in PD. TRPC1 was shown to protect PD-related neuronal death. Intriguingly, both activation and inhibition of TRPV1, reported by different studies, improved motor function through regulation of distinctive molecular and cellular mechanisms. Oxidative stress, due to accumulation of high levels of ROS, is a pathological factor as well as a conspicuous pathological feature in PD (Prasad and Hung, 2020). Vaidya and Sharma also discussed the role of TRPM2 and TRPM7, both known to be sensitive to activation by ROS, in mediating PD-related neuronal death.

TRPV IN BBB DYSFUNCTION AND RELATED BRAIN PATHOLOGIES

Endothelial cells play a key role in forming blood brain barrier (BBB), and BBB dysfunction results in infiltration of peripheral immune cells into the brain to exacerbate neuroinflammation and thereby ischemic stroke and neurodegenerative diseases (Sweeney et al., 2018). TRPV4 is known to regulate endothelial barrier function via mediating Ca^{2+} influx into endothelial cells. Consistently, Rosenkranz et al. demonstrated that pharmacological inhibition of TRPV4 in mouse brain microvascular endothelial cells improved endothelial barrier function. Such an effect was however obliterated in endothelial cells after exposure to interferon-γ and tumour necrosis factor-α that down-regulated the TRPV4 expression. In mice, TRPV4-KO failed to prevent BBB dysfunction and associated experimental autoimmune encephalomyelitis, a model of multiple sclerosis, or MCAO/R-induced brain damage, suggesting loss of TRPV4-mediated regulation of BBB function under inflammation. Luo et al. examined and revealed a different profile of TRPV1-4 expression in brain endothelial cells of rat and human origins, ranking from high to low: TRPV4 > TRPV2 > TRPV3 > TRPV1 in rat endothelial cells, and TRPV2 >> TRPV4 > TRPV1 > TRPV3 in human endothelial cells. Thus, TRPV4 and TRPV2 represent the predominant TRPV in human and rat brain endothelial cells, respectively. Such species difference may complicate testing drugs using rat models of BBB dysfunction and related brain pathologies.

TRPM3 IN DEVELOPMENTAL DISORDERS

TRPM3 is activated by endogenous neurosteroid pregnenolone sulphate. TRPM3 expression is documented in several brain regions, including hippocampus and choroid plexus, and in cerebellar Purkinje neurons and oligodendrocytes. Held and Tóth have reviewed the potential role of TRPM3 in brain physiological and pathophysiological functions, and highlighted genetic alterations in the TRPM3 gene in patients with development and intellectual disabilities. Particularly interesting is the identification of *de novo* gain-of-function mutations in patients with developmental and epileptic encephalopathies. Deletions in the TRPM3 gene were found in patients with Kabuki syndrome, a multisystem disorder including intellectual disability, and in autism patients. However, it remains unclear how alteration of TRPM3 affects brain development and other functions.

TRP IN PSYCHIATRIC DISORDERS

Psychiatric disorders such as depression and anxiety disorders are caused by diverse and complex factors, both genetic and environmental. As proposed in neurodegenerative diseases, oxidative stress is a critical pathological factor, and Ca^{2+} signalling is disrupted, in psychiatric disorders. Nakao et al. have overviewed the redox-sensitive Ca^{2+}-permeable TRP channels in neurons and glial cells, including TRPC4, TRPC5, TRPV1, TRPM2, and TRPA1, in regulating cell functions. They have provided a concise summary of the studies that showed using a battery of behaviour tests that anxiety-like behaviours in mice were attenuated by genetic deletion (TRPC4, TRPC5, TRPV1, TRPM2, or TRPA1) or pharmacological inhibition (TRPC4/TRPC5). They also discussed the possible mechanisms by which these channels mediate oxidative stress-induced Ca^{2+} signalling and subsequent alterations in neuronal connectivity, synaptic plasticity and glial cell function, leading to psychiatric disorders.

TRP IN CHRONIC PAIN AND ITS INHIBITION BY RESOLVINS

Chronic pain occurs as a result of neuronal tissue inflammation and/or damage. Increased expression and/or activation of TRP channels in nociceptive neurons can enhance the excitability of nociceptive neurons and thereby intensify the pain signals. Resolvins are a class of lipid mediators generated during the resolution phase of acute inflammation. Roh et al. discussed the studies showing analgesic effects of different resolvins against

inflammatory pain, through activating distinct cognate receptors to inhibit nociceptive TRP channels, including TRPV1, TRPV3, TRPV4, and TRPA1. The role of such inhibitory mechanisms in neuropathic pain is unknown.

TRP IN BRAIN TUMOURS

Malignant glioma including glioblastoma is the most common group of primary brain tumours and exhibits resistance to treatment and high recurrence. Chinigò et al. have conveyed the literature informing the expression of TRP channels and their potential role in brain tumours. The expression in glioma tissues and cells was shown to be upregulated (TRPC1, TRPC6, TRPV4, TRPM2, TRPM7, TRPM8, and TRPML2), or down-regulated (TRPV1, TRPV2, and TRPML1). Such alterations in some cases exhibited correlations with glioma progression and overall patient survival. Moreover, some of these channels (TRPC1, TRPC6, TRPV4, TRPM7, TRPM8, and TRPML2) appear pro-tumorigenic and, conversely, others (TRPV1, TRPV2, TRPM2, TRPM3, TRPA1, and TRPML1) are anti-tumorigenic. Chinigò et al. have proposed diverse signalling pathways, downstream of TRP channel-mediated elevation in intracellular Ca^{2+}, in the regulation of glioma cell proliferation, migration and invasion as well as cell death.

TRP IN REGULATING Ca^{2+} AND H^{+} HOMEOSTASIS AND INTERACTING WITH OTHER SIGNALLING MECHANISMS

Disruption in Ca^{2+} homeostasis has been alluded by multiple articles as an important mechanism in the pathogenesis of brain pathologies. Plasma membrane Ca^{2+}-ATPase drives Ca^{2+} efflux and H^{+} influx and thus plays a vital role in modulating intracellular Ca^{2+} and H^{+} homeostasis. Hwang et al. have reviewed the role in neurodegenerative diseases of the plasma membrane Ca^{2+}-ATPase in concerted actions with Ca^{2+}-permeable TRP channels in altering Ca^{2+} and H^{+} homeostasis in neuronal and glial cells.

Hermann et al. investigated contribution of dinucleotide nicotinic acid adenine phosphate (NAADP)-induced Ca^{2+} release in mouse hippocampal neurons to the Ca^{2+} response evoked by glutamate, the major excitatory neurotransmitter in the brain. They proposed that NAADP induces Ca^{2+} release from intracellular acidic stores by activating TRPML1 and two-pore channels, and also from ER by activating ryanodine receptors, and showed that inhibition of NAADP signalling reduced glutamate-evoked Ca^{2+} response, particularly the sustained component. The study demonstrated substantial contribution of NAADP to glutamate-induced Ca^{2+} signalling in hippocampal neurons.

The activity of small Rho GTPases is critical for actin-based cytoskeletal remodelling. Ca^{2+} is an intracellular signal upstream of Rho GTPases. Lavanderos et al. have introduced the role of Rho GTPases in the regulation of axon growth, dendritic spine development, synapse formation, glial cell migration, and endothelial permeability, which are vital in sustaining brain structure and function. They further presented the potential Rho GTPase-dependent mechanisms that mediate Ca^{2+}-permeable TRP channels in various brain conditions, including AD (TRPC6 and TRPV1), ischemic bran damage (TRPM7), glioma and neuroblastoma (TRPC6, TRPV1, TRPM7, and TRPM8).

Manchanda et al. extended their previous work to study TRPV1 in regulating the generation of endocannabinoids. Treatment of human embryonic kidney 293 cells expressing TRPV1 with capsaicin increased the levels of 2-acyl glycerols and reduced the levels of N-acyl ethanolamines, depending upon temperature. These results support the interesting notion that the TRPV1 and the receptor for endocannabinoids cross-talk in response to changes in temperature.

Collectively, the insightful evaluation of the literature and the new information presented in this Research Topic have evolved a better understanding of the TRP channels in the pathogenesis of multiple brain pathologies, and also raised many outstanding questions that need further researches in order to gain comprehensive and mechanistic insights into these pathological conditions and provide the proof of concept of targeting the TRP channels and/or related mechanisms as potential therapeutic strategies.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing, the revision of this Editorial Article, and approved it for publication.

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Contribution of NAADP to Glutamate-Evoked Changes in Ca^{2+} Homeostasis in Mouse Hippocampal Neurons

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger that evokes calcium release from intracellular organelles by the engagement of calcium release channels, including members of the Transient Receptor Potential (TRP) family, such as TRPML1, the (structurally) related Two Pore Channel type 1 (TPC1) and TPC2 channels as well as Ryanodine Receptors type 1 (RYR1; Guse, 2012). NAADP evokes calcium release from acidic calcium stores of many cell types (Guse, 2012), and NAADP-sensitive Ca^{2+} stores have been described in hippocampal neurons of the rat (Bak et al., 1999; McGuinness et al., 2007). Glutamate triggers Ca^{2+}-mediated neuronal excitotoxicity in inflammation-induced neurodegenerative pathologies such as Multiple Sclerosis (MS; Friese et al., 2014), and when applied extracellularly to neurons glutamate can elevate NAADP levels in these cells. Accordingly, glutamate-evoked Ca^{2+} signals from intracellular organelles were inhibited by preventing organelle acidification (Pandey et al., 2009). Analysis of reported RNA sequencing experiments of cultured hippocampal neurons revealed the abundance of Mcoln1 (encoding TRPML1), Tpcn1, and Tpcn2 (encoding TPC1 and TPC2, respectively) as potential NAADP target channels in these cells. Transcripts encoding Ryr1 were not found in contrast to Ryr2 and Ryr3. To study the contribution of NAADP signaling to glutamate-evoked calcium transients in murine hippocampal neurons we used the NAADP antagonists Ned-19 (Naylor et al., 2009) and BZ194 (Dammermann et al., 2009). Our results show that both NAADP antagonists significantly reduce glutamate-evoked calcium transients. In addition to extracellular glutamate application, we studied synchronized calcium oscillations in the cells of the neuronal cultures evoked by addition of the $GABA_A$ receptor antagonist bicuculline. Pretreatment with Ned-19 (50 μM) or BZ194 (100 μM) led to an increase in the frequency of bicuculline-induced calcium oscillations at the cost of calcium transient amplitudes. Interestingly, Ned-19 triggered a rise in intracellular calcium concentrations 25 min after bicuculline stimulation, leading to

the question whether NAADP acts as a neuroprotective messenger in hippocampal neurons. Taken together, our results are in agreement with the concept that NAADP signaling significantly contributes to glutamate evoked Ca^{2+} rise in hippocampal neurons and to the amplitude and frequency of synchronized Ca^{2+} oscillations triggered by spontaneous glutamate release events.

Keywords: hippocampal neurons, glutamate, NAADP, Ca^{2+} homeostasis, neuronal excitotoxicity

INTRODUCTION

In neurons, changes of free intracellular calcium ion concentration [$(Ca^{2+})_i$] regulate many physiological functions such as neuronal plasticity, gene transcription and synaptic transmission (Ureshino et al., 2019). Defects in Ca^{2+} homeostasis are a reasonable cause for cell death and, eventually, neurodegeneration (Wojda et al., 2008; Szydlowska and Tymianski, 2010). Under these conditions, the homeostatic system, which assures low $[Ca^{2+}]_i$ in the range of 100 nM (in resting conditions), is defective (Nedergaard and Verkhratsky, 2010; Zundorf and Reiser, 2011). The tight equilibrium in $[Ca^{2+}]_i$ is usually maintained by a large number of ion channels and transporters in the plasma membrane as well as in the intracellular compartments of the cell.

Exaggerated rise in $[Ca^{2+}]_i$, which can be triggered by glutamate accumulation, e.g., after hypoxia, leads to apoptosis and necrotic cell death. Glutamate-evoked changes in intracellular Ca^{2+} homeostasis involve the activation of ionotropic NMDA, AMPA, and kainate (KA) receptors, as well as the mechanisms downstream of the activation of metabotropic glutamate receptors (mGluRs; Reiner and Levitz, 2018). The underlying processes of Ca^{2+}-overload mediated toxicity may include over-activation of several types of enzymes, e.g., proteases of the calpain family, phosphatase calcineurin, nitric oxide synthases, endonucleases (leading to DNA fragmentation), and phospholipid scramblase leading to phosphatidyl-choline PS exposure in the outer leaflet of the plasma membrane (Orrenius et al., 2003; Hardingham and Bading, 2010). In acute (e.g., following cerebral ischemia) or chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple sclerosis (MS), glutamate elevations evoke an impairment of mitochondrial function, survival-promoting gene expression and the maintenance of structural integrity of neurons that all act in concert during the development of neurodegeneration (Bading, 2017). Impaired Ca^{2+} homeostasis in familial forms of AD is thought to occur from a dysfunction of presenilins, which act as Ca^{2+} release channel in the ER, a subsequent Ca^{2+} accumulation in the ER, and downregulation of neuronal store-operated Ca^{2+} entry (Popugaeva et al., 2017). Furthermore, Aß42/40 proteins fragments, which arise by proteolysis from the amyloid precursor protein (APP) and aggregate to neurotoxic oligomers, can form Ca^{2+}-permeable pores in the plasma membrane and modulate activity of voltage dependent calcium channels and NMDA receptors. Elevated ER Ca^{2+} content leads to enhanced Ca^{2+} release via D-*myo*-inositol 1, 4, 5-trisphosphate receptors (IP_3R), and ryanodine receptors (RYR) as well as Ca^{2+} accumulation in the cytosol (Popugaeva et al., 2017). In PD the impaired Ca^{2+} homeostasis leading to increased vulnerability in dopaminergic neurons of the substantia nigra is characterized by an activity-related oscillatory intracellular Ca^{2+} load potentially caused by the altered activity of multiple Ca^{2+} conducting channels including store-operated channels, ionotropic glutamate receptors, and voltage gated Ca_V1 channels (Duda et al., 2016). Notably, the application of dihydropyridines as negative allosteric modulators of Ca_V1 Ca^{2+} channels is associated with a decreased risk and progression of PD (Surmeier et al., 2017).

Glutamate-evoked neurotoxicity is also a key process in the pathogenesis of MS, and glutamate levels are elevated in the CSF and brains of MS patients. A growing body of evidence has suggested that the inflammatory insults in MS determine neurodegeneration by alterations of ion exchange mechanisms and here in particular Ca^{2+} handling (Friese et al., 2014). Dysregulation of cellular Ca^{2+} homeostasis evoked by glutamate was previously considered to be a result of Ca^{2+} entry via ionotropic glutamate receptors; however, our recent studies have identified that the neuronally expressed Na^+- and Ca^{2+}-permeable acid sensing ion channel-1 (ASIC1) and Ca^{2+}-activated transient receptor potential melastatin 4 (TRPM4) crucially contribute to maladaptive cation handling under inflammatory conditions (Friese et al., 2007; Schattling et al., 2012). Specifically, our studies showed that neuronal Ca^{2+} overload with excessive activation of Ca^{2+}-dependent processes may be one component of neuronal injury. However, the constituents of channels in both the plasmalemmal and intracellular organelles contributing to cytosolic Ca^{2+} rise, as well as the mechanisms by which intracellular Ca^{2+} homeostasis is orchestrated, e.g., by providing a sufficient buffering capacity during neuroinflammation, remain to be poorly understood.

The cellular Ca^{2+} homeostasis can be modulated not only by Ca^{2+} channels in the plasma membrane, but also by Ca^{2+} release from intracellular Ca^{2+} stores. Organelles that function as Ca^{2+} stores with a major impact on cytosolic Ca^{2+} homeostasis include (i) the mitochondrion, (ii) the endoplasmic reticulum, and (iii) the lysosomes that can buffer and release Ca^{2+}. Over the last years, numerous studies have revealed that glutamate stimulation in neurons evokes, in addition to IP_3, the synthesis of Nicotinic acid adenine dinucleotide phosphate (NAADP) and Ca^{2+} release from acidic stores. NAADP-sensitive Ca^{2+} stores have been described in brain microsomes (Bak et al., 1999) and in lysosome-related organelles in hippocampal neurons of the rat (Bak et al., 1999; McGuinness et al., 2007). Extracellular glutamate application is able to elevate cellular NAADP levels in hippocampal neurons, and glutamate-evoked Ca^{2+} signals from intracellular organelles were inhibited by

antagonizing acidification of lysosomes (Pandey et al., 2009). A recent study has also shown that Ca^{2+} entry via voltage gated Ca^{2+} channels triggers Ca^{2+} release from the lysosome via an NAADP-sensitive channel and, subsequently, fusion of the lysosome with the plasma membrane (Padamsey et al., 2017). In addition, metabotropic glutamate receptor 1 (mGluR1) has been reported to couple to NAADP signaling eliciting Ca^{2+} release from acidic stores via TPC channels during synaptic plasticity (Foster et al., 2018). In non-neuronal cells the NAADP-evoked Ca^{2+} release depends on ryanodine receptors, such as on Ryanodine Receptors type 1 (RYR1) in *T* cells (Diercks et al., 2018), and on TRPML1 channels in fibroblasts (Zhang et al., 2011).

In this study we aimed to investigate the contribution of NAADP signaling to glutamate evoked calcium transients in murine hippocampal neurons following extracellular glutamate application using NAADP antagonists. A second set of experiments was performed under conditions of facilitated spontaneous glutamate release events by pharmacological inhibition of $GABA_A$ receptors that, while being active, exhibit an inhibitory influence on neurotransmitter exocytosis. The results of our experiments reveal that the NAADP antagonists Ned-19 (Naylor et al., 2009) and BZ194 (Dammermann et al., 2009), respectively, significantly reduced glutamate evoked calcium transients in neurons upon extracellularly applied glutamate and that these NAADP antagonists increase the frequencies of bicuculline-evoked calcium oscillations at the cost of lower calcium transient amplitudes supporting the concept that NAADP signaling significantly contributes to glutamate evoked Ca^{2+} rise in hippocampal neurons.

MATERIALS AND METHODS

Isolation of Hippocampal Neurons From Mouse Embryos

Pregnant females (E16.5) from timed mating were euthanized by CO_2 inhalation. Embryos were isolated after ovarian section. After removal of the placenta and the uterus the embryos were placed in a Petri dish with 25 ml ice cold Hanks' Balanced Salt Solution (ThermoFisher). Hippocampi were dissected from the embryo's head under a stereo microscope (8× magnification, Stemi SV4, Carl Zeiss) and digested with trypsin/EDTA (Sigma-Aldrich) and warmed to 37°C. For each independent cell preparation hippocampi from at least 4 Embryos were pooled. Cells were dissociated by repeated trituration steps using a Pasteur pipette and filtered using a cell strainer (40 μm, Corning). Thereafter, cells were centrifuged (1.700*g*, 2 min), and resuspended in Neurobasal medium (Neurobasal medium, ThermoFisher: 21103049, supplemented with 1% Penicillin-Streptomycin, 45 μM ß-Mercaptoethanol, 1× B27 Supplement, and 0.5 mM GlutaMAX-I). Cells were then plated on Poly-D-lysine coated coverslips (382.000 cells per coverslip, diameter 18 mm) and cultured in 12 well plates at 37°C and 5% CO_2 in Neurobasal medium supplemented with 1% Penicillin-Streptomycin, 45 μM ß-Mercaptoethanol, 1 × B27 Supplement, and 0.5 mM GlutaMAX-I. At day 3 after plating, cells were treated with Ara-C (500 nM) to prevent proliferation of glial cells. Medium was changed at day 6 or 7 and microfluorimetric calcium imaging experiments were performed at day 13 to 15 *in vitro* (d.i.v.).

All animal procedures were approved and performed according to the regulations of the Regierungspräsidium Karlsruhe and the University Heidelberg (*T* 25/16) and conformed to the guidelines from the directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Fluorimetric $[Ca^{2+}]_i$ Measurements

Cultured hippocampal neuronal cells were loaded with a fluorescent Ca^{2+} indicator Fura-2 by incubation in Physiological Salt Solution (PSS) supplemented with 2 μM Fura-2 AM (Thermo Fisher Scientific, Darmstadt, Germany) and 0.1% Pluronic F-127 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 30 min at room temperature and prior to imaging were washed with PSS. The PSS had the following composition (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 20, and Glucose 10. For the preparation of high potassium solution (60 mM), NaCl was equimolarly replaced by KCl. The imaging set-up was built on a base of a fluorescence microscope Axio Observer A1 equipped with a Fluar 20×/0.75 objective (both Zeiss, Germany). Excitation at 340 nm and 380 nm (exposure time 100 ms) was achieved using a polychrome V monochromator (Till Photonics, Germany) and the emitted light with a cut off filter >500 nm was collected by a CMOS camera (ORCA-flash 4.0, Hamamatsu Photonics, Japan). The acquisition frequency was 1 Hz. The monochromator and the CMOS camera were controlled by the ZEN 2.0 Pro (Zeiss, Germany) acquisition software. Regions of interest (ROIs) were placed at cell somata and the fluorescence signals were background corrected. The time course of $[Ca^{2+}]_i$ changes was presented as F340/F380 ratio. All experiments were performed at room temperature.

Glutamate and Bafilomycin A1 were purchased from Sigma-Aldrich, Ned-19 was purchased from Santa Cruz Biotechnology. BZ194 was synthesized as described (Dammermann et al., 2009). After 5 min of baseline recording glutamate or bicuculline were applied and changes in $[Ca^{2+}]_i$ were monitored for 15 (glutamate) or 25 min (bicuculline) treatment. Thereafter, high-potassium solution (60 mM) was applied to identify excitable cells.

The change of basal $[Ca^{2+}]_i$ (**Figures 5J,N,R**) was calculated from the F 340/380 ratios at the time point 30 min subtracted by the F340/380 values at the time point 5 min.

Gene Expression Analysis

Gene expression analysis was performed using the data of high throughput mRNA sequencing. Raw gene counts were taken from the gene expression omnibus (GEO) database under the accession numbers GSE104802 (Mao et al., 2018) and GSE142064 (unpublished[1]).For the unstimulated control samples of each dataset transcripts per kilobase million (TPM) were calculated and normalized to the mean mRNA expression

[1]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142064

value of the depicted genes from each dataset. Data processing and visualization were performed within the R environment (Version 1.2.5001).

Statistical Analysis

Analysis of $[Ca^{2+}]_i$ peak amplitudes and area under the curve (AUC) was performed using Origin PRO software (Origin Lab 2015). Oscillation peaks observed under bicuculline treatment were classified as "low" or "high" if their absolute ratio amplitude was <0.15 or ≥ 0.15, respectively. The Fura-2 measurements from each independent cell preparation (made out of at least 4 embryos) were performed at least three times. 50–100 hippocampal neurons were measured simultaneously during each independent measurement. For calculation of a mean preparation trace, all the cells recorded with a particular protocol from a particular independent cell preparation were pooled. For the determination of significant differences of mean values obtained from two groups, a two-sample Student's *t*-test was used ($p < 0.05$ for significance). *N* indicates the number of independent cell preparations and n indicates the number of measured cells unless otherwise stated.

RESULTS

Current Concepts About Glutamate-Evoked and NAADP-Mediated Calcium Homeostasis in Neurons and Potential NAADP Target Channels

In addition to ionotropic NMDA, AMPA, and KA receptors, glutamate activates mGluRs to elicit downstream signaling events in the cell (Reiner and Levitz, 2018). It was shown that stimulation of hippocampal neurons with glutamate triggers NAADP synthesis probably via activation of mGluR1 receptors and mediates calcium release from lysosomal stores via two-pore-channels (Bak et al., 1999; McGuinness et al., 2007; Pandey et al., 2009; Foster et al., 2018). The prerequisite for this process is the loading of acidic stores with calcium ions which depends on the activity of the V-ATPase (**Figure 1**) and acidification that then allows Ca^{2+} uptake via H^+/Ca^{2+} exchange. Recently, experimental evidence was reported that calcium entry into neurons mediated by voltage gated calcium channels can trigger calcium release from acidic stores via NAADP sensitive channels. In addition to Two Pore Channel type 1 (TPC1) and TPC2 channels (Rahman et al., 2014; Grimm et al., 2017), TRPML1 (Zhang et al., 2011), and ryanodine receptors such as RYR1 have been reported as a NAADP target channels in other cell types (Guse, 2012; Diercks et al., 2018). The current concepts of NAADP-mediated calcium release and potential NAADP target channels that also may operate in hippocampal neurons are summarized in **Figure 1A** based on two independent RNAseq expression analyses in primary cultured neurons from mouse hippocampus GSE104802 (Mao et al., 2018) and GSE142064 (unpublished). Our analysis of these studies revealed that transcripts of Mcoln1 (encoding TRPML1 channels) are abundantly expressed in murine hippocampal neuron cultures whereas Mcoln2 and Mcoln3 transcripts are virtually not detectable. Within the TPC channel family, Tpcn1 transcripts (encoding TPC1) were detected (**Figure 1B**) and Tpcn2 transcripts (encoding TPC2) were also present, however, to a lower extent. Amongst the ryanodine receptors, transcripts encoding Ryr2 and Ryr3 were readily detected, whereas expression of Ryr1 seems to be negligible in hippocampal neurons in both studies.

In the current study we aim to analyze the contribution of NAADP-mediated calcium release from acidic stores in primary hippocampal neurons. Representative images of cultured hippocampal neurons 14 days after isolation, which were analyzed in our study, are shown in **Figure 1C** and demonstrate a dense and complex network of synaptic connections between the neurons. Cell bodies are 13–20 μm in size, from which numerous axonal structures originate similarly as has been described in previous publications (Mao and Wang, 2001). We have studied a possible involvement of NAADP-mediated Ca^{2+}-release in $[Ca^{2+}]_i$ elevation events registered after extracellular application of glutamate or during endogenous glutamate release events triggered by inhibition of $GABA_A$ receptors. To this end, we tested different concentration of glutamate (1, 3, and 10 μM) and measured the time course of intracellular calcium rise using Fura-2-based microfluorimetry. The rise of $[Ca^{2+}]_i$ triggered by 1 μM glutamate was transient and completely returned to the baseline ∼5 min after the beginning of stimulation (**Supplementary Figure 1A**), whereas the responses elicited by application of 3 μM glutamate had a higher amplitude and a much longer decay phase with a half-decay time from about ∼8 min (**Supplementary Figure 1B**). Following application of 10 μM glutamate the calcium plateau did not revert within a time frame of 15 min (not shown). Thus, protocols using the application of 3 μM glutamate were used in the following to test its modulation by interference with NAADP antagonists.

Glutamate Evoked $[Ca^{2+}]_i$ Rise in Hippocampal Neurons Is Reduced by NAADP Antagonists

After Fura-2 loading, hippocampal neurons were pre-incubated with Ned-19 (50 μM) for 5 min before starting the recording. 5 min after beginning of the recording, the cells were stimulated with glutamate (3 μM) for 15 min. Finally, to exclude possible recording of $[Ca^{2+}]_i$ in glial cells, we stimulated the cells with high-potassium solution (60 mM) as a positive control for excitable cells. Representative recordings are shown in **Figure 2B** and demonstrate an instantaneous $[Ca^{2+}]_i$ rise followed by a continuous and more variable decay phase. In average, the fluorescence ratio declined during 15 min after glutamate application to ∼30% of initial amplitude (**Figure 2D**, black traces).

The treatment with Ned-19 resulted in a significant reduction of the amplitude of glutamate-evoked $[Ca^{2+}]_i$ rise (**Figures 2C–E**). The F340/F380 ratio was reduced by ∼27%. We also observed a ∼54% reduction of the AUC of the fluorescence ratio for the time period of 15 min after glutamate application (**Figure 2F**). After application of high

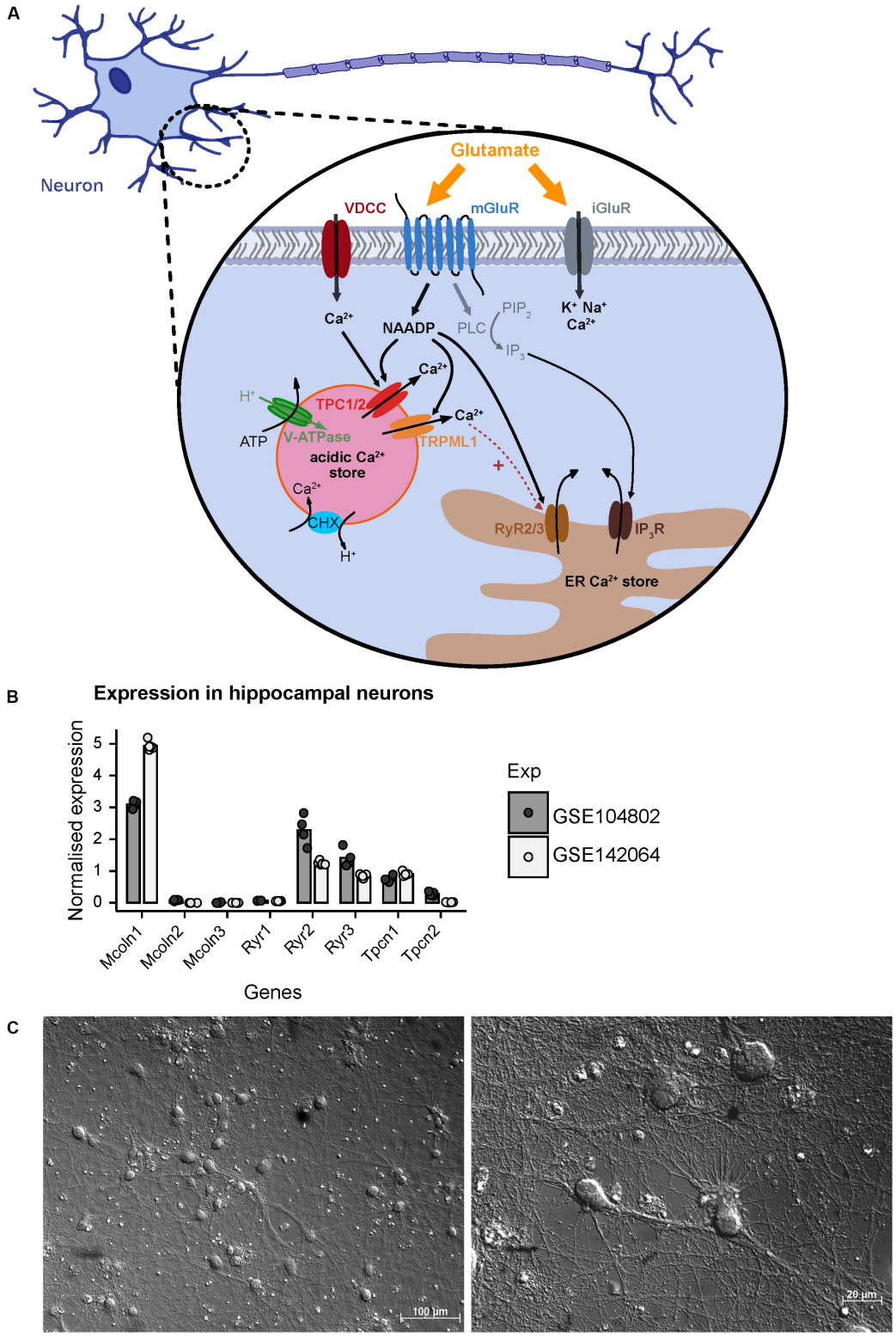

FIGURE 1 | A model based on current literature summarizing regulators of NAADP-mediated Ca^{2+} homeostasis following extracellular glutamate action in hippocampal neurons of mice. **(A)** Glutamate activates ionotropic NMDA, AMPA, and KA receptors (iGluR) resulting in Ca^{2+} influx across the plasma membrane. The activation of metabotropic glutamate receptors (mGluR) induces the synthesis of inositol trisphosphate (IP_3) which mediates Ca^{2+} release from the endoplasmic reticulum (ER). In the CNS and particularly in hippocampal neurons glutamate induces the synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) and

(Continued)

FIGURE 1 | Continued
subsequent Ca^{2+} release (Bak et al., 1999; McGuinness et al., 2007; Pandey et al., 2009). NAADP can be produced following activation of mGuR1 receptors and is known to mediate Ca^{2+} release into the cytosol via activation of two-pore channels 1/2 (TPC1/2) residing in the membrane of endo-lysosomal Ca^{2+} stores in many cell types including neurons (Bayraktar et al., 1990; Patel, 2015; Grimm et al., 2017; Foster et al., 2018) resulting in Ca^{2+} flux into the cytoplasm. Recently, it was also shown that Ca^{2+} entry via voltage gated Ca^{2+} channels triggers Ca^{2+} release from the lysosome via an NAADP-sensitive channel and, subsequently, fusion of the lysosome with the plasma membrane (Padamsey et al., 2017). In some cells the NAADP-evoked Ca^{2+} release depends on ryanodine receptors (RyR) such as the RyR1 in T cells (Diercks et al., 2018), and also TRPML1 channels were proposed as NAADP target channels (Zhang et al., 2011; Li et al., 2013). **(B)** Normalized mRNA expression of depicted genes in unstimulated primary hippocampal neuronal cultures. Transcripts per kilobase million (TPM) were calculated from GSE104802 (Mao et al., 2018; *n* = 4) and GSE142064 (*n* = 5) and were normalized to the mean expression from each dataset. **(C)** Representative images of primary hippocampal neurons of a C57BL/6N mice after 14 days *in vitro* as used in this study; photomicrography was performed using a transmitted light microscope with differential interference contrast (upper figure: objective EC Plan-Neofluar 20×/0.50 M27; lower figure: Plan Apochromat 63×/1.4 Oil DIC M27). Scale bars 100 μM, 20 μM.

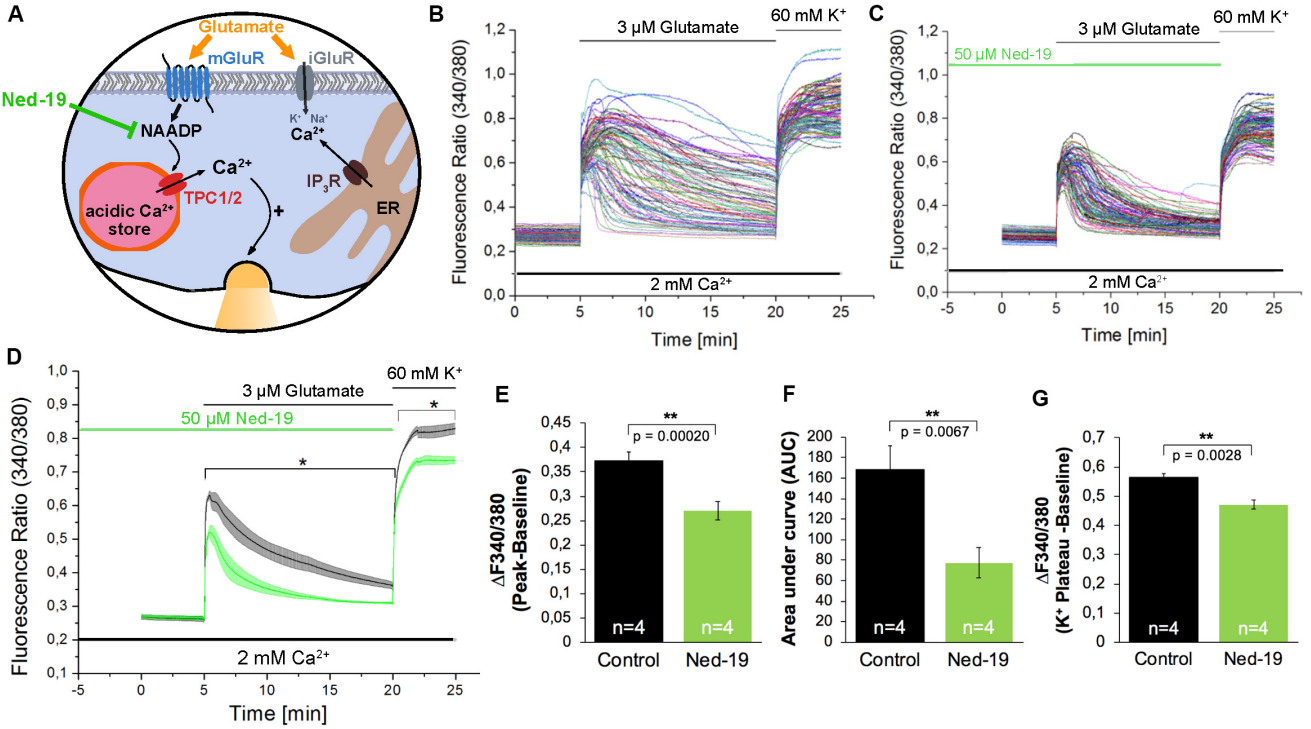

FIGURE 2 | The NAADP antagonist Ned-19 reduces the glutamate-induced Ca^{2+} increase in hippocampal neurons. **(A)** Ned-19 acts as a NAADP antagonist (Naylor et al., 2009). **(B,C)** Representative traces of the glutamate-induced changes in the F340/380 fluorescence intensity values in hippocampal neurons without **(B)** and with **(C)** pretreatment with Ned-19 (50 μM). **(D)** Average traces are given as the arithmetic mean ± SEM of the F340/380 fluorescence intensity values without (black, *n* = 1057 cells) and with (green, *n* = 659 cells) Ned-19 (50 μM) pre-incubation. Data from four independent C57Bl/6N preparations (*N* = 4) are shown. During all experiments, the cells were remained in physiological solution containing 2 mM Ca^{2+}. For measurements with Ned19, the cells were pre-incubated for 5 min. After 5 min of baseline recording, the cells were stimulated with glutamate (3 μM) for 15 min. For the last 5 min, the cells were stimulated with high potassium solution (60 mM K^+). The amplitude of the glutamate-induced calcium increase **(E)** as well as the AUC **(F)** and the amplitude of the high-potassium induced calcium transients **(G)** were analyzed. **: $p < 0{,}01$; *: $p < 0{,}05$.

potassium solution all cells in both conditions reacted with fast $[Ca^{2+}]_i$ rise reaching a plateau phase about 2 min after the stimulation. However, the F340/F380 fluorescence ratio amplitude of this high potassium-induced responses was reduced by ∼16% in cells pretreated with the NAADP antagonist Ned-19 (**Figure 2G**).

In addition to Ned-19, we used BZ194 as an independent NAADP antagonist. BZ194 was shown to effectively reduce NAADP-dependent calcium transients at a concentration of 500 μM (Nebel et al., 2013) and (**Figure 3A**). As it is evident from the representative traces (**Figures 3B,C**) and the average traces (**Figure 3D**), it is obvious that BZ194 pretreatment at the concentration at 500 μM does not prevent immediate $[Ca^{2+}]_i$ rise although the peak ratio levels were reduced significantly by ∼38% (**Figure 3E**), and the AUC of the $[Ca^{2+}]_i$ transients as a measure for the total calcium elevation over the period of stimulation is largely reduced by ∼82% (**Figure 3F**). Similarly, like in case of Ned-19 (50 μM) pretreatment the amplitude of high potassium-evoked $[Ca^{2+}]_i$ transients was also reduced. The observed reduction was ∼12% (**Figure 3G**). As the NAADP antagonist BZ194 was shown to affect calcium release in a dose-dependent manner in *T* lymphocytes and cardiomyocytes

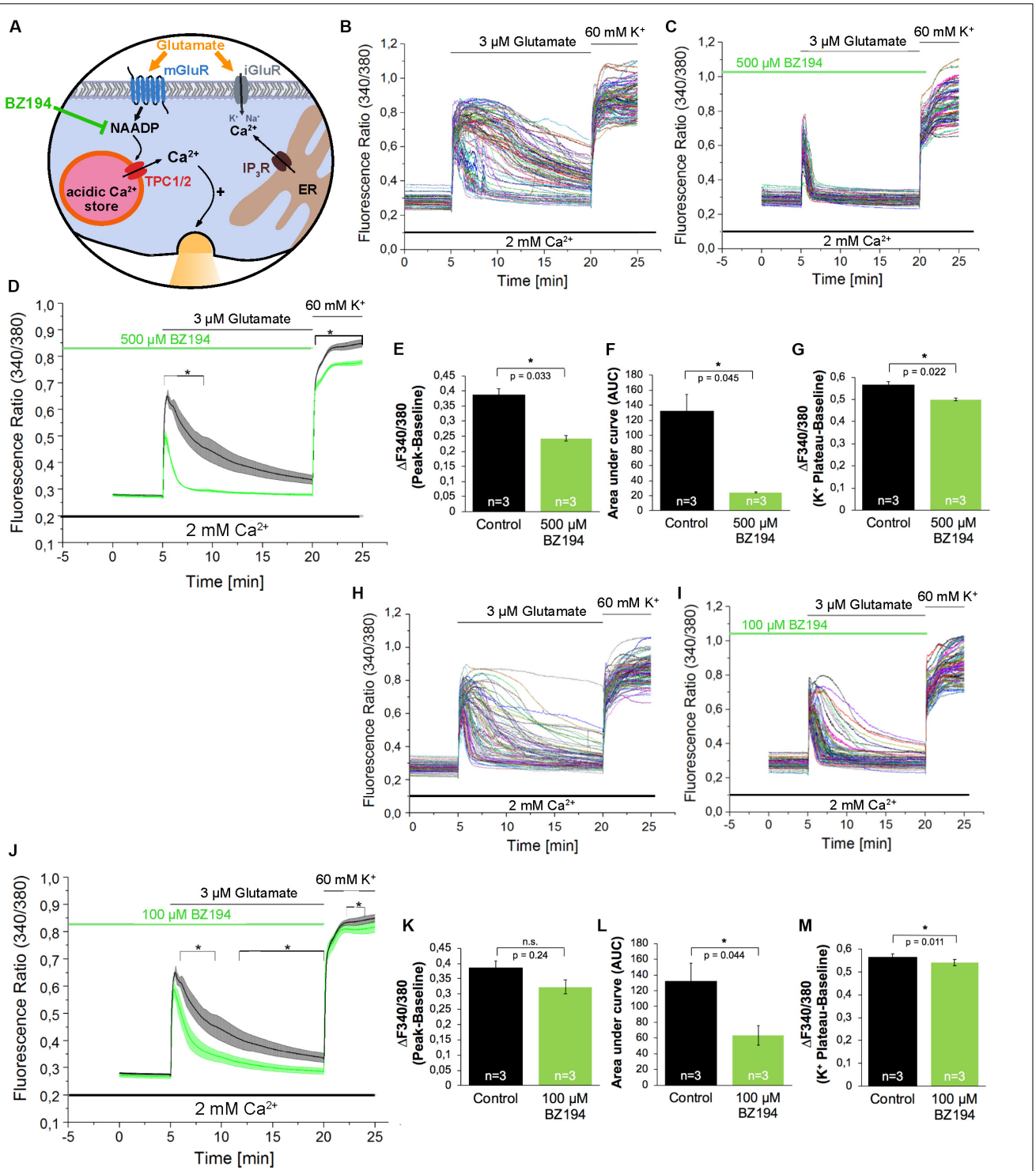

FIGURE 3 | The effect of the NAADP antagonist BZ194 on the time course of the glutamate-induced Ca^{2+} increase in hippocampal neurons. **(A)** BZ194 acts as a NAADP antagonist (Dammermann et al., 2009). **(B,C,H,I)** Representative traces of the glutamate-induced changes in the F340/380 fluorescence intensity values of hippocampal neurons without **(B,H)** and with 500 µM **(C)** or 100 µM **(I)** pre-incubation with BZ194. **(D,J)** Average traces are given as the arithmetic mean ± SEM of the averaged F340/380 fluorescence intensity values without (black, n = 719 cells in **D** and **J**) and with (green) 500 µM (**D**, n = 650 cells) or 100 µM (**J**, n = 611 cells) BZ194 pre-incubation. Data from independent C57Bl/6N preparations (n = 4) are shown. During all experiments, the cells were remained in physiological solution containing 2 mM Ca^{2+}. For measurements with BZ194, the cells were pre-incubated for 5 min. After 5 min recording, the cells were stimulated with glutamate (3 µM) for 15 min. For the last 5 min, the cells were stimulated with high potassium solution (60 mM K^+). Finally, the amplitude of the glutamate-induced calcium increase **(E,K)** as well as the AUC **(F,L)** and the amplitude of the high-potassium induced calcium transients **(G,M)** were analyzed. **: $p < 0{,}01$; *: $p < 0{,}05$.

(Dammermann et al., 2009; Nebel et al., 2013) we performed similar experiments with BZ194 pretreatment in hippocampal neurons using a lower concentration (100 μM, **Figures 3H–M**). Under these conditions the inhibition of glutamate evoked $[Ca^{2+}]_i$ rise was less pronounced as compared to BZ194 applied at the concentration of 500 μM. The AUC of the $[Ca^{2+}]_i$ transient was reduced by ∼52% (**Figure 3L**) and the amplitude of glutamate evoked $[Ca^{2+}]_i$ rise was partially reduced in average, but was not significantly different from the non-treated cells (**Figure 3K**). High potassium-evoked calcium rise was still significantly reduced at some time points but the reduction was only ∼5% in average (**Figure 3M**).

The acidification of endo-lysosomes depends on the activity of the Vacuolar-type H^+-ATPase (V-ATPase; **Figure 4A**), and by its inhibition using Bafilomycin A1 (Bowman et al., 1988) the calcium accumulation in the acidic stores can be prevented indirectly by inhibition of lysosomal acidification in many cell types (Yoshimori et al., 1991; Shen et al., 2012). Pre-incubation with Bafilomycin A1 (100 nM) for 60 min only slightly reduced the amplitude of glutamate evoked $[Ca^{2+}]_i$ transients by ∼16% (**Figures 4B–E**) and the AUC of these responses was not significantly reduced under the conditions tested (**Figure 4F**). Nevertheless the fluorescence ratio amplitude of the high potassium solution-evoked responses was significantly reduced by ∼23% (**Figure 4G**).

Calcium Transients Triggered by Bicuculline-Evoked Glutamate Release Events Are Modulated by NAADP Antagonists

In the next set of experiments, we aimed to investigate whether NAADP not only affects the calcium transients evoked by extracellular application of glutamate, but also by glutamate release events that occur endogenously in cells of our neuronal cultures. To facilitate such glutamate release events the cells were treated with bicuculline, a $GABA_A$ receptor antagonist, to prevent the inhibitory influence of these chloride channels on exocytosis of neurotransmitters (Hardingham et al., 2002). To this end, bicuculline (300 μM) was applied 5 min after recording of the resting $[Ca^{2+}]_i$. Representative traces of individual cells as well as the average traces are shown in **Figures 5A–F**. The bicuculline (300 μM) evoked changes in $[Ca^{2+}]_i$ appear as repetitive Ca^{2+} oscillations, were evoked within the first minute after bicuculline application and appeared synchronized between all tested cells of the culture dish. To assess whether NAADP antagonists affect the properties of these synchronized calcium transients, we quantified the oscillation frequency (**Figures 5G,K,O**), the AUC (**Figures 5I,M,Q**) as well as the amplitude of the corresponding calcium transients. To this end we compared calcium transients with high and low amplitudes (see method section for details) in bicuculline-evoked calcium transients with and without NAADP antagonist pretreatment (**Figures 5H,L,P**). In these experiments, cells were pretreated with Ned-19 in a concentration of 50 μM (**Figures 5A,B**), or 30 μM (**Figures 5C,D**) for 5 min. BZ194 (100 μM, **Figures 5E,F**) was pre-incubated for 60 min. The quantitative analysis of the time course of bicuculline-evoked calcium transients showed that pre-incubation with Ned-19 (50 μM) and BZ194 (100 μM), respectively, significantly altered the properties of bicuculline-evoked calcium transients, whereas Ned-19 at the concentration of 30 μM had no significant effect. In fact, the oscillation frequency was significantly increased by both NAADP antagonists (**Figures 5G,O**). Ned-19 (50 μM) pretreatment led to a significant increase in the number of calcium transients with a lower amplitude at the cost of transients with a higher amplitude although the reduction of the proportion of higher amplitudes itself was not statistically significant (**Figure 5H**). In average, there was a tendency toward an increase of the AUC upon Ned-19 (50 μM) pretreatment (**Figure 5I**). We noticed that the $[Ca^{2+}]_i$ continuously increased in most experiments particularly at the end of the observation time between 25 and 30 min after bicuculline application when cells were pretreated with Ned-19 (50 μM; **Figures 5A,B**, right panels). Therefore, we quantified the average change in $[Ca^{2+}]_i$ at the end of the bicuculline treatment compared to the calcium levels before bicuculline applications in cells without (black) and with Ned-19 (50 μM, green) pretreatment (**Figure 5J**). We found that Ned-19 treatment caused a significant calcium accumulation over this observation time period under bicuculline action and this increase in resting $[Ca^{2+}]_i$ was also observed to some degree when Ned-19 pretreatment was performed at the concentration of 30 μM, however, a similar effect was not observed with BZ194 (100 μM) pretreatment.

DISCUSSION

Over the last two decades, the concept of generation of NAADP in neurons following stimulation with glutamate has been first developed in brain microsomes (Bak et al., 1999) and then in lysosome-related organelles in hippocampal neurons (McGuinness et al., 2007). The generation of NAADP upon extracellular glutamate application has been directly measured (Pandey et al., 2009), however, the contribution of lysosomal stores due to glutamate evoked Ca^{2+} release relied on the depletion of lysosomal calcium stores by the dipeptide glycyl-l-phenylalanine 2-naphthylamide (GPN) that prevents organellar acidification. Yet, a recent study has demonstrated that GPN-evoked increase in $[Ca^{2+}]_i$ is not due to selective targeting of GPN on lysosomes, but increases cytosolic pH and subsequently Ca^{2+} release from the ER (Atakpa et al., 2019). However, our results using NAADP antagonists show that NAADP signaling contributes to glutamate-evoked $[Ca^{2+}]_i$ rise after extracellular glutamate application in hippocampal neurons. We also find that the nature of calcium transients, which occur in synchronous manner in all neurons throughout culture of hippocampal neurons after endogenous glutamate release, also depends on NAADP signaling as revealed by the use of the NAADP antagonists.

To analyze the contribution of NAADP-evoked signaling events during glutamate evoked $[Ca^{2+}]_i$ rise, in the present

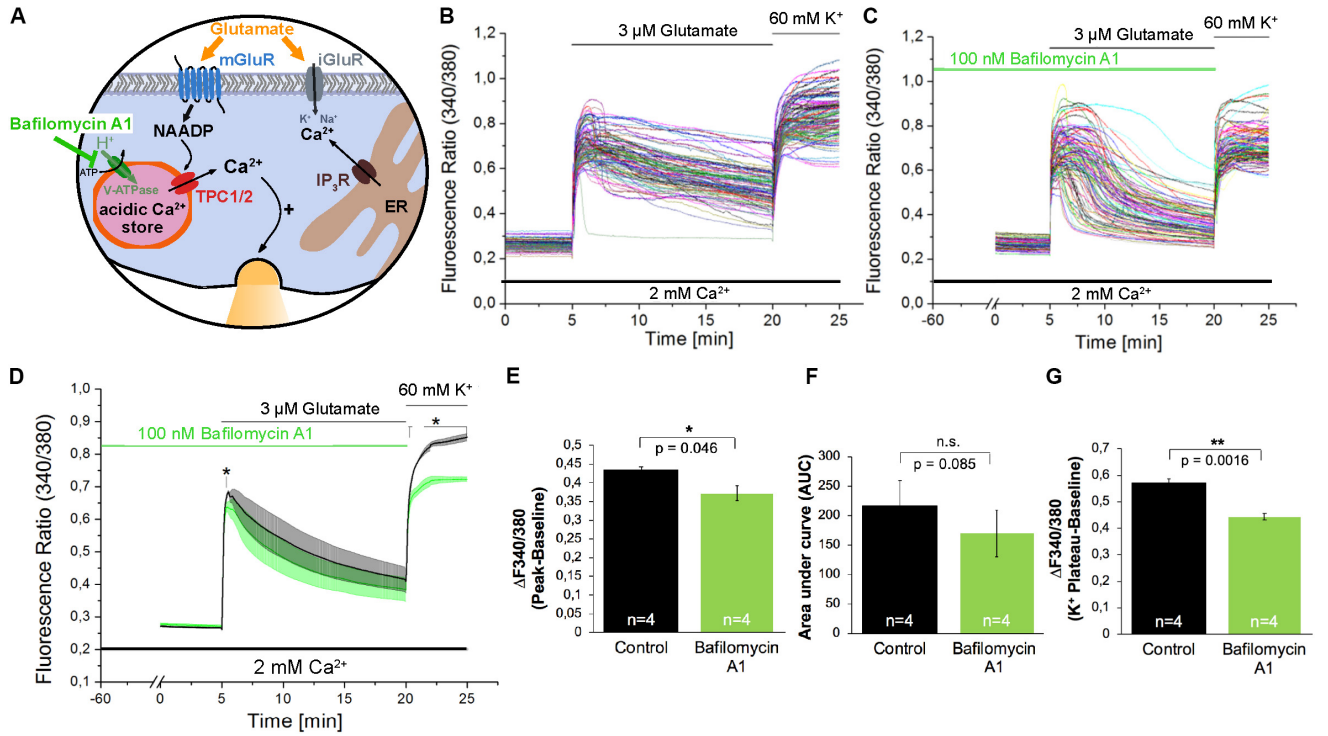

FIGURE 4 | The V-ATPase blocker Bafilomycin A1 does not affect glutamate-induced Ca^{2+} increase in hippocampal neurons. **(A)** Model depicting the action of Bafilomycin A1 as inhibitor of H^+ transport into acidic organelles. **(B,C)** Representative traces of the glutamate-induced changes in the F340/380 fluorescence intensity values in hippocampal neurons without **(B)** and with **(C)** pretreatment with Bafilomycin A1 (100 nM). **(D)** Average traces are given as the arithmetic mean ± SEM of the averaged F340/380 fluorescence intensity values without (black, n = 745 cells) and with (green, n = 849 cells) Bafilomycin A1 (100 nM) pre-incubation. Data from independent C57Bl/6N preparations (N = 4) are shown. During all experiments, the cells were remained in physiological solution containing 2 mM Ca^{2+}. For measurements with Bafilomycin A1, the cells were pre-incubated for 60 min. After 5 min of baseline recording, the cells were stimulated with glutamate (3 µM) for 15 min. For the last 5 min, the cells were stimulated with high potassium solution (60 mM K^+). The amplitude of the glutamate-induced calcium increase **(E)** as well as the AUC **(F)** and the amplitude of the high-potassium induced calcium transients **(G)** were analyzed. **: $p < 0{,}01$; *: $p < 0{,}05$.

study, we used two independent NAADP antagonists, Ned-19 (Naylor et al., 2009), and BZ194 (Dammermann et al., 2009). Pre-incubation with Ned-19 (50 µM) reduced peak amplitudes of glutamate evoked $[Ca^{2+}]_i$ rise by ∼27%, after pretreatment with BZ194 (500 µM) peak amplitudes of glutamate-evoked calcium transients decreased by ∼38%. In previous research, Ned-19 was used at this concentration in other cell types to antagonize NAADP action (Esposito et al., 2011), and BZ194 at this concentration was found to significantly reduce the Isoproterenol-evoked $[Ca^{2+}]_i$ rise from acidic stores in cardiomyocytes (Nebel et al., 2013). Pre-incubation with Bafilomycin A1, a V-ATPase blocker, which erases calcium accumulation in acidic stores indirectly by preventing their acidification (Bowman et al., 1988; Yoshimori et al., 1991), resulted only in a ∼16% reduction of the amplitude of glutamate-evoked Ca^{2+} release. In a previous study, the Bafilomycin A1 effect on glutamate evoked $[Ca^{2+}]_i$ rise amplitude was more pronounced reaching ∼50% reduction of the Ca^{2+} transient amplitude, however, in the aforementioned study, Bafilomycin A1 was used in a much higher concentration (1 µM vs. 100 nM in this study) and glutamate was applied at a concentration of 10 µM to stimulate the cells (Pandey et al., 2009). Another reason behind this discrepancy might also be that this study analyzed calcium transients in the absence of extracellular calcium levels, which was not pursued in our study, as the removal of extracellular calcium ions repeatedly triggered spontaneous calcium transients in mouse hippocampal neurons (data not shown). In addition, the fact that glutamate evokes Ca^{2+} entry also via ionotropic glutamate receptors in the presence of extracellular Ca^{2+}, as well as via voltage-gated calcium channels (Bading et al., 1993; Hardingham et al., 2001), can also contribute to the differences in the effectivity of Bafilomycin A1 between the two studies. Nevertheless, our study, using two independent NAADP antagonists and Bafilomycin A1, provide evidence about the contribution of NAADP-mediated calcium release and acidic stores to the amplitude of glutamate-evoked global calcium rise in hippocampal neurons of the mouse. Additional evidence comes from the analysis of the AUC of the glutamate-evoked $[Ca^{2+}]_i$ rise as Ned-19 (50 µM) pretreatment reduced AUC by ∼54%, and pretreatment with BZ194 reduced AUC by ∼82% and ∼52% when used at concentrations of 500 µM and 100 µM, respectively. Bafilomycin A1 did not affect this parameter, which can be attributed to the differences in the kinetics, and/or in the ability to evoke alkalization of acidic calcium stores by

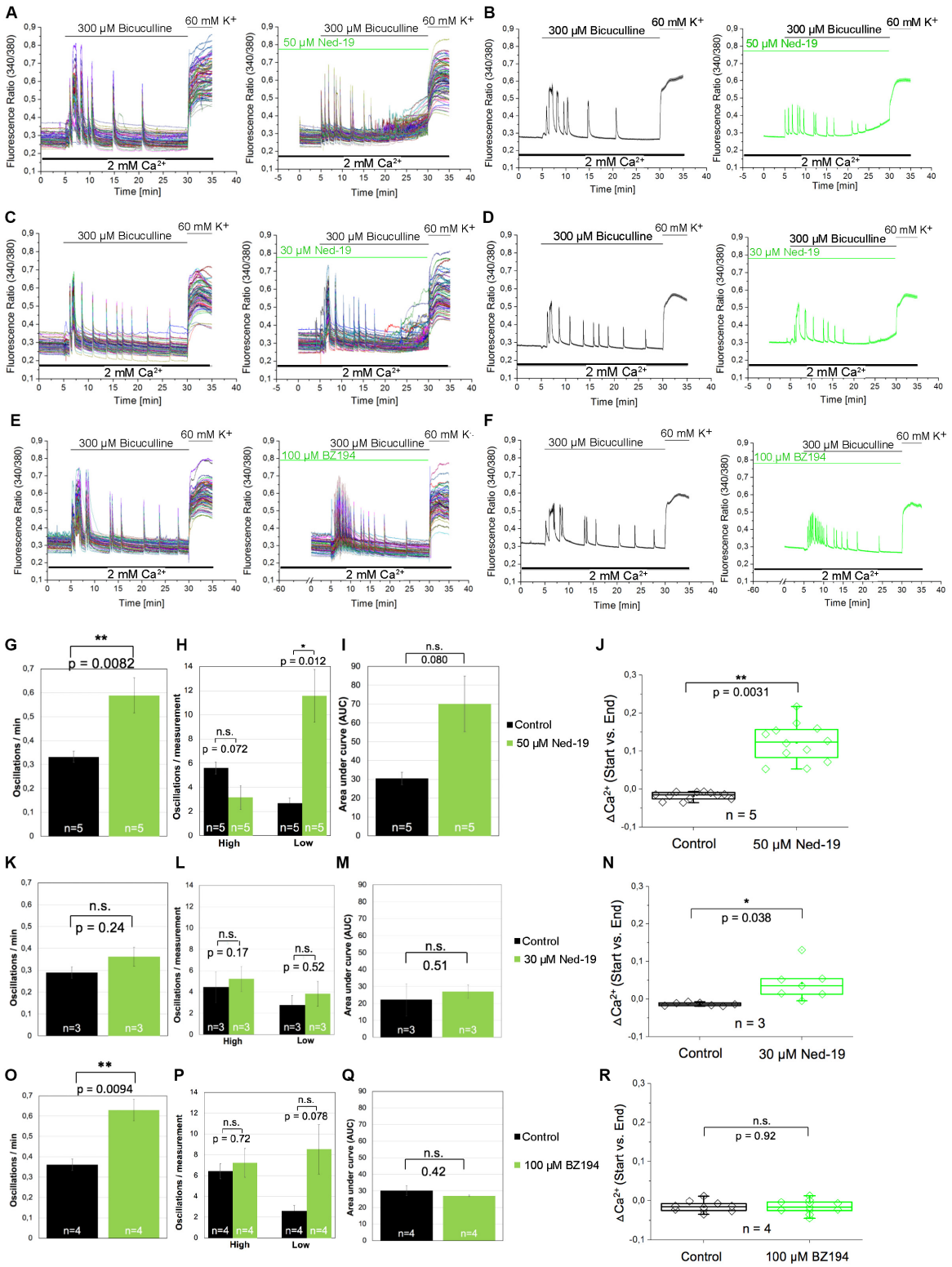

FIGURE 5 | The effect of the NAADP antagonists Ned19 and BZ194 on Ca^{2+} transients triggered by glutamate release events following inhibition of $GABA_A$ receptors in hippocampal neurons. **(A,C,E)** Representative traces of the bicuculline-induced changes in the F340/380 fluorescence intensity values in hippocampal neurons without (left) and with (right) preincubation with Ned-19 (50 µM; **A** or 30 µM; **C**) or BZ194 (100 µM; **E**). **(B,D,F)** Average traces are given as the arithmetic mean ± SEM without (black) and with (green) the corresponding NAADP antagonists (50 µM Ned-19, $n = 89$ cells, **B**; 30 µM Ned-19, $n = 86$ cells, **D**;

(Continued)

FIGURE 5 | Continued
and 100 μM BZ194, *n* = 79 cells, **F**). During all experiments, the cells were remained in physiological solution containing 2 mM Ca^{2+}. For measurements with Ned19, the cells were pre-incubated for 5 min; for measurements with BZ194, the cells were pre-incubated for 60 min. After 5 min recording, the cells were stimulated with bicuculline (300 μM) for 25 min. For the last 5 min, the cells were stimulated with high potassium solution (60 mM K^+). For each antagonist, several independent preparations (50 μM Ned19: *N* = 5: upper line; 30 μM Ned19: *N* = 3: middle line; and 100 μM BZ194: *N* = 4: lower line) were pooled. The oscillation frequency **(G,K,O)**, the number of high and low peaks **(H,L,P)** as well as the AUC **(I,M,Q)** were determined for each preparation without (black) and with (green) pre-incubation of the respective NAADP antagonist. **(J,N,R)** The change of the intracellular Ca^{2+} levels before and 25 min after bicuculline application (ΔCa^{2+}) was determined for each measurement without (black) und with (green) pre-incubation of the respective NAADP antagonist. For each p-value: *n* = 5 (50 μM Ned19); *n* = 3 (30 μM Ned19), and *n* = 4 (100 μM BZ194). **: $p < 0{,}01$; *: $p < 0{,}05$.

Bafilomycin A1 in the mouse and rat's hippocampal neurons. There could be some differences in effectivity of Bafilomycin A1 depending on cell type and time window (Klionsky et al., 2008).

Stimulation of the neuronal cultures with a high potassium solution led to an instantaneous increase that in contrast to glutamate stimulation was characterized by a continuous plateau. This response is similar to previously reported findings (Verkhratsky and Petersen, 2010). Interestingly, the high potassium-evoked $[Ca^{2+}]_i$ rise, which is supposed to be mediated by voltage gated calcium channels, was significantly reduced by both NAADP antagonists, Ned-19 and BZ194, as well as by pretreatment with the V-ATPase inhibitor Bafilomycin A1. A possible explanation for this result is that calcium entry via voltage-gated calcium channels can lead to a subsequent activation of Ca^{2+} release from NAADP-sensitive lysosomal calcium stores (Padamsey et al., 2017). An alternative explanation can be that endo-lysosomal calcium release supports the trafficking of *N*-type calcium channels into the plasma membrane (Hui et al., 2015). In agreement with the results reported by Padamsey et al. (2017) and Hui et al. (2015), the functional inactivation of endo-lysosomal calcium stores, which we performed in the present study using NAADP antagonists and Bafilomycin A1, would disrupt mutually facilitating interaction between lysosomal Ca^{2+} release and voltage-dependent calcium entry resulting in the reduced depolarization-induced calcium entry (see **Figures 3D,J**, **4D**). However, it remains to be determined in further research whether the Ca^{2+} release from the acidic stores precedes the activation of voltage-gated channels or vice versa.

In order to corroborate the relevance of NAADP signaling found after extracellular application of glutamate in micromolar concentrations we aimed to study conditions that are more similar to those occurring *in vivo* where glutamate is released locally by exocytotic release, e.g., in the synaptic cleft or even extrasynaptically. To this end, we performed experiments in which we applied the $GABA_A$ receptor antagonist bicuculline. This experimental strategy has been commonly used to remove the inhibitory influence of the $GABA_A$-mediated chloride currents on the exocytosis in glutamatergic neurons. Bicuculline-evoked calcium signals were characterized by $[Ca^{2+}]_i$ oscillations synchronized through all neuronal cells in the cell culture dish. Both Ned-19 (50 μM) and BZ194 (100 μM) pretreatments resulted in a significant increase in the frequency of bicuculline-evoked $[Ca^{2+}]_i$ oscillations, accompanied by a significant increase in the occurrence of calcium transients with a lower amplitude. While further research is needed to identify physiological and pathophysiological consequences of such changes in the nature of these glutamate-evoked $[Ca^{2+}]_i$ oscillations, it is already known that a precise regulation of Ca^{2+} release from acidic stores via two-pore channels is essential for regulation of synaptic plasticity, since deletion of TPC channels leads to a reversal from long-term potentiation to long-term depression in mice (Foster et al., 2018).

Potential NAADP Target Channels in Hippocampal Neurons

To get an idea which channels might be present in our cell system to mediate glutamate-evoked calcium release we analyzed the expression of candidate NAADP target channels in two independent RNA seq experiments that were performed in murine hippocampal neurons, i.e., GSE104802 (Mao et al., 2018) and GSE142064 (unpublished). The analysis suggests that both TPC channel subtypes, TPC1 and TPC2, were expressed in murine hippocampal neurons and might operate as NAADP target channels upon glutamate stimulation similarly like TRPML1. The causal contribution of any of these channels could be revealed by studying glutamate evoked Ca^{2+} transients in hippocampal neurons that are deficient for the corresponding channels or by patch clamp recordings performed in the endo-lysosomes of these cells as was done before in other cell types (Chen et al., 2017). Alternatively, agonists and antagonists that specifically target individual channel subtypes, as recently reported for members of these channel families (Plesch et al., 2018; Gerndt et al., 2020), might be useful tools to pinpoint the contribution of distinct channel entities in this process. TRPML1 protein expression in cultured mouse embryonic hippocampal neurons was previously demonstrated using Western blotting analysis (Zhang et al., 2017). RYR1, which operates as an NAADP target channel early during *T* cell activation (Wolf et al., 2015; Diercks et al., 2018), seems to be not expressed in cultured murine hippocampal neurons. In contrast to RYR2 and RYR3, we did not find RYR1 expression in mouse embryonic hippocampal neurons. Similar results were reported in the study analyzing developmental changes in the expression of the three ryanodine receptor mRNAs in the mouse brain (Mori et al., 2000). These authors report that in the hippocampal CA1 region RYR1 mRNA appeared only at P1 and reached its peak level at P7. Also in the adult mouse hippocampal CA region, RYR1 had the lowest expression level compared to RYR2 and RYR3. A contribution of RYR2 and/or RYR3 for intracellular Ca^{2+} rise downstream of NAADP generation has not been reported so far, although these receptors appear to be regulated by cyclic ADP-ribose (Fritz et al., 2005; Dabertrand et al., 2007). However, RYR2 and RYR3 should

be studied in the future with similar approaches as discussed for TPC and TRPML1 channels above.

Taken together, the results of this study provide evidence that NAADP-sensitive calcium stores contribute to the alteration in cellular calcium homeostasis in neurons evoked by glutamate. Whether calcium release from these acidic stores aggravates excitotoxicity in disease models with inflammatory neuronal degeneration such as Multiple Sclerosis needs to be elaborated in further research by the inhibition of endo-lysosomal channels with specific antagonists or their genetic deletion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Regierungspräsidium Karlsruhe and the University Heidelberg.

AUTHOR CONTRIBUTIONS

MF and VT: Conceptualization. JH and MW: Data curation. JH, VT, MW, VK, and MF: Formal analysis. MAF and MF: Funding acquisition. JH, VT, and MF: Investigation. JH, MB, DS, MW, AS, SR, and VT: Methodology. VT, MAF, and MF: Project administration. CM and AG: Resources. VT, MAF, and MF: Supervision. JH, MW, and MF: Visualization. JH, VT, and MF: Writing – original draft. Writing – review and editing, all authors.

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TRPV4-Mediated Regulation of the Blood Brain Barrier Is Abolished During Inflammation

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Blood-brain barrier (BBB) dysfunction is critically involved in determining the extent of several central nervous systems (CNS) pathologies and here in particular neuroinflammatory conditions. Inhibiting BBB breakdown could reduce the level of vasogenic edema and the number of immune cells invading the CNS, thereby counteracting neuronal injury. Transient receptor potential (TRP) channels have an important role as environmental sensors and constitute attractive therapeutic targets that are involved in calcium homeostasis during pathologies of the CNS. Transient receptor potential vanilloid 4 (TRPV4) is a calcium permeable, non-selective cation channel highly expressed in endothelial cells. As it is involved in the regulation of the blood brain barrier permeability and consequently cerebral edema formation, we anticipated a regulatory role of TRPV4 in CNS inflammation and subsequent neuronal damage. Here, we detected an increase in transendothelial resistance in mouse brain microvascular endothelial cells (MbMECs) after treatment with a selective TRPV4 inhibitor. However, this effect was abolished after the addition of IFNγ and TNFα indicating that inflammatory conditions override TRPV4-mediated permeability. Accordingly, we did not observe a protection of *Trpv4*-deficient mice when compared to wildtype controls in a preclinical model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), and no differences in infarct sizes following transient middle cerebral artery occlusion (tMCAO), the experimental stroke model, which leads to an acute postischemic inflammatory response. Furthermore, Evans Blue injections did not show differences in alterations of the blood brain barrier (BBB) permeability between genotypes in both animal models. Together, TRPV4 does not regulate brain microvascular endothelial permeability under inflammation.

Keywords: TRPV4, transendothelial resistance, blood brain barrier, experimental autoimmune encephalomyelitis, stroke

INTRODUCTION

The blood-brain barrier (BBB) is responsible for maintaining the separation of the central nervous system (CNS) from the blood and thereby controls the entry of neurotoxic metabolites, ions, pathogens and blood cells (Zhao et al., 2015). The cells of the BBB communicate with cells of the CNS but also with circulating immune cells allowing a tight adaption to maintain the highly regulated CNS internal milieu (Banks, 2016). The major component of the BBB are pericytes, astrocytes and endothelial cells that control the passage of molecules by a crosstalk between tight and adherens junctions (Obermeier et al., 2013; Keaney and Campbell, 2015; Tietz and Engelhardt, 2015).

Diminished BBB integrity results in increased vascular permeability and is essential for the development of cerebral edema (Sweeney et al., 2018). Similarly, a breakdown of the BBB facilitates the entry of immune cells into the CNS and is crucial for neuroinflammation (Man et al., 2007; Bennett et al., 2010; Ransohoff and Engelhardt, 2012). Proinflammatory cytokines evoke an upregulation of cell adhesion molecules on endothelial cells which can bind to integrins on leukocytes and thereby facilitate transendothelial leukocyte migration (Minagar and Alexander, 2003; Keaney and Campbell, 2015). Attenuating BBB permeability reduces the number of invading immune cells and the extent of a subsequent edema, which then ameliorates neuronal loss. Interventional studies in animal models of multiple sclerosis (Göbel et al., 2019; Viñuela-Berni et al., 2020) and stroke (Casas et al., 2017; Pan et al., 2020) where the BBB permeability was decreased as a result of treatment showed improved neurological outcomes. However, until now no clinically approved drug exists, highlighting the need to identify new effective targets.

A promising candidate which is known to regulate the integrity of several endo- and epithelial barriers, is the transient receptor potential vanilloid 4 (TRPV4), which is a calcium-permeable non-selective cation channel and a member of the transient receptor potential (TRP) superfamily of cation channels (Liedtke et al., 2000; Strotmann et al., 2000; Wissenbach et al., 2000). TRP channels play an important role as environmental sensors and constitute an attractive gene family that could be involved in CNS pathologies (Schattling et al., 2012; Gelderblom et al., 2014; Kanju and Liedtke, 2016). *Trpv4* is expressed widely including endothelial cells and astrocytes. TRPV4 is polymodally activated (Nilius et al., 2004; Moore et al., 2018) by multiple extracellular mechanic stimuli such as cellular shear, stretch and cell compression (Michalick and Kuebler, 2020), by osmotic changes (Liedtke et al., 2000; Liedtke, 2005), by thermal cues (Tominaga and Caterina, 2004), by UVB radiation (Moore et al., 2013), by endogenous bioactive lipids such as arachidonic acids metabolites (Watanabe et al., 2003) and others. However, the molecular mechanisms of TRPV4 activation could differ from cell-type to cell-type and are overall not completely understood. In endothelial cells, TRPV4 functions as mechanoreceptor in response to shear stress (Matthews et al., 2010). Upon TRPV4 activation calcium fluxes into the cell and activates calcium-activated potassium channels. This can result in increased barrier permeability (Morty and Kuebler, 2014). It was shown that TRPV4 inhibition prevents and resolves pulmonary edema (Hamanaka et al., 2010; Thorneloe et al., 2012; Michalick et al., 2017), reduces the infarct size in myocardial ischemia (Dong et al., 2017), decreases the vascular endothelial permeability in murine colitis (Matsumoto et al., 2018) and diabetic retinal injury (Arredondo Zamarripa et al., 2017; Ríos et al., 2019). Furthermore, TRPV4 also regulates the integrity of the blood-cerebrospinal fluid barrier (Narita et al., 2015) and TRPV4 inhibition reduced BBB disruption and consecutive edema in a mouse model of intracerebral hemorrhage and thereby ameliorated neurological symptoms (Zhao et al., 2018). These observations imply an important role of TRPV4 in the BBB.

Therefore, modifications of TRPV4 could constitute an attractive therapeutic target in diseases with neuroinflammatory pathways that rely on temporarily impaired BBB integrity for influx of disease-enhancing immune cells. Here we analyzed whether TRPV4 inhibition has an impact on transendothelial resistance under homeostatic and inflammatory conditions and whether deletion of *Trpv4* affects the clinical outcome in mouse models of multiple sclerosis and stroke. We show that inflammation overrules the homeostatic TRPV4-mediated regulation of the BBB, and that *Trpv4* deficiency does not modify the outcome in these disease models.

MATERIALS AND METHODS

Mice

All mice [C57BL/6J wild-type, The Jackson Laboratory; *Trpv4*$^{-/-}$ mice on a C57BL/6J genetic background (Liedtke and Friedman, 2003)] were kept under specific pathogen-free conditions in the central animal facility of the Universitätsklinikum Hamburg-Eppendorf (UKE). Animals were housed in a facility with 55–65% humidity at 24 ± 2°C with a 12-h light/dark cycle and had free access to food and water.

EAE Induction

Mice were anaesthetized with isoflurane 1–2% v/v oxygen and immunized subcutaneously with 200 μg myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) peptide (peptides & elephants) in complete Freund's adjuvant (BD) containing 4 mg ml^{-1} *Mycobacterium tuberculosis* (BD). 200 ng pertussis toxin (Merck) was injected intravenously on the day of immunization and 48 h later. Animals were scored daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; 5, premorbid or dead. Animals reaching a clinical score ≥ 4 were sacrificed according to the regulations of the Animal Welfare Act. The experimenters were blinded to the genotype until the end of the experiment, including data analysis. Sex- and age-matched adult animals (8–12 weeks of age) were used in all experiments. For *Trpv4*$^{-/-}$ mice and WT controls, three independent EAE experiments were conducted. The data were pooled for final analysis. For analysis of the disease course and weight we only included animals which received a score ≥ 1 until day 20 and survived until day 30. For analysis of onset we included all mice which received a score ≥ 1.

Determination of Blood Brain Barrier Permeability in EAE

BBB integrity was measured at the peak (day 13 after immunization) of EAE (n = 5 per genotype). Mice were intravenously injected with 200 µl of 2% Evans Blue (Millipore Sigma) dissolved in 0.1 M phosphate buffer saline (PBS). 2 h later mice were anesthetized intraperitoneally with 100 µl solution (10 mg ml^{-1} esketamine hydrochloride (Pfizer), 1.6 mg ml^{-1} xylazine hydrochloride (Bayer) dissolved in water) per 10 g of body weight and perfused with PBS. Brain, spinal cord and kidney were resected, dried for 24 h at 50°C and placed into formamide (Sigma) the volume of which was adjusted to the tissue weight. For the complete Evans Blue extraction, samples were incubated 24 h at 55°C. Amount of dye was determined by colorimetric analysis as absorbance coefficient at 610 nm. The data for brains and spinal cords were normalized to the kidneys values (Radu and Chernoff, 2013).

Mouse Tissue Preparation and Histopathology of EAE Mice

Mice were anesthetized intraperitoneally with 100 µl solution (10 mg ml^{-1} esketamine hydrochloride (Pfizer), 1.6 mg ml^{-1} xylazine hydrochloride (Bayer) dissolved in water) per 10 g of body weight. Afterward mice were perfused with 4% paraformaldehyde (PFA) and cervical spinal cord was resected, dehydrated and cast in paraffin. CD3 staining (rabbit IgG, Abcam ab16669) was visualized by the avidin-biotin technique with 3,3-diaminobenzidine according to standard procedures of the UKE Mouse Pathology Facility. The slides were analyzed using a NanoZoomer 2.0-RS digital slide scanner and NDP.view2 software (Hamamatsu). Quantification of infiltrating CD3 cells was done with ImageJ software (NIH), using the same settings across experimental groups.

In vivo Stroke Model

Mice were anesthetized using isoflurane 1–2% v/v oxygen and we injected buprenorphine 0.03 mg/kg body weight intraperitoneally (i.p.) every 12 h for 24 h as analgesia. We conducted transient middle cerebral artery occlusion (tMCAO) for 45 min using the intraluminal filament method (6-0 nylon, Docoll) as described before (Gelderblom et al., 2014). We monitored mice for heart rate, respiratory rate, oxygen saturation, rectal body temperature, and cerebral blood flow by using transcranial temporal laser Doppler technique. Only mice with a sufficient decrease in the ipsilateral laser Doppler flow (below 20 % when compared to the contralateral site) were included in the study, to ensure an appropriate occlusion of the middle cerebral artery. Animals were scored immediately after reawakening and daily for clinical signs by the following system (Bederson Score): 0, no deficit; 1, preferential turning; 2, circling; 3, longitudinal rolling; 4, no movement; 5, death. For *Trpv4*$^{-/-}$ mice and WT controls, two independent stroke experiments were conducted. The data were pooled for final analysis. Mortality did not differ between the groups. To reduce the variability of our outcome parameters caused by sex-differences only male mice (12–16 weeks of age) were used throughout the study.

Analysis of Infarct Size by TTC Staining

Only mice with a Bederson score greater or equal than one after reawakening and a sufficient occlusion of the middle cerebral artery during MCAO as measured by laser Doppler technique were included in stroke size analysis. Mice were sacrificed 3 days after reperfusion using isoflurane and decapitation. Brains were harvested and cut into 1 mm slices (Braintree Scientific, 1 mm) followed by vital staining using 2% (wt/vol) 2,3,5-triphenyl-2-hydroxy-tetrazolium chloride (TTC) in phosphate buffer. We determined infarct volumes blinded for genotype by using NIH ImageJ software.

Determination of Blood Brain Barrier Permeability in Stroke

BBB integrity following stroke was assessed in mice (n = 6 per experimental group) that were intraperitoneally injected with 200 µl of 2% Evans Blue (Millipore Sigma, Cat. No. E2129) dissolved in 0.1 M phosphate buffer saline (PBS) directly after tMCAO. 24 h later mice were processed as described above. We additionally analyzed Evans Blue staining after 72 h of tMCAO.

Primary Endothelial Culture and Transendothelial Electrical Resistance (TEER)

Mouse brain microvascular endothelial cells (MbMECs) were isolated as previously described (Weidenfeller et al., 2005; Ruck et al., 2014) and incubated in a humidified incubator with 5% CO_2 at 37°C. Fresh, puromycin free MbMEC medium was added 4 days after isolation. Two days later, when cells reached confluence, they were harvested by trypsinization and seeded for subsequent TEER experiments onto pre-coated transwell inserts (pore size 0.4 µm; Corning) at 2×10^4 cells per insert. TEER measurements were performed and analyzed using the cellZscope 24-cell module and cellZscope v2.2.2 software, respectively (nanoAnalytics GmbH) as described before (Kuzmanov et al., 2016). Automated TEER and cell layer capacitance (Ccl) measurements were performed every hour for 3 to 4 days until the MbMEC monolayer reached full confluence, as determined by stable Ccl below 1 µF/cm^2 and TEER at its maximum plateau for at least 6 h. At that time point, MbMECs were either kept naïve or inflammation was induced by addition of 50 U/ml IFNγ and 50 U/ml TNFα. Additionally, either vehicle or GSK2193874 (Sigma), a TRPV4-specific inhibitor, which has been shown to inhibit TRPV4-dependent calcium influx in endothelial cells and exerts potent *in vivo* activity (Thorneloe et al., 2012; Cheung et al., 2017), were applied and TEER measurements were resumed for another 24 h.

Expression Analysis

For analysis of *Trpv4* mRNA expression on MbMECs, total RNA was extracted from naïve MbMECs or MbMECs inflamed for 24 h with 50 U/ml IFNγ and 50 U/ml TNFα by using a Quick RNA Micro Prep Kit (Zymo Research); cDNA was synthesized from 300 ng of total RNA by using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific), all performed according to the manufacturers' instructions. For quantitative

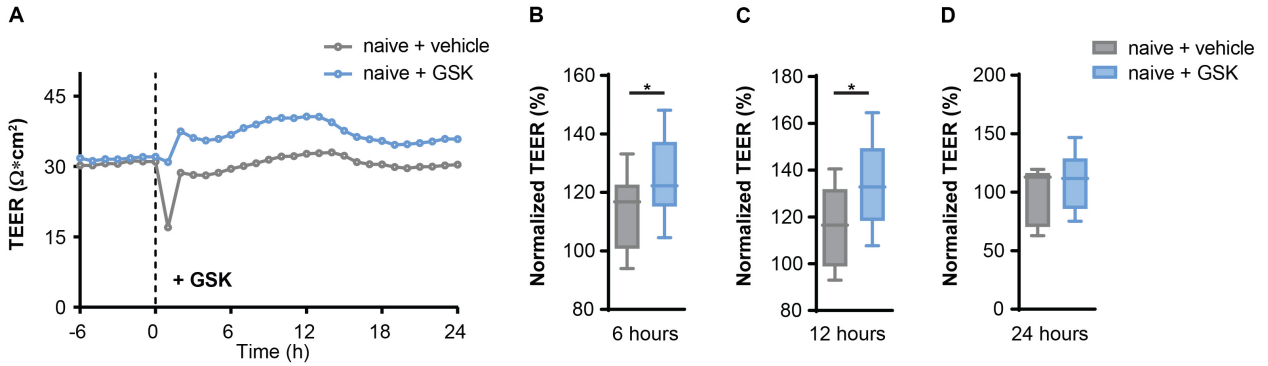

FIGURE 1 | TRPV4 inhibition increases transendothelial electrical resistance (TEER) in mouse brain microvascular endothelial cells (MbMECs) under homeostatic conditions. **(A)** TEER of MbMECs treated with 5 nM of GSK2193874 in comparison to vehicle-treated MbMECs. One representative experiment out of 12 is shown. **(B–D)** Quantification of TEER of MbMECs treated with 5 nM of GSK2193874 (n = 12) in comparison to vehicle treated MbMECs (n = 12) at 6, 12, and 24 h. TEER was normalized to the time-point of GSK treatment (0 h). Data are presented as box plots. Statistical analysis was performed by two-tailed Student's t-test. $^*P < 0.05$.

RT-PCR (RT-qPCR), a mouse *Trpv4* TaqMan Gene Expression Assay (Mm00499025_m1) with 18S rRNA as endogenous control was used. RT-qPCR was performed by using the StepOnePlus System (Applied Biosystems). Data were analyzed using the $\Delta\Delta$CT method followed by relative quantification ($2^{-\Delta\Delta CT}$).

Study Approval

All animal care and experimental procedures were performed according to institutional guidelines and conformed to requirements of the German Animal Welfare Act. All animal experiments were approved by the local ethics committee (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz in Hamburg; G22/13 and 59/17. We conducted all procedures in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

Statistics

Experimental data were analyzed using Prism 8 software (GraphPad) and are presented as mean values ± SEM. Statistical analyses were performed using the appropriate test indicated in the figure legends. D'Agostino and Pearson test was used to analyze normality. Unless stated otherwise, differences between two experimental groups were determined by unpaired, two-tailed Mann-Whitney or Students t-test. Significant results are indicated by asterisks: $^*P < 0.05$.

RESULTS

TRPV4 Inhibition Increases Transendothelial Resistance

First, we validated a regulative role of TRPV4 for BBB permeability. We measured the transendothelial electrical resistance (TEER) of mouse brain microvascular endothelial cells (MbMECs) in the absence and presence of the specific pharmacological TRPV4 inhibitor GSK2193874, which has been previously shown to have potent efficiency in ameliorating lung edema (Thorneloe et al., 2012). Six and 12 h after addition of GSK2193874 we detected a significant increase of the TEER, which was not anymore detectable after 24 h (**Figures 1A–D**). Thus, TRPV4 inhibition by GSK2193874 induces an increased endothelial cell barrier integrity.

Inflammation Overrides TRPV4-Mediated Permeability of the Blood Brain Barrier

Since TRPV4 inhibition leads to an increased resistance in MbMECs, we next investigated whether this could be confirmed under inflammatory conditions. We analyzed TRPV4 functionality after the exposure of tumor necrosis factor-α (TNFα) and interferon-γ (INFγ) that are key cytokines during neuroinflammation (Becher et al., 2017). Notably, in the presence of TNFα and INFγ the observed effect of TEER increase by inhibiting TRPV4 activity under homeostatic conditions was abolished (**Figures 2A–D**).

As changes in cytokine-mediated *Trpv4* expression could be an explanation for the abolished regulation, we next analyzed *Trpv4* expression in MbMECs under the different conditions. Indeed, we observed a significant reduction of *Trpv4* mRNA in MbMECs after exposure to TNFα and INFγ in comparison to homeostatic conditions (**Figure 2E**). We concluded that inflammatory cytokines induce a TRPV4 loss-of-function in MbMECs by attenuating the gene expression of *Trpv4*.

Blood Brain Barrier Permeability in EAE Is Not Altered by TRPV4

As TRPV4 inhibition leads to an increased resistance of endothelial cells, which is abrogated under inflammatory conditions, we next investigated whether TRPV4-mediated BBB regulation is indeed overruled in EAE. For this, we induced EAE in *Trpv4*-deficient ($Trpv4^{-/-}$) mice and compared their disease course to wild-type (WT) controls. We did not see any differences in hallmark clinical phenotypes such as disease onset (**Figure 3A**), disease disability score (**Figure 3B**) or body weight changes (**Supplementary Figure 1A**).

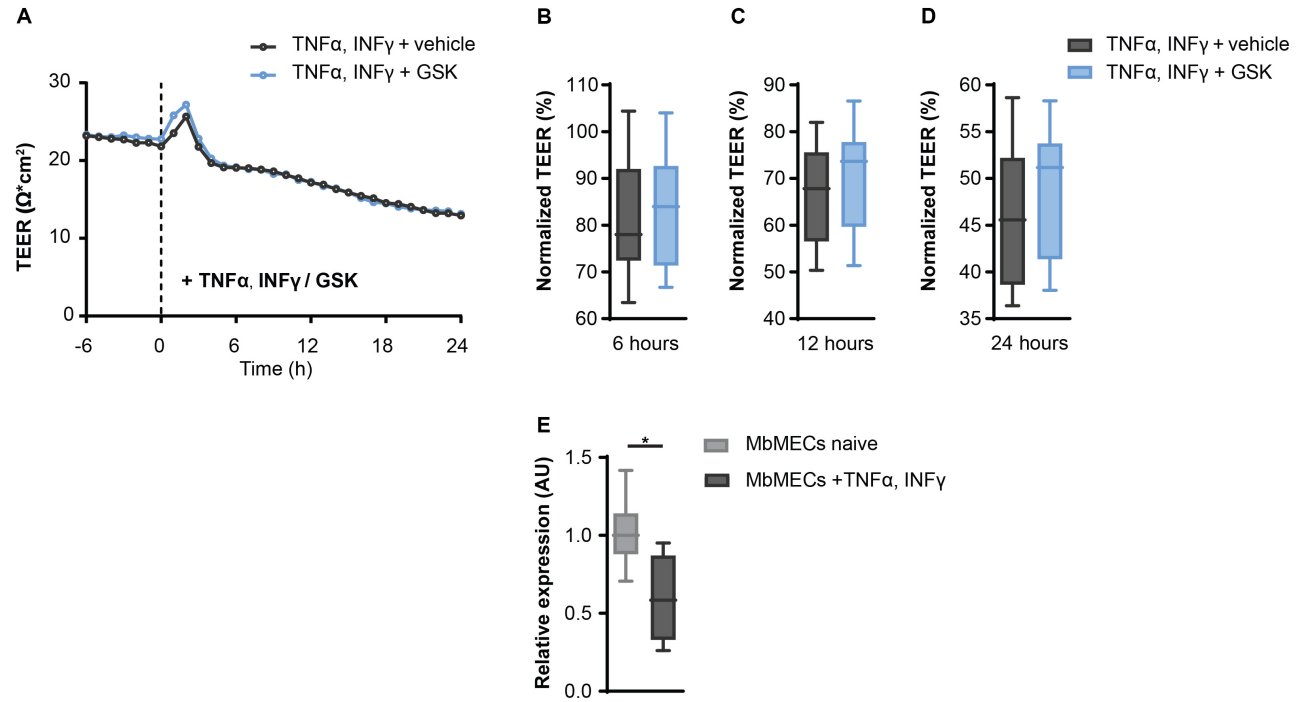

FIGURE 2 | Inflammatory conditions abolish the effect of TRPV4 inhibition on transendothelial electrical resistance (TEER) in mouse brain microvascular endothelial cells (MbMECs). **(A)** TEER of MbMECs treated with 5 nM of GSK2193874 in comparison to vehicle-treated MbMECs, both were exposed to 50 U/ml TNFα and INFγ. One representative experiment out of 14 is shown. **(B–D)** Quantification of TEER of MbMECs treated with 5 nM of GSK2193874 (n = 14) in comparison to vehicle-treated MbMECs (n = 14) at 6, 12, and 24 h, both were exposed to 50 U/ml TNFα and INFγ. TEER was normalized to the time-point of GSK treatment (0 h). **(E)** Relative *Trpv4* mRNA expression assessed by qPCR in MbMECs exposed to 50 U/ml TNFα and INFγ (n = 4) in comparison to homeostatic conditions (n = 8). Data are presented as box plots. Statistical analysis was performed by two-tailed Student's t-test **(A)** and **(B–D)** or two-tailed Mann Whitney test **(E)**. $^*P < 0.05$.

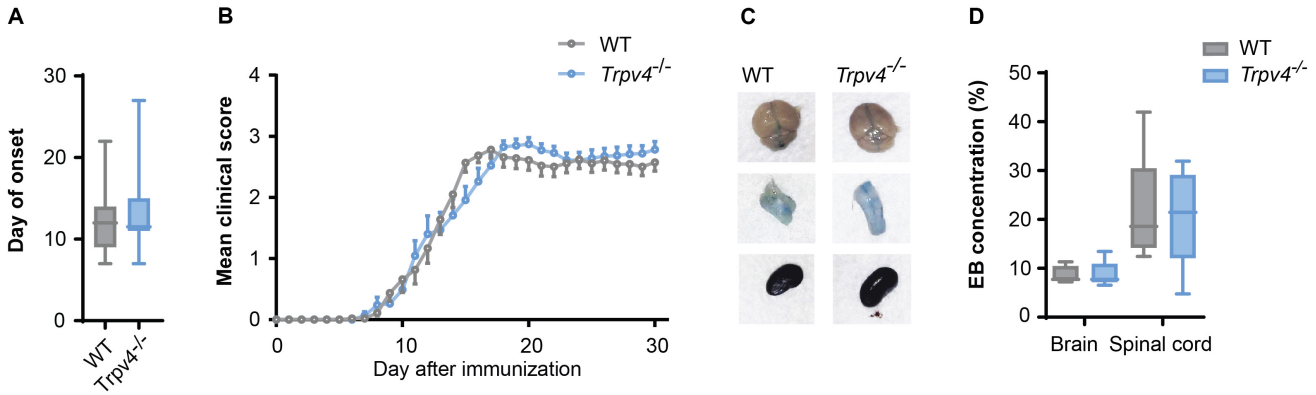

FIGURE 3 | No difference between *Trpv4*$^{-/-}$ mice and WT controls on the disease course and BBB permeability in EAE. **(A)** Day of onset of WT (n = 33) and *Trpv4*$^{-/-}$ mice (n = 32) during the course of EAE. **(B)** Clinical scores of WT (n = 27) and *Trpv4*$^{-/-}$ mice (n = 23) undergoing EAE. **(C)** Evans Blue (EB) quantification in brain and spinal cord of WT (n = 5) and *Trpv4*$^{-/-}$ (n = 5) mice at day 13 after EAE immunization. Evans Blue concentration was normalized to the concentration of the right kidney of the corresponding animal. **(D)** Representative images of brain, spinal cord and kidney of WT- and *Trpv4*$^{-/-}$-EAE mice 2 h after Evans Blue injection. Data in **(A,C)** are presented as box plots, in **(B)** as mean values ± s.e.m. Statistical analysis was performed by two-tailed Student's t-test in **(A)** by two-tailed Mann Whitney test in **(B,C)**.

Additionally, we assessed the BBB permeability by analyzing Evans Blue staining at the peak of disease. As this form of EAE mainly affects the spinal cord we observed higher levels of Evans Blue in the spinal cord than the brain at day 13 after immunization, without detecting a significant difference in the amount of Evans Blue in *Trpv4*$^{-/-}$ mice in

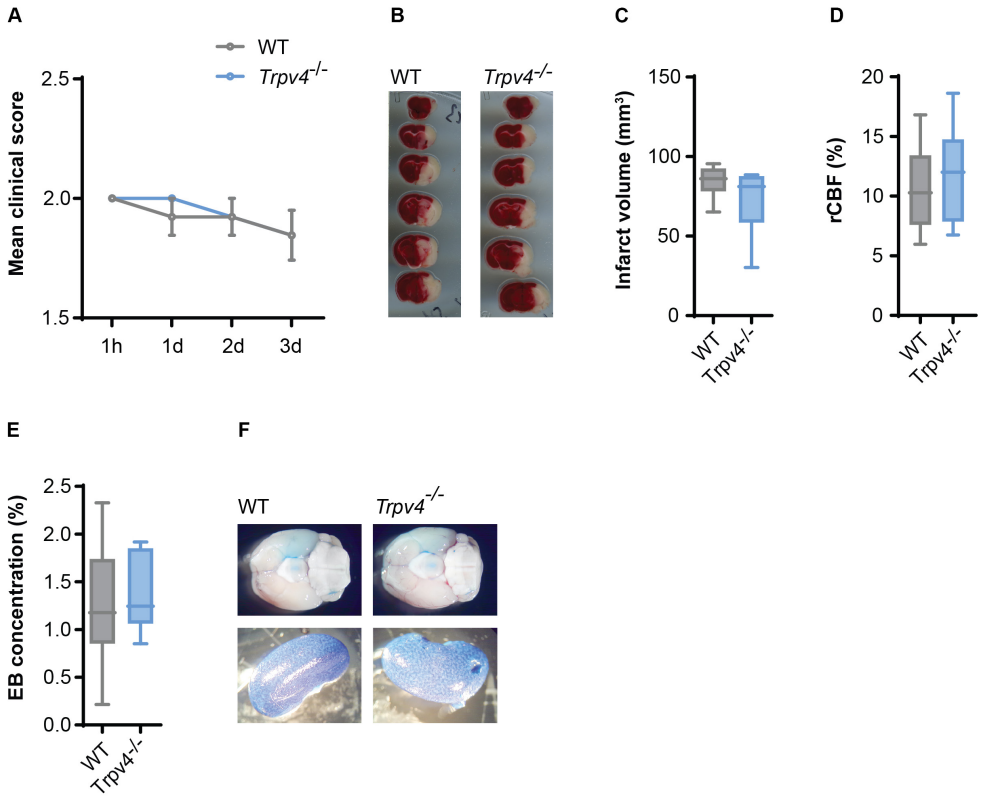

FIGURE 4 | No difference between *Trpv4*$^{-/-}$ mice and WT controls on disease course and BBB permeability in tMCAO. **(A)** Clinical scores of WT (n = 13) and *Trpv4*$^{-/-}$ mice (n = 13) after tMCAO; h = hours, d = days. **(B)** Representative TTC staining of WT and *Trpv4*$^{-/-}$brains 3 days after tMCAO. **(C)** Quantification of infarct volume of WT (n = 8) and *Trpv4*$^{-/-}$ (n = 9) 3 days after tMCAO. **(D)** Regional cerebral blood flow (rCBF) of WT (n = 13) and *Trpv4*$^{-/-}$ mice (n = 13) was measured during occlusion by laser Doppler and normalized to contralateral hemispheres. **(E)** Evans Blue (EB) quantification in brains of WT (n = 6) and *Trpv4*$^{-/-}$ (n = 6) mice 24 h after tMCAO. Evans Blue concentration was normalized to the concentration of the right kidney of the corresponding animal. **(F)** Representative images of brains and kidneys of WT and *Trpv4*$^{-/-}$ mice 24 h after tMCAO and Evans Blue injection. Data in **(A)** are presented as mean values ± s.e.m, in **(C–E)** as box plots. Statistical analysis was performed by two-tailed Mann Whitney test for (A) and two-tailed Student's t-test for **(C–E)**.

comparison to WT mice (**Figures 3C,D**). Moreover, we could not detect any differences in the numbers of infiltrating T cells (**Supplementary Figures 1B,C**).

Blood Brain Barrier Permeability in tMCAO Is Not Altered by TRPV4

Given the absence of a phenotype and a difference in BBB permeability in *Trpv4*$^{-/-}$ mice subjected to EAE, we decided to investigate the effects on experimental stroke, another model with significant impact of BBB disruption on clinical outcome but with more mildly and acute inflammation.

Therefore, we induced cerebral ischemia by tMCAO in *Trpv4*$^{-/-}$ and WT control mice and assessed their infarct sizes and neurological scores. After three days we were not able to detect any differences in the disease score between the two groups (**Figure 4A**) or in the body weight (**Supplementary Figure 2A**). There was also no difference in the volume of infarcted tissue (**Figures 4B,C**). Notably, both genotypes showed the same regional cerebral blood flow in the tMCAO model assessed by laser Doppler (**Figure 4D**) and were not different in physiological parameters (**Supplementary Figures 2B–E**).

Moreover, Evans Blue staining of the brain was assessed 24 h (**Figures 4E,F**) and 72 h (data not shown) after tMCAO, revealing lack of any differences between the two groups.

DISCUSSION

Here we show that TRPV4 inhibition increases endothelial resistance under homeostatic conditions, however, this effect was lost during inflammatory conditions. TRPV4 activation led to a disintegration of tight junctions (Narita et al., 2015) and TRPV4 inhibition decreases the vascular endothelial permeability and thereby counteracts pulmonary edema induced by heart failure (Thorneloe et al., 2012). Our data support these previously established concepts, as we detected a higher TEER in MbMECs after inhibiting TRPV4. Of note, inflammatory cytokines abolished this effect and led to a downregulation of *Trpv4* in MbMECs. Consistently, previous data showed that proinflammatory cytokines (e.g., TNF-α and IL-1β) had inhibitory effects on TRPV4-stimulated transepithelial ion flux and permeability changes in the choroid plexus, whereas anti-inflammatory cytokines (e.g., IL-10 and IL-4) showed no

effect (Simpson et al., 2019). However, in this study the cytokines reduced only the functional but not the transcriptional regulation of TRPV4. Thus, it is likely a contribution of transcriptional regulation, alteration of TRPV4 channel function by trafficking and/or post-translational modification, and other complex regulation. Hence, further studies exploring the impact of inflammation on *Trpv4* expression and TRPV4 ion channel function in endothelial cells and their respective barrier are required.

In our study, we were not able to detect any differences in the *Trvp4*-deficient mice in EAE and tMCAO. Immune cell infiltration, microglia activation and increased level of proinflammatory cytokine release into the CNS accounts for the pathogenesis of both disease models (Lopes Pinheiro et al., 2016) but in comparison to EAE with abundant immune cell infiltration into the spinal cord, tMCAO is accompanied by less robust immune cell invasion of the cortex. We could not detect any difference of BBB permeability in *Trvp4*-deficient in comparison to WT control mice in both models.

We conclude that the inflammation overrides the possible protective effects of TRPV4. In line with our data, TRPV4 inhibition showed no protective effect in TNF-α induced sepsis, whereas it does in LPS induced sepsis (Dalsgaard et al., 2016). Therefore, it needs to be further specified which mechanisms and conditions lead to the abolishment of TRPV4 induced regulation of BBB permeability during inflammation.

Our lack of an *in vivo* phenotype might be rooted in several factors so that we are not claiming a decisively confirmed lack of a role of *Trpv4* in the examined animal models. *Trpv4* is also expressed on the other side of the endothelial side of the BBB, in astrocytes, in particular in astrocytic endfeet abutting the capillary (Shibasaki et al., 2007; Benfenati et al., 2011; Dunn et al., 2013; Filosa et al., 2013). Absence of astrocytic TRPV4 could alter the astrocytic contribution to the BBB. In that case the net result of absence of TRPV4 in endothelial cells and astrocytes could be lack of a phenotype in *Trpv4*-deficient mice because of opposite effects. In addition, there are other relevant cell migratory types such as macrophages that also express *Trpv4* and might contribute to the phenotype in various ways (Hamanaka et al., 2010; Scheraga et al., 2016; Michalick and Kuebler, 2020). Importantly, *Trpv4*-deficient mice have a genetically encoded absence of *Trpv4* in all cells at all developmental stages. For critical structures and function such as the BBB, this means compensatory gene expression to back up TRPV4 function might not be unlikely, plus cells that normally express *Trpv4* and have regulated expression of *Trpv4* under stress/injury can have a very different response, which is not directly rooted in absence of TRPV4, but rather in developmental compensation of gene expression. This uncertainty could be further addressed by studies in mice with lineage-specific gene targeting so that the deletion of *Trpv4* can be induced (Moore et al., 2013). Such dedicated studies can be combined with acute application of selective inhibitor molecules with systemic and compartmentalized application.

Beyond *Trpv4* expression in endothelial cells and astrocytes of the BBB, the channel appears to function in multiple roles and multiple lineages of the CNS (Lee and Choe, 2014; Kanju and Liedtke, 2016). For example, in a cuprizone-induced mouse model of demyelination a TRPV4 antagonist, RN-1734, alleviated demyelination and inhibited glial activation (Liu et al., 2018). Intracerebroventricular injection of HC-067047, a specific TRPV4 antagonist, reduced brain infarction 24 h after tMCAO (Jie et al., 2016). It appears therefore puzzling that TRPV4 activation using the selective activator 4α-PDD, induced angiogenesis and neurogenesis and thereby contributed to functional recovery from ischemic stroke in mice (Chen et al., 2017). These could be off-target effects of the different "specific" chemicals, but perhaps could highlight different signaling functions of TRPV4 in different lineages at different phases of ischemic injury of the CNS, characterized by different stages of BBB injury.

In summary, we demonstrate that TRPV4 inhibition leads to an increase of transendothelial resistance under homeostatic conditions, while this effect could not be observed under inflammatory conditions. In our *in vivo* models of EAE and acute cerebral ischemia, *Trpv4*-deficient mice were devoid of a phenotype, especially regarding BBB injury. These results build the foundation for a future quest for the role of TRPV4 in the BBB with focus on relevant constituting and TRPV4-expressing cells such as endothelial cells. However, our data do not support therapeutic TRPV4 channel inhibition in patients with multiple sclerosis or ischemic stroke.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Behörde Für Soziales, Familie, Gesundheit und Verbraucherschutz in Hamburg; G22/13 and 59/17.

AUTHOR CONTRIBUTIONS

SR and AS conducted and designed the experiments, analyzed the data, and wrote the manuscript. OS, VV, LE, and KH conducted the experiments and analyzed the data. BS, SM, and MF designed the experiments. WL interpreted the data and wrote the manuscript. MG and MAF designed the experiments, conceived, supervised, and funded the study, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00849/full#supplementary-material

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REVIEW
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TRP Channels Regulation of Rho GTPases in Brain Context and Diseases

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Neurological and neuropsychiatric disorders are mediated by several pathophysiological mechanisms, including developmental and degenerative abnormalities caused primarily by disturbances in cell migration, structural plasticity of the synapse, and blood-vessel barrier function. In this context, critical pathways involved in the pathogenesis of these diseases are related to structural, scaffolding, and enzymatic activity-bearing proteins, which participate in Ca^{2+}- and Ras Homologs (Rho) GTPases-mediated signaling. Rho GTPases are GDP/GTP binding proteins that regulate the cytoskeletal structure, cellular protrusion, and migration. These proteins cycle between GTP-bound (active) and GDP-bound (inactive) states due to their intrinsic GTPase activity and their dynamic regulation by GEFs, GAPs, and GDIs. One of the most important upstream inputs that modulate Rho GTPases activity is Ca^{2+} signaling, positioning ion channels as pivotal molecular entities for Rho GTPases regulation. Multiple non-selective cationic channels belonging to the Transient Receptor Potential (TRP) family participate in cytoskeletal-dependent processes through Ca^{2+}-mediated modulation of Rho GTPases. Moreover, these ion channels have a role in several neuropathological events such as neuronal cell death, brain tumor progression and strokes. Although Rho GTPases-dependent pathways have been extensively studied, how they converge with TRP channels in the development or progression of neuropathologies is poorly understood. Herein, we review recent evidence and insights that link TRP channels activity to downstream Rho GTPase signaling or modulation. Moreover, using the TRIP database, we establish associations between possible mediators of Rho GTPase signaling with TRP ion channels. As such, we propose mechanisms that might explain the TRP-dependent modulation of Rho GTPases as possible pathways participating in the emergence or maintenance of neuropathological conditions.

Keywords: TRP channels, Rho GTPases, actin cytoskeleton, TRP interactome, GEFs, GAPs

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INTRODUCTION

Neurological diseases encompass broad pathophysiological events, characterized by alterations such as hypo- or hyper-connectivity of synapses, tumoral growth, and loss of neuronal networks (Forrest et al., 2018; La Rosa et al., 2020; Liu Y. et al., 2020). Nevertheless, in most of the cases the precise cellular and molecular mechanisms involved in the pathogenesis of these disorders have

not been fully described. Ion channels have been widely described as pivotal molecular entities that contribute to brain physiology and are associated to the development of various brain diseases, thus emerging as highly potential pharmacological targets (Bagal et al., 2013). Several members of the TRP ion channel family play key roles in the regulation of cellular and tissue structures, such as membrane protrusions, synapses, endothelial barriers and glial architecture (Goswami and Hucho, 2007; Goswami et al., 2007a; Gorse et al., 2018; Zhao et al., 2018; Cornillot et al., 2019), whose abnormal activity is intimately linked to neurodevelopmental and neurodegenerative disorders (Morelli et al., 2013). Despite that, how TRP channels participate in brain physiology and brain-affecting diseases is still not fully understood. The concerted activity of Rho GTPases has been described as a signaling node that governs structural maintenance and dynamic changes of cell architecture, mainly through the control and integration of cytoskeleton rearrangements and membrane trafficking. Similarly to TRP channels, Rho GTPases command neurophysiological processes such as dendritic spines morphology, axon cone growth, glial cells migration, blood-brain barrier permeability and vascular tone (Wu et al., 2005; Stankiewicz and Linseman, 2014; Pennucci et al., 2019). Thus, coordinated TRP channels and Rho GTPases activity might be a conserved mechanism for the modulation of changes in physiological and pathophysiological contexts.

Rho GTPases in Actin-Based Processes in Brain

Ras Homologs (Rho) proteins are a family of small GTPases belonging to the Ras superfamily. Since the discovery of the first Rho protein (Madaule and Axel, 1985), twenty members have been identified in mammals, currently grouped in 8 subfamilies (Vega and Ridley, 2008). These proteins cycle between an "*active*" GTP-bound state, and an "*inactive*" GDP-bound state. The basis for the cyclic activity of Rho GTPases lies in 3 regulators: (1) Guanine Exchange Factor (GEF) proteins, which catalyze swapping of GDP to GTP, allowing the transition of Rho proteins into their *"active"* state; (2) GTPases-Activating Proteins (GAP), which increase the intrinsic GTPase activity of the guanosine nucleotide-binding protein, promoting GTP hydrolysis and leading to the *"inactive"* state (Bos et al., 2007); and (3) Guanine Dissociation Inhibitors (GDI), which sequester the inactive form of Rho GTPases to prevent their activation by GEFs (Dovas and Couchman, 2005) (**Figure 1**).

Rho GTPases are crucial for the regulation of the actin cytoskeleton (Rottner et al., 2017) and processes involving cellular motility such as contractility, migration, and membrane protrusions (Machacek et al., 2009). RhoA, Rac1 and Cdc42 are the most studied and distinguished members of this family (Ridley and Hall, 1992; Ridley et al., 1992; Wedlich-Soldner et al., 2003). In neurons, actin cytoskeleton remodeling is essential for several processes including neuronal growth (Marsh and Letourneau, 1984), dendrite development and synapse formation (Sin et al., 2002). These events are indispensable for proper establishment and plasticity of brain circuits. Thus, neurons require accurate actin cytoskeleton regulation, which in turn depends on the fine spatiotemporal and coordinated control of Rho GTPases activity (Murakoshi et al., 2011). Accordingly, the modulation of Rho GTPases has been described to occur in a time/length scale of minutes/micrometers (Pertz, 2010). Canonically, Rac1 activation facilitates the formation of dendritic spines (Luo et al., 1996) and membrane ruffles, while RhoA inhibits these processes and promotes spine shortening (Tashiro, 2000). Moreover, while Glutamate-promoted Cdc42 activity is confined to stimulated dendritic spines, active RhoA spreads along the dendrites (Murakoshi et al., 2011). Thus, spatiotemporal regulation of Rho GTPases is a key point in spine structural plasticity (Hedrick et al., 2016). In addition to their role in neurons, Rho GTPases also play pivotal functions on brain vessels and glial cells. For instance, Rho GTPases regulate contractility and permeability of endothelial brain vessels (Kutcher et al., 2007; Kruse et al., 2019), as well as coordinate different morphological changes on glial cells (reviewed in Zeug et al., 2018). Therefore, these proteins are an essential component for maintaining brain homeostasis and deregulation of their activity lead to several neurological pathologies (Huang et al., 2017).

The precise coordination of Rho GTPases activity highly depends on GEFs, GDIs and GAPs defined by establishing differential protein–protein interactions (PPIs) of Rho GTPases and their modulators. For instance, Rac1 regulation is mediated by selective association with other proteins such as Tiam1 (Rac-GEF), Bcr (Rac-GAP) (Um et al., 2014), and Vilse (Rac-GAP) (Lim et al., 2014). Moreover, RhoA activity is regulated by the interaction of Graf1c (RhoA-GAP) with the tyrosine kinase Pyk2 in postsynaptic neurons, resulting in spine retraction (Lee et al., 2019). Furthermore, Rho GEFs that contain the PDZ-domain interact with lysophosphatidic acid, which promotes RhoA activation (Yamada et al., 2005). Hence, GAP and GEF differential localization and/or interactions are crucial elements for the spatiotemporal regulation of Rho GTPases. Consistently, mutations on these proteins (GAP/GEF) lead to several congenital malformations or developmental neuropathologies (O'Brien et al., 2000; Bai et al., 2015; Pengelly et al., 2016). In this context, the existence of over 70 GEFs and over 70 GAPs adds a further level of complexity in the regulation of Rho GTPases (Müller et al., 2020). In line with this, GEFs and GAPs are tightly regulated by protein interaction partners, second messengers such as Ca^{2+}, and posttranslational modifications (PTM) such as phosphorylations (Bos et al., 2007) (**Figure 1**).

Rho GTPases are also modulated by posttranslational modifications (PTMs). Prenylation of the carboxyl-terminal is the most frequent modification, which entails the addition of a 15- or 20- carbon isoprenoid to a cysteine residue immersed in a *CAAX* motif (Roberts et al., 2008; Reddy et al., 2020). This PTM causes Rho GTPase targeting to the plasma membrane (Hodge and Ridley, 2016), leading to GTP-bound Rho GTPases interaction with their effectors and regulators (Reddy et al., 2020). Moreover, several Rho GTPases phosphorylations, ubiquitylation, sumoylation, and their differential upstream enzymes and outcomes have been reported (extensively reviewed in Hodge and Ridley, 2016).

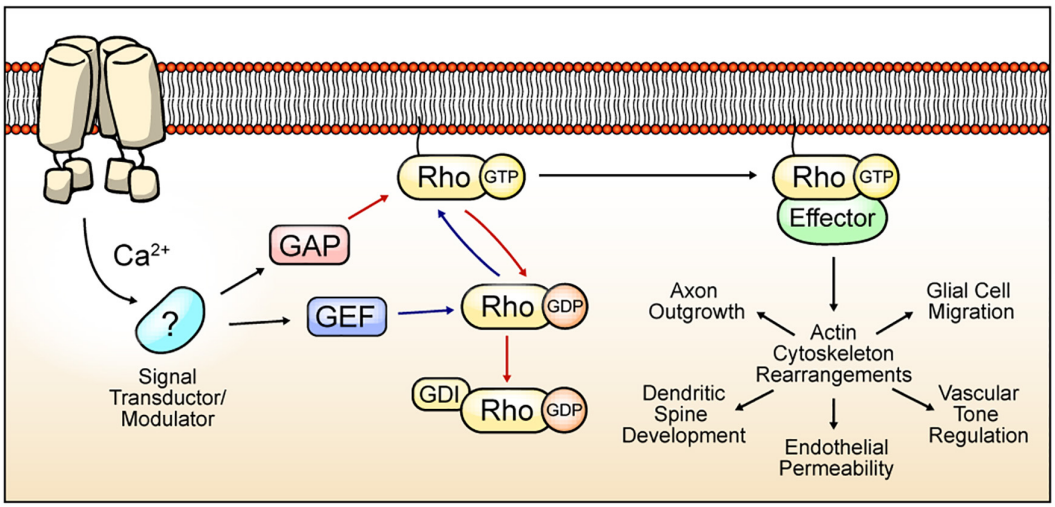

FIGURE 1 | Cyclic Rho GTPase modulation, functions, and TRP channel-mediated regulation hypothesis. Rho GTPases cycle between an active form bound to GTP (blue arrows) and an inactive form that is attached to GDP (red arrows). Binding to guanosine nucleotide is induced by GEF proteins. GAP proteins promote GTPase activity, and GDI proteins preclude GDP exchange to GTP. The active state of Rho GTPase promotes the rearrangement of the actin cytoskeleton by the modulation of several effectors. The actin cytoskeleton mediates multiple processes depending on the cell types. In neurons, axon outgrowth and dendritic spine dynamics regulation. In brain vasculature, Rho GTPases regulate endothelial permeability and vascular tone regulation. Rho GTPases regulates migration in glial cells. TRP channels-mediated Ca^{2+} influx regulates signal transductors/modulators, leading to changes in GAP, GEF, or GDI activity, thus promoting Rho GTPases activation/inactivation and actin cytoskeleton regulation.

The activity of several Rho GTPases is linked to depolarization and Ca^{2+} influx in neurons (Hirata et al., 1992; Szászi et al., 2005). This Ca^{2+} influx is related to Voltage-gated Ca^{2+} Channels (VGCCs), although intracellular Ca^{2+} reservoirs and Store-Operated Ca^{2+} Entry (SOCE) also might have an important role in the activation of Rho GTPases as has been widely reported (Jin et al., 2005; Saneyoshi and Hayashi, 2012; Bollimuntha et al., 2017). In this context, most of the mechanisms described involved in Ca^{2+}-dependent Rho GTPase modulation rely on Calmodulin (CaM) and Calmodulin-dependent Kinases (CaMKs) as transductors of Ca^{2+} signals through the regulation of GEFs, GAPs, and GDIs. For example, neurotrophins induce intracellular long-range Ca^{2+} waves, increasing RhoA activity during axon formation (Takano et al., 2017). Interestingly, Ca^{2+} waves promote CaMKI activation, leading to recruitment and interaction with GEF-H1, promoting RhoA activation (Takano et al., 2017). Also, CaMKII phosphorylates GEFs and GAPs of Rac1 and Cdc42, such as the Rac1 GEF Tiam1, and RICS, a Cdc42/Rac1 GAP (Okabe et al., 2003). CaMKII-dependent phosphorylation of the Rac1 GEF Kalirin-7 promotes cytoskeleton rearrangement and leads to the plasticity of dendritic spines (Xie et al., 2007).

In this context, the correlation between local/broad Ca^{2+} signals and the activation of different Rho GTPases is an interesting issue, but the molecular entities responsible for these Ca^{2+} signals have not been fully identified. TRP channels are implicated in a myriad of cellular processes associated with actin-based events in migratory cells, through the regulation of Rho GTPases activity (Canales et al., 2019). These channels also play an important role in brain physiology (Dussor et al., 2014; Zeng et al., 2016). As such, TRP channels might serve as key hubs for signaling transduction related to Rho GTPases activities, regulating several features of brain function and architecture.

TRP CHANNELS

The TRP ion channel family comprises six subfamilies of non-selective cationic channels in mammals, corresponding to TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV (Clapham et al., 2001). All members present six transmembrane domains (S1 – S6) and cytoplasmic amino- and carboxyl-terminal segments of varying length, and a pore-loop between S5 and S6 domains (**Table 1**) (Hellmich and Gaudet, 2014). The first identified TRP channel in mammals was named as classical or canonical (TRPC). The remaining subfamilies have been named depending on the designation of the first identified member of the respective subfamily, which relies on activation properties, function and/or featured domains (Montell, 2005; Venkatachalam and Montell, 2007).

Transient receptor potential channels can be activated by multiple stimuli, such as temperature, pH changes, and membrane mechanical stress (Clapham, 2003). Moreover, the activity of these channels can be modulated by PIP_2 (Nilius et al., 2008; Rohacs, 2014), PTMs such as phosphorylation (Cerda et al., 2015; Xu et al., 2019; Liu X. et al., 2020) and interacting proteins (Singh et al., 2002; Zhu, 2005; Cho et al., 2014; Rivas et al., 2020). Monomers can form heterotetrameric functional channels with distinctive properties in relation with their homotetrameric counterparts (Chubanov et al., 2004; Ma et al., 2011; Kim et al., 2019). Heteromultimerization can occur not only among members of the same TRP subfamily but

TABLE 1 | TRPA, TRPC, TRPV, TRPM and TRPML channels features.

TRP subfamily	Size	P_{Ca}^{2+}/P_{Na}^{+}	Domains N-term	Domains C-term	Phosphoinositides-Binding	Localization
TRPA	∼1000 aa	0.8–1.4	Ankyrin-repeats	TRP-box-like motif Inositol phosphate binding region Coiled-coil region	PIP_2 – Activation	Plasma Membrane
TRPC	<1000 aa	0.5 – 9	Ankyrin-repeats Coiled-coil regions	TRP-box motif Calmodulin-binding CIRB domains PDZ domains	PIP_2-Activation/Inhibition	Plasma Membrane
TRPV	<900 aa	1 - >100	Ankyrin-repeats	TRP-box motif Calmodulin-binding	PIP_2 – Activation/Inhibition PG-Activation (V1) PI-Activation (V1) PS-Activation (V1)	Plasma Membrane
TRPM	<1500 aa	0.5–10 (Except M4/5 which have <0.05)	Melastatin family channel homology region	TRP-box motif Coiled-Coil region Nudix (M2) Kinase (M6/7)	PIP_2 – Activation	Plasma Membrane Melanosomes (M1)
TRPML	<600 aa	∼1	–	EF-Hand motifs	PIP_2 – Activation	Endolysosome vesicles

also with those of different subfamilies (Ma et al., 2011). These features and the wide expression of these channels in multiple tissues and cell types, grant a high level of complexity to their role in diverse physiological and pathophysiological processes (Venkatachalam and Montell, 2007). In this context, several TRP channels are implicated in cellular processes related to central nervous system (CNS) functioning such as the modulation of neuronal excitability (Mickle et al., 2016; Hong et al., 2020b), maturation or establishment of subcellular structures such as dendrites (Tai et al., 2008), excitatory synapses (Zhou et al., 2008), axonal outgrowth (Jang et al., 2014), frontal cortex postnatal development (Riquelme et al., 2018) and brain blood flow regulation (Earley et al., 2004; Cornillot et al., 2019). Also, current evidence suggests that TRP channels could regulate downstream processes such as gene expression or more lasting effects, like Long Term Potentiation (LTP). Hence, it is not surprising to find CNS pathophysiological conditions associated with TRP channels activity (**Table 2**). TRP channels have been associated to stroke (Zhang and Liao, 2015), CNS ischemia-reperfusion damage (Gauden et al., 2007; Chen et al., 2017; Leiva-Salcedo et al., 2017), status epilepticus (Kim et al., 2013; Phelan et al., 2017), Alzheimer's and Parkinson's diseases (Zhang et al., 2013; Hong et al., 2020b), and progression of neoplasms of neuronal (Chen et al., 2014; Middelbeek et al., 2015) or glial (Lepannetier et al., 2016; Ou-Yang et al., 2018) origin.

Several reports suggest a role for TRP channels on Rho GTPases regulation. In this context, interactome maps based on different Protein–Protein Interaction databases suggest associations between TRP channels with Rho GTPases-related proteins. For instance, the TRIP database, a curated database from individual studies that report protein interactions with TRP channels, provides insightful information regarding the TRP-Rho GTPase interactions (Shin et al., 2011). Other general interactome databases such as BioGRID (Oughtred et al., 2019) and BioPlex (Schweppe et al., 2018), generated from high-throughput interactome datasets and curated individual studies, complement these interactions. The resulting interactome map reveals that the TRP-interacting proteins network reveals several Rho GTPases-related regulators, such as Src, PLC-γ1, Akt, PKC and the GTPase Gαq (Shin et al., 2011) (**Figure 2**). In this context, the activity or expression of TRPC3 (Kitajima et al., 2011; Numaga-Tomita et al., 2016), TRPC5 (Tian et al., 2010) TRPC6 (Singh et al., 2007; Tian et al., 2010), TRPM4 (Cáceres et al., 2015), TRPM7 (Su et al., 2011), TRPV1 (Li J. et al., 2015; Li et al., 2015a), and TRPV4 (Ou-Yang et al., 2018; Zhao et al., 2018) increase the activity of several Rho GTPases (**Table 3**). Also, there is evidence showing that TRPM8 (Sun et al., 2014), TRPV2 (Laragione et al., 2019), and TRPV4 (Thoppil et al., 2016) channels inhibit Rho GTPases (**Table 3**). These opposite effects could rely on multiple mechanisms that give versatility to Ca^{2+} signals-dependent responses such as specific localization in membrane subdomains or cellular substructures of the channels, leading to differential and dynamic interactions with Ca^{2+}-regulated proteins that might modulate Rho GTPases. This regulation might occur *via* activation of Ca^{2+}-dependent kinases proteins such as PKC and CaM kinases, leading to direct regulatory phosphorylations on Rho GTPases (Hodge and Ridley, 2016). Despite the above, the elevated number of GAPs and GEFs (Müller et al., 2020) and the diverse outcomes on cytoskeleton rearrangement elicited by TRP channels (Kuipers et al., 2012; Canales et al., 2019) might point to an indirect TRP channels-dependent regulation of Rho GTPases through these modulators (GEFs and GAPs).

In the following sections, based on established and inferred interactions, we will discuss and hypothesize possible mechanisms for TRP channels participation *via* Rho GTPases in different neuropathologies.

TRPC Channels

TRPC is the founding subfamily of the mammalian TRP channel family, since the first TRP channel found in mammals was named "canonical" owing to its similarities to the TRP channel from *Drosophila* (Montell and Rubin, 1989; Clapham et al., 2001). The mammalian TRPC subfamily can be divided into two subgroups based on the percentage of identity between their sequences: (1) TRPC1/TRPC2/TRPC4/TRPC6 and (2) TRPC3/TRPC6/TRPC7.

TABLE 2 | TRP channels-associated pathologies and possible Rho GTPases-dependent mechanisms.

TRP Channel	TRP Channel activity deregulation-associated disease	Possible Rho GTPase-dependent mechanism for TRP channel role in disease
TRPC3	Mwk phenotype (Wu et al., 2019), Cerebellar ataxia (Dulneva et al., 2015)	Aberrant spine remodeling/morphology due to deregulation of CaMKII/Tiam1 axis
TRPC5	Substance abuse (Pomrenze et al., 2013) – Excitotoxicity (Phelan et al., 2013)	Rac1 and DR-2-mediated regulation of RhoA
TRPC6	Glioma (Ding et al., 2010). AD (Lessard et al., 2005; Feng, 2017)	Glioma: Promotion of FAK activation, cell proliferation and migration AD: Loss of TRPC6 expression leads to the activation of Rac1, promoting the amyloidogenic pathway
TRPV1	AD (Balleza-Tapia et al., 2018; Du et al., 2020), Glioblastoma (Nabissi et al., 2016)	AD: Protective effect due to induction of axonal filopodia or dendritic spines Glioblastoma:Increment of cell invasion through RhoA activity
TRPV2	Possible participation in neurodegenerative processes	Rac1/RhoA-mediated neuritogenesis and tubulogenesis modulation
TRPV4	ICH, TBI (Zhao et al., 2018), AD (Zhang et al., 2013) Glioma progression (Ou-Yang et al., 2018)	RhoA-mediated endothelial dilation and vascular tone regulation Increased cell migration due to enhanced Rac1 activity
TRPM4	IS (Leiva-Salcedo et al., 2017; Chen et al., 2019), glutamatergic toxicity (Schattling et al., 2012), SCI (Gerzanich et al., 2009)	Rac1-mediated induction of NADPH oxidase and ROS production
TRPM7	IS (Chen et al., 2015), Neuroblastoma (Middelbeek et al., 2015)	IS: RhoA upregulation and subsequent induction of cell death. Neuroblastoma: RhoA-dependent cytoskeleton remodeling Endothelial Barrier disruption
TRPM8	Migraines (Gavva et al., 2019; Ling et al., 2019), Glioblastoma (Zeng et al., 2019)	Regulation of cerebral arterial vasodilation
TRPML1	Mucolipidosis type IV (Bargal et al., 2000)	Control of Rho GTPases-dependent membrane trafficking

ICH, intracerebral hemorrage; IS, ischemic stroke; TBI, traumatic brain injury; SCI, spinal cord injury; AD, Alzheimer's disease.

Of note, TRPC2 is a pseudogene in *Homo sapiens* (Wang et al., 2020). These channels can heteromultimerize with members of the corresponding (TRPC) or other sub-families, which yields functional non-selective ion channels with a wide range of relative P_{Ca}/P_{Na} (**Table 1**) (Chen et al., 2020). All these channels are responsive to GPCR/RTK-induced PLC activation (Wang et al., 2020). Some members also respond to ER-Ca^{2+} stores depletion *via* STIM1, such as TRPC1, TRPC3, TRPC4, and TRPC5 (Yuan et al., 2007; Lee et al., 2014). Moreover, TRPC channels activity is regulated by $PIP(4,5)_2$ and PIP_2-derivates such as DAG, IP3, PI(4)P and PI. Structurally, TRPC channels are composed by intracellular N-terminal and C-terminal domains, and six membrane-spanning domains. Expression of TRPC channels has been reported in various tissues, such as kidney, salivary glands, hippocampus, pancreatic β cells, heart, and vascular smooth muscle, and therefore participate in a wide variety of physiological processes (Chen et al., 2020).

TRPC3

TRPC3 channels are characterized by their coupling to tyrosine kinase and G protein coupled receptors activation, acting as mediators of Ca^{2+} signals induced by these receptors (Ambudkar and Ong, 2007). TRPC3 channels are especially abundant in the brain, mainly in the cerebellum, caudate nucleus, putamen and striatum (Riccio et al., 2002). These channels participate in Purkinje cells physiology in the cerebellum (Hartmann et al., 2008). Indeed, mGluR1 promotes TRPC3-dependent rises of local Ca^{2+} signals, leading to slow membrane depolarization at Purkinje cells dendrites (Hartmann et al., 2008). Accordingly, changes in the expression or mutations of TRPC3 gene cause detrimental consequences on motor functions such as the 'Moonwalker' (Mwk) phenotype (Wu et al., 2019). Mice with Mwk phenotypes display gait and limb incoordination (Becker, 2017). Interestingly, TRPC3 upregulation in Mwk animals impairs the development of dendrites (Becker et al., 2009). Furthermore, the mutant variant R672H of the human TRPC3 gene leads to cerebellar ataxia (Dulneva et al., 2015). Consequently, TRPC3 activity deregulation might induce structural alterations during the progress of cerebellar ataxia by modulating dendrite development.

There is not a direct evidence for TRPC3-mediated regulation of Rho GTPases in neurons or brain tissues. Nevertheless, data obtained from other models, as well as TRPC3/Rho GTPases associated pathways, might suggest a functional relationship between these proteins. For instance, BDNF induces Rac1 and Cdc42 activation, but not RhoA, through activation of TrkB receptor (Hedrick et al., 2016). Moreover, BDNF-induced TrkB activation leads to TRPC3 activation, which is necessary to induce spine remodeling (Amaral and Pozzo-Miller, 2007), suggesting that TRPC3 activation might be a mediator for BDNF-dependent activation of Rac1 and Cdc42, although further studies would be needed to confirm this. Interestingly, TRPC3 inhibition in heart mouse model induces a reduction of CaMKII and Rac1 activity (Kitajima et al., 2011). CaMKII-mediated Tiam1 phosphorylation leads in turn to Rac1 activation (Fleming et al., 1999; Buchanan et al., 2000; Tolias et al., 2005). These data suggest that TRPC3 activation by BDNF might induce Tiam1 CaMKII-dependent phosphorylation (**Table 3**), resulting in dendritic spine remodeling by Rac1 activity (**Figure 3**). Furthermore, downstream effectors might include PAK1, since

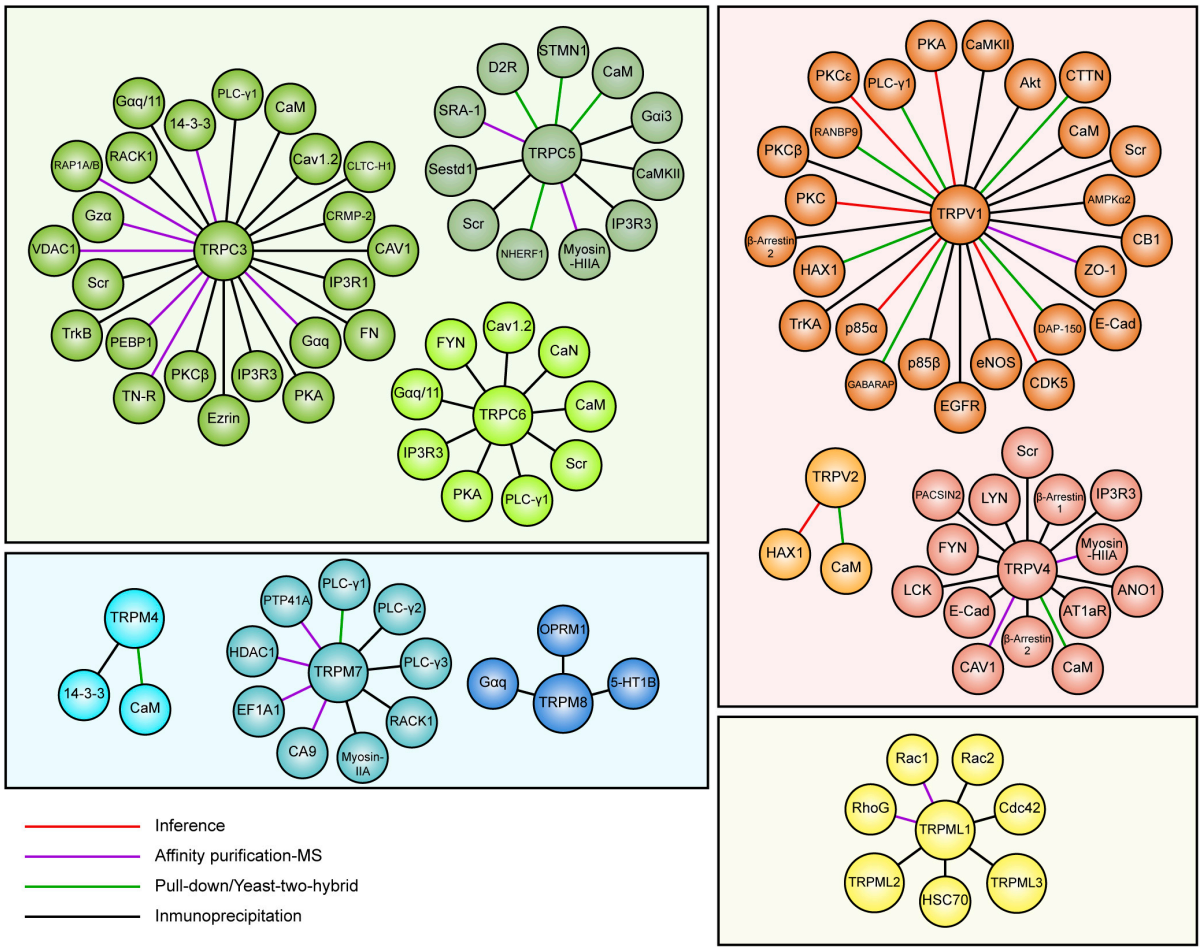

FIGURE 2 | Map of curated TRP ion channel interactors associated with Rho GTPases pathways. Data obtained from the TRIP database showing interactors of TRPC3, TRPC5, TRPC6, TRPV1, TRPV2, TRPV4, TRPM4, TRPM7, TRPM8, and TRPML1. TRP channels members are grouped and color-coded by families.

its activation by Rac1 that leads to actin remodeling through LIMK1 activity (Edwards et al., 1999). Likewise, CaMKII-ß regulates dendritic spine formation in Purkinje cells through a mechanism that involves mGluR1 and PKC activation. This CaMKII-ß-mediated effect requires IP_3R activation (Sugawara et al., 2017). Moreover, CaMKII has an important role in the production of LTP by promoting the insertion of AMPA receptors in the post synaptic region (Lisman et al., 2012). Importantly, our curated search in the TRIP database showed that TRPC3 interacts physically and functionally with the IP_3R isoforms (Kim J.Y. et al., 2006), and also with BDNF receptor TrkB (Li et al., 1999) (**Figure 2**). Thus, we propose that TRPC3 activation by BDNF/TrkB or mGluR1 in Purkinje might elicit spine remodeling through CaMKIIß activation and subsequent Rac1 activation. This proposed pathway could be relevant for pathologies that entail degeneration of neuronal processes. For example, morphology of dendritic spines is heavily altered in conditions of aberrant CaMKII/Tiam1/Rac1 activity, which might collaborate to intellectual disability of patients bearing mutations of the ATRX-encoding gene (Shioda et al., 2011). These data is consistent with a possible role for TRPC3 in this pathology due to the above-mentioned role of this channel in BDNF-dependent spine remodeling, which might open new avenues of studies for novel TRPC3 functions in brain pathophysiology.

TRPC5

TRPC5 is another non-selective cation channel that belongs to the TRPC subfamily. TRPC5 activity is related to many sensorial features such as touch and hearing (Sexton et al., 2016), satiety sensation (Gao et al., 2017), and internal physiological pH chemosensitivity (Cui et al., 2011). TRPC5 regulates neurites length (Greka et al., 2003), neurite outgrowth during neuron differentiation (Heo et al., 2012), neurite retraction (Hui et al., 2006), and axonal outgrowth (Oda et al., 2020). Moreover, several studies link the function of TRPC5 to dynamic behavioral processes that are tightly related to structural changes in synapsis, such as fear (Riccio et al., 2009), addictive behavior (Pomrenze et al., 2013), and tolerance to opioids (Chu et al., 2020) (**Table 2**). Thus, TRPC5 activity has been intimately associated to LTP

TABLE 3 | Rho GTPases regulated by TRP channels.

TRP Channel	Effect on Rho GTPase Regulation	Downstream Rho GTPase	Possible mechanism for GTPase modulation by TRP channel
TRPC3	Positive	Rac1 (Kitajima et al., 2011)	CaMKII-induced Rac1 GEF Tiam1 phosphorylation/activation*
TRPC5	Positive	Rac1 (Tian et al., 2010)	Interaction with SESTD1*
	Negative	Rhoa A (Tian et al., 2010)	Rac1 positive modulation, leading to RhoA inhibition*
TRPC6	Positive	RhoA (Singh et al., 2007; Tian et al., 2010; Jiang et al., 2011)	Calcineurin-dependent regulation of RhoA*
	Negative	Rac1 (Tian et al., 2010)	Downregulation of Rac1 GEF Tiam1 by TRPC6-mediated NMDA receptor inhibition*
TRPV1	Positive	RhoA (Li J. et al., 2015; Li et al., 2015a)	TRPV1-mediated Inhibition of AMPA receptor endocytosis*
TRPV2	Negative	Rac1/RhoA (Laragione et al., 2019)	PKA activation by TRPV2-driven cAMP increases, leading to RhoA inhibition*
TRPV4	Positive	RhoA (Zhao et al., 2018)	PKC-dependent RhoA activation (Brain endothelial cells, Zhao et al., 2018)
		Rac1 (Ou-Yang et al., 2018)	Increasing AKT phosphorylation levels (U87 glioma cells, Ou-Yang et al., 2018)
TRPM4	Positive	Rac1 (Cáceres et al., 2015)	Modulation of Ca^{2+} signals – Vm depolarization-dependent phosphatidylserine translocation*
TRPM7	Positive	RhoA, Rac1, CDC42 (Su et al., 2011)	RhoA: Modulation by p116RIP* Rac1: S1P1 activation through Mg^{2+} influx (Endothelial cells [Zhu et al., 2019])
TRPM8	Negative	RhoA (Sun et al., 2014)	Regulation of mitochondrial function and ROS production, leading to modulation of RhoA/ROCK (Vascular Smooth Muscle cells, Xiong et al., 2017)
TRPML1	Undetermined	Possible modulation of Rac1, Rac2, Cdc42 and RhoG	Interaction with Rac1, Rac2, Cdc42 and RhoG*

**Proposed mechanisms.*

modulation (Phelan et al., 2013), suggesting a participation on synaptic plasticity and in turn, on brain physiology and pathophysiology. The morphological changes mediated by TRPC5 overlap with Rho GTPases-dependent signaling, since this has also been related to structural modifications required for memory and learning (Diana et al., 2007). Thus, TRPC5 might promote structural dynamic changes through the modulation of Rho GTPases. Interestingly, enhanced Rac1 activity has been reported upon TRPC5 overexpression (Tian et al., 2010), although, the underlying mechanisms remain unclear. Interestingly, TRPC5 interacts with Rho GTPases modulators, such as SESTD1 (**Figure 2**) (Miehe et al., 2010). SESTD1 is a putative scaffold protein that diminishes dendritic spine density by abolishing the interaction between Rac1 and the Rac-GEF Trio8 (Lee C.C. et al., 2015). Nevertheless, it is not known whether this mechanism could affect the retraction or extension of dendritic spines. In this context, we suggest that SESTD1 interaction with TRPC5 might decrease its available levels to block the Rac1-Trio8 association, causing an indirect TRPC5-dependent increased Rac1 activity. This is consistent with the negative regulatory effect of TRPC5 on neurites growth as discussed above. Rac1 activity promotes the growth of neurites (Woo and Gomez, 2006). Therefore, a possible negative effect of TRPC5 upon neurite extension through Rac1 modulation seems paradoxical. However, it has been recently reported that either hypo or hyperactivity of Rac1 could lead to neuritogenesis impairment, giving arise to intellectual disability (Zamboni et al., 2018). On the other hand, TRPC5 interacts with CaMKII (Puram et al., 2011). This could be relevant since CaMKII is an activator of RhoA and Cdc42, leading to the induction of dendrites growth (Murakoshi et al., 2011). Consistently, TRPC5 promotes dendrite growth through CaMKII activity (He et al., 2012), suggesting that TRPC5 might control this process through CaMKII/RhoA/Cdc42 axis. As mentioned before, CaMKII is important for the production of LTP response (Lisman et al., 2012), which suggests a TRPC5 participation in processes related to memory and learning.

TRPC5 activity is also associated with cell viability. These channels have been proposed to exert either a protective role (Hong et al., 2020a) or promote neuronal loss depending on its PTM status in neurodegenerative disorders such as Huntington's disease (Hong et al., 2015). Moreover, TRPC5 promotes cell death induced by oxidative stress (Park et al., 2019) and excitotoxicity during epileptic seizures (Phelan et al., 2013). Dopamine D2 receptor (D2R) activity has been reported to enhance cell vulnerability by inducing neurite retraction and axon collapse *via* RhoA/ROCK activity (Deyts et al., 2009). TRPC5 interacts with D2R (**Figure 2**) (Hannan et al., 2008) and as such, TRPC5 channels might induce cell death in a RhoA-dependent manner, although this still needs to be determined. Interestingly, Rac1 activity is associated with cell death promotion during oxidative stress caused by neurovascular ischemia (Smith et al., 2017; Karabiyik et al., 2018). Moreover, Rac1 activity might also have a protective role in epileptic excitotoxicity (Posada-Duque et al., 2015). In contrast, RhoA activation has been linked to Ca^{2+}-dependent neuronal loss elicited by excitotoxicity (Semenova et al., 2007). This dichotomy between protective and degenerative roles of Rho GTPases upon ischemic injury has been widely reviewed elsewhere (Posada-Duque et al., 2014). As such, TRPC5 might have a dual participation in both dynamics of protrusive processes and neuronal death. These effects might depend on differential TRPC5-dependent spatiotemporal regulation of Rho

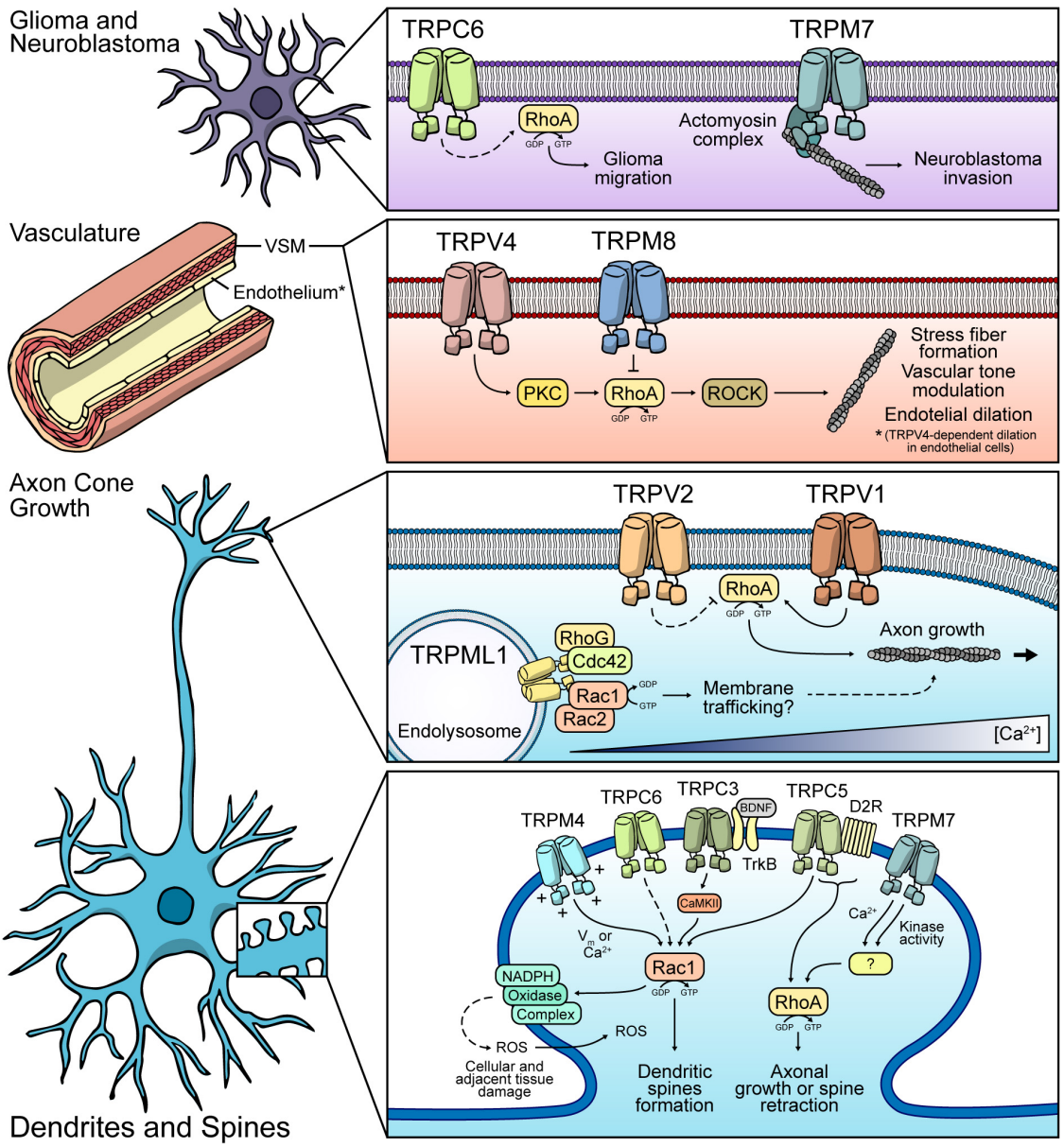

FIGURE 3 | Possible mechanisms of Rho GTPases modulation by TRP channels. Glia and Neuroblastoma: TRPC6 might modulate RhoA in glial cells, thus regulating cell migration. TRPM7 participates in neuroblastoma invasion. Vasculature: TRPV4 and TRPM8 display the opposite effect over RhoA. Both channels modulate vasculature tone. TRPV4 promotes endothelial dilation in a RhoA-dependent fashion. Axon cone: TRPML1-dependent regulation of protein trafficking *via* modulation of Rac1/Cdc42 might occur. TRPV1 promotes RhoA activity and modulates axonal growth. TRPV2 might have an inhibitory role of RhoA. Dendrites and spines: TRPM4, TRPC3, TRPC6, and TRPC5 might induce Rac1 activity, leading to dendritic spines formation. Under ischemic conditions, these channels might have a Rac1-dependent role in ROS production, leading to neuronal cell death. TRPM7 and TRPC5 are proposed to regulate RhoA, favoring the retraction of dendritic spines or axonal growth.

GTPases, a process that could be further analyzed with new tools to study more exactly ion channel-related spatiotemporal events.

TRPC6

TRPC6 shares close sequence and structural homology with TRPC3. TRPC6 can also be modulated by tyrosine kinase receptors and G protein-coupled receptors, being especially sensitive to activation by diacylglycerol (DAG) (Hofmann et al., 1999). TRPC6 is widely expressed in the peripheral nervous system (Riccio et al., 2002). TRPC6 is located in excitatory post-synaptic neurons and regulates the number of spines and spatial learning and memory (Zhou et al., 2008) *via* CaMKIV activation and CREB regulation, promoting both neurite and dendrite growth (Tai et al., 2008; Heiser et al., 2013). The transcription factor CREB is associated with the late phase of LTP. Its activation results in changes in gene expression that

promote synapsis development and structural rearrangements (Frey et al., 1993; Nguyen et al., 1994). This suggests that TRPC6 contributes to the late phase of LTP. Interestingly, CaMKIV has been associated with actin cytoskeleton remodeling, since it regulates LIM Kinase 1 (LIMK1) activity to promote neurite growth (Takemura et al., 2009). In turn, LIMK1 phosphorylated cofilin to favor actin filaments stabilization (Yang et al., 1998). This suggests that TRPC6 activity might affect synapsis formation through the CaMKIV-LIMK1-cofilin pathway. Moreover, several reports suggest a protective role for TRPC6 from excitotoxicity by inhibiting NMDA receptor activity (Li et al., 2012; Shen et al., 2013). Although no direct interaction has been reported between TRPC6 and Rho GTPases, our analysis of the curated list of interactors of TRP channels from the TRIP database (**Figure 2**) reveals that TRPC6 interacts with several proteins that regulate Rho GTPases function such as Calcineurin (Ly and Cyert, 2017). Furthermore, several studies report the contribution of TRPC6 in RhoA regulation in non-excitable cells (Singh et al., 2007; Tian et al., 2010).

Similarly to TRPC3, TRPC6 has been involved in different brain disorders such as neuronal damage in stroke, Aβ-production in Alzheimer's disease and especially in the development and progression of glioma (Ding et al., 2010). TRPC6-mediated effects in the brain have been described as a mix between positive and negative. One of the most studied TRPC6-related roles in cerebral pathologies is in the development and progression of gliomas. TRPC6 is overexpressed in glioma samples compared to normal brain tissue. Additionally, its activity is essential for G2/M phase transition and glioma progression (Ding et al., 2010). Moreover, TRPC6 maintains the stability of HIF-1α in glioma cells under hypoxia (Li et al., 2015b) and mediates Notch-Driven glioblastoma growth and invasiveness (Chigurupati et al., 2010). In this context, the RhoA/ROCK signaling pathway is upregulated in glioma cells, promoting cell migration and proliferation (Zohrabian et al., 2009). Interestingly, RhoA/ROCK pathway is activated by TRPC6 in other tissues such as the kidney (Yang et al., 2013), suggesting that this relation between TRPC6 and RhoA/ROCK might also play a role in glioma progression. Moreover, upregulation of TRPC6 and subsequent increased Ca^{2+} influx in podocytes lead to an aberrant activation of focal adhesion kinase (FAK), which is necessary for focal adhesion assembly and disassembly in migratory cells (Nakuluri et al., 2019). RhoA activity can promote the activation of FAK (Al-Koussa et al., 2020), suggesting an additional mechanism for TRPC6-RhoA mediated tumoral progress in glioma (**Figure 3**). Although there are several possible mechanisms for TRPC6-mediated regulation of the RhoA/Rock pathway in glioma progression, this still needs to be confirmed in the context of this neoplastic disease.

As discussed above, TRPC6 also regulates several beneficial processes in the neurons such as survival, synaptogenesis, learning and memory, all of which are altered in Alzheimer's Disease (Feng, 2017). Overexpression of presenilin 2 and/or Alzheimer's-disease-related presenilin 2 variants, decreased TRPC6-mediated Ca^{2+} entry in HEK293 cells (Lessard et al., 2005). Moreover, TRPC6 expression and activity are diminished in neurons from Alzheimer's-disease patients. Also, pharmacological activation of TRPC6 inhibited the elevation of Aβ and phospho-tau in neurons of patients (Tao et al., 2020), indicating a possible protective role of TRPC6 in Alzheimer's-disease. Moreover, upregulation of Rac1 appears to be important in driving the progress of Alzheimer's-disease by promoting the production of the amyloid precursor protein and the amyloidogenic pathway (Aguilar et al., 2017). Importantly, loss of TRPC6 expression leads to the activation of Rac1 in the kidney (Tian et al., 2010), which suggests that the lower expression of this channel observed in the brain of Alzheimer's-disease patients could also promote the pathology by activating Rac1 (**Figure 3**).

TRPV Channels

The TRPV subfamily comprises six members, of which the temperature-sensitive TRPV1 is the founder and the most studied. TRPV1 was first described as an ion channel activated by the vanilloid capsaicin, which explains the name of the subfamily (Caterina et al., 1997, 1999; Güler et al., 2002). The members of the TRPV subfamily share a similar architecture. These channels contain an ankyrin-repeat domain (ARD) and follow six transmembrane helix domains in the amino-terminal region. Cytoplasmatic carboxyl-terminal contains the TRP domain and other binding sites for lipid regulators, all of them with a resolved structure (Yuan, 2019). In addition to the canonical role of TRPV channels in temperature and pain sensing, other functions have been attributed to these channels, wherein the regulation of Rho GTPases emerges. Other characteristics are described in **Table 1** and have been extensively summarized previously (Bevan et al., 2014; Garcia-Elias et al., 2014; Kojima and Nagasawa, 2014).

TRPV1

TRPV1 was the first and most studied member of the vanilloid subfamily of TRP ion channels (Caterina et al., 1997; Benítez-Angeles et al., 2020). TRPV1 can be activated by exogenous agonists such as capsaicin and resiniferatoxin (RTX) (Caterina et al., 1997), as well as high temperature (>42°C), pH and membrane potential changes (Caterina et al., 1997; Piper et al., 1999; Gunthorpe et al., 2000), mechanical forces (Ho et al., 2014), ROS (Starr et al., 2008), arachidonic acid (Chu et al., 2003), and endocannabinoids (Ross, 2003). Initial studies identified TRPV1 in sensory neurons, describing its participation in the regulation of pain transduction. TRPV1 is also expressed in brain cortex, hippocampus, cerebellum, substantia nigra, hypothalamus, midbrain, olfactory bulb and astrocytes (Mezey, 2000; Marinelli et al., 2003; Kim et al., 2005; Kim S.R. et al., 2006; Cavanaugh et al., 2011; Park et al., 2012; Nam et al., 2015). Interestingly, multiple studies associate TRPV1 activity to cytoskeleton remodeling, cellular migration (Ho et al., 2014; Miyake et al., 2015) and cell morphology (Goswami and Hucho, 2007; Goswami et al., 2007b; Li et al., 2015a). For example, mechanical stress-elicited TRPV1-dependent Ca^{2+} signals contribute to astrocyte migration (Ho et al., 2014). Also, mitochondrial TRPV1 enhances microglial cell migration (Miyake et al., 2015). Moreover, TRPV1 localizes in multiple dynamic structures of neurons such as the growth cone, neurites and axonal filopodia, displaying differential roles among these

structures. TRPV1 activation results in rapid retraction of growth cones in sensory neurons dependent on microtubule depolymerization (Goswami et al., 2007b). Also, a recent study established that Ca^{2+} influx mediated by TRPV1 is necessary for NGF deprivation-induced axonal degeneration (Johnstone et al., 2019). Interestingly, RhoA activity is necessary for axonal growth cone collapsing and proper polarity establishment for axon guidance (Wu et al., 2005; Loudon et al., 2006). Furthermore, RhoA also controls microtubules protrusion during axonal growth (Dupraz et al., 2019). Importantly, mechanical stress-induced TRPV1 activity participates in guidance during neurite extension of spiral ganglion neurons in a RhoA/ROCK-dependent fashion (**Figure 3**) (Li et al., 2015a). Thus, along with its participation on microtubule dynamics, TRPV1-dependent modulation of RhoA-ROCK might also cooperate in guidance during axonal growth (**Figure 3**). Since Ca^{2+} is crucial for axon specification and outgrowth in CNS neurons (Davare et al., 2009; Nakamuta et al., 2011), these TRPV1-dependent proposed effects might occur in dorsal root ganglion (DRG) and CNS neurons as well, although further studies would be needed to confirm this.

TRPV1 regulates axonal filopodia formation, a key process for axonal branching (Spillane and Gallo, 2014). Interestingly, this process is not dependent on the conductive activity of the channel, but only on its C-terminal domain, which interacts with microtubules (Goswami et al., 2007a). TRPV1-induced filopodia resembles those formed upon ROCK and Myosin II inhibition (Loudon et al., 2006). These data contrast with the role of TRPV1 on the regulation of RhoA since this Rho-GTPase canonically inhibits axonal branching (Spillane and Gallo, 2014). We hypothesize that such discrepancy might be due to the structural features rather than the conductive activity of the TRPV1 channels on axon filopodia initiation, leading to a differential mechanism on cytoskeleton regulation.

The complex role of TRPV1 in the modulation of neuronal structures might be involved in neurodegenerative pathologies such as Alzheimer's disease. Indeed, a protective effect of TRPV1 in Alzheimer's disease has been reported (Balleza-Tapia et al., 2018; Du et al., 2020). One of the proposed mechanisms consists in TRPV1-mediated inhibition of the AMPA receptor endocytosis, which is a key modulator of spine development *via* GEF-H1 (Kim et al., 2004; Kang et al., 2009). Thus, TRPV1-dependent hampering of AMPAR endocytosis might be a putative mechanism for TRPV1 positive modulation of RhoA, thus contributing to the development of Alzheimer's disease (**Table 3**).

TRPV1 has also been proposed as a negative prognosis marker for glioblastoma (Nabissi et al., 2016), in which invasiveness is highly dependent on RhoA activity since it regulates several matrix metalloproteinases in this type of neoplasms (Al-Koussa et al., 2020). Nevertheless, further studies are needed to detail the participation of TRPV1 in the development of neoplastic pathologies associated with neuronal or glial cells in the brain.

TRPV2

TRPV2 is a member of the vanilloid subfamily of TRP ion channels that is activated by high temperatures (>52°C) (Caterina et al., 1999). TRPV2 is also activated by hypoosmolality (Muraki et al., 2003), cell stretching (Sugio et al., 2017) and chemical stimuli such as 2-aminoethoxydiphenyl borate (2-APB) (Hu et al., 2004), cannabinoids (Qin et al., 2008), and probenecid (Bang et al., 2007). TRPV2 is found in DRG neurons (Caterina et al., 1999), trigeminal motor nucleus (Park et al., 2011), hypothalamus and hindbrain regions (Wainwright et al., 2004; Nedungadi et al., 2012), and astrocytes (Shibasaki et al., 2013). TRPV2 localizes in subcellular domains enriched in filamentous actin, such as the growth cone, filopodia, lamellipodia and neurites, where interacts with soluble actin (Yadav and Goswami, 2019). Furthermore, inhibition of TRPV2 produces retraction of the growth cone (Yadav and Goswami, 2019) (unlike TRPV1), while its activation enhances axon outgrowth (Shibasaki et al., 2010) and growth cone motility (Sugio et al., 2017), along with inducing rapid membrane ruffling, changes in lamellipodial and filopodial dynamics, and rapid translocation of leading edges during neuritogenesis (Yadav and Goswami, 2019). Although the downstream signaling pathway of TRPV2 activity has not been elucidated yet, TRPV2 induces an increase in the cAMP levels in neurites and their branching points (Yadav and Goswami, 2019). TRPV2-dependent increase of cAMP leads to PKA activation, which inhibits RhoA (**Figure 3**) (Ellerbroek et al., 2003; Oishi et al., 2012). Therefore, the remodeling effect on the actin cytoskeleton by TRPV2 activity might be through modulation of the Rho GTPase family *via* cAMP/PKA (Howe, 2004) (**Table 3**). Given this data, we hypothesize that these mechanisms might lead to a TRPV2-dependent inhibition of the growth cone retraction. Further studies are needed to determine these mechanisms. Furthermore, PKA activates Rac1 (Goto et al., 2011) and Cdc42 (Leemhuis et al., 2004), crucial proteins for the formation of lamellipodia and filopodia, respectively. Moreover, both GTPases activate PAK1 to promote the axonal growth cone (Toriyama et al., 2013). Thus, these Rho GTPases might also be molecular targets that explain the effects of TRPV2 activity on lamellipodia and filopodia dynamics.

TRPV4

TRPV4 is a major regulator of muscle- and endothelium-dependent vascular tone in blood vessels from multiple organs such as the intestine, lungs and brain (White et al., 2016). TRPV4 responds to shear stress and lipids, such as arachidonic acid and epoxyeicosatrienoic (EET) acid (White et al., 2016), which are essential vasoactive substances required for vascular dilation/contraction (Sudhahar et al., 2010). The participation of TRPV4 in the modulation of brain vessels is related to the activity of the channel in endothelial cells (White et al., 2016) and vascular smooth muscle cells (Chan et al., 2019). For instance, chronic cerebral hypoperfusion produces a myogenic tone decrease of brain parenchymal arterioles, an effect that was not observed in $TRPV4^{-/-}$ mice (Chan et al., 2019). Additionally, TRPV4 loss-of-function is associated with diminished endothelial dilation, decreased cerebral perfusion and impaired cognitive function in aged rats (Diaz-Otero et al., 2019). Moreover, TRPV4 promotes PKC-dependent RhoA activation, leading to stress fibers formation during internal cerebral hemorrhage (ICH) in endothelial cells (**Table 2** and **Figure 3**), presumably by ROCK activation and MLCP inhibition (Zhao et al., 2018). TRPV4 inhibition ameliorates damage produced by ICH, suggesting

that TRPV4 promotes blood brain barrier disruption in ICH conditions by inducing the formation of endothelial intercellular gaps in a way that involves RhoA activation and stress fiber formation (Zhao et al., 2018). Long-range Ca^{2+} signals caused by Ca^{2+}-Induced Ca^{2+} Release (CICR) induce RhoA activation after neurotrophin-3 treatment (Takano et al., 2017). TRPV4 promotes CICR in neurons in ICH model, most likely by regulating the IP_3R (Shen et al., 2019). Moreover, the interplay between TRPV4 and IP_3R also occurs at astrocytes endfeet, where TRPV4-dependent Ca^{2+} entry induces Ca^{2+} oscillations mediated by IP_3R, leading to neurovascular coupling and vasodilation (Dunn et al., 2013). In this regard, we propose that the propagation of Ca^{2+} waves in these cells might contribute to RhoA's spatiotemporal regulation and subsequent cytoskeleton remodeling.

TRPV4 promotes Rac1 activity in glioblastoma U87 cells leading to increased cell migration through TRPV4-dependent Akt phosphorylation/activation (Ou-Yang et al., 2018) (**Table 3**). Akt activation induces the phosphorylation of the Rac1 GEF Tiam1, which promotes Rac1 activation, driving tumorigenesis (Zhu et al., 2015). In this context, CaMKII has been shown to phosphorylate Akt in ovarian (Gocher et al., 2017) and prostate cancer cells (Schmitt et al., 2012). Thus, local Ca^{2+} signals elicited by these ion channels might promote Ca^{2+}/CaM binding to CaMK II, leading to Akt-mediated Rac1 activation through Tiam1, suggesting participation of TRPV4-mediated Rac1 regulation in glioblastoma progression.

TRPM Channels

Eight members of this subfamily have been identified. These channels have differential ion selectivity between their members. For instance, TRPM4 and TRPM5 are monovalent cationic channels (Launay et al., 2002; Hofmann et al., 2003), while TRPM7 and TRPM8 mediate divalent cationic (Nadler et al., 2001) and non-selective cationic currents (McKemy et al., 2002). TRPM channels share a Melastatin homology region (MHR) domain located at the amino-terminal region, a transmembrane domain similar to other TRP members, and a versatile carboxyl-terminal that varies between the TRPM members. For instance, TRPM7 contains a functional α-kinase domain in the carboxyl-terminal (Nadler et al., 2001), while TRPM4 and TRPM8 possess coiled-coil domains and binding motifs for different regulatory molecules (Fleig and Penner, 2004). The structures of all the TRPM channels discussed in this review have been recently described (Huang et al., 2020). Thus, the TRPM subfamily is a highly versatile subgroup of TRP channels, due to the particular structures and biophysical properties (Fleig and Penner, 2004). TRPM features have been summarized in **Table 1** and extensively reviewed (Fleig and Penner, 2004; Huang et al., 2020).

TRPM4

TRPM4 is activated by intracellular Ca^{2+} increases (Venkatachalam and Montell, 2007), but, unlike other members of the TRP family, TRPM4 (along with TRPM5) is only permeable to monovalent ions. Despite that, TRPM4 regulates Ca^{2+} signals in different cell types, such as fibroblasts (Cáceres et al., 2015), mastocytes (Shimizu et al., 2009), dendritic cells (Barbet et al., 2008), and lymphocytes (Launay et al., 2004). TRPM4 expression has been described in hypothalamus (Teruyama et al., 2011), hippocampal CA1 area (Menigoz et al., 2016), preBötzinger nucleus (Picardo et al., 2019), and medial prefrontal cortex (Riquelme et al., 2018). Interestingly, TRPM4 increases Rac1 activity in MEF cells (Cáceres et al., 2015). Even though, the Ca^{2+}-permeable ion channels regulated by TRPM4 that could explain changes in Ca^{2+} oscillations have not been identified yet, functional association between NMDA receptors and TRPM4 in postsynaptic dendrites of CA1 hippocampal neurons has been shown (Menigoz et al., 2016). Indeed, TRPM4 activity-mediated postsynaptic depolarization allows proper lifting of Mg^{2+} block from the NMDA receptor, leading to an increased Ca^{2+} entry and initiation of LTP (Menigoz et al., 2016). Moreover, glutamatergic excitotoxicity is decreased in TRPM4$^{-/-}$ mice, further suggesting a functional interaction between NMDA and TRPM4 (Schattling et al., 2012). Interestingly, Ca^{2+} entry mediated by NMDA receptors, and subsequent CaMKII-dependent Tiam1 phosphorylation, induces dendritic/spine development (Tolias et al., 2005). Thus, interplay between TRPM4 and NMDA receptors might be important in Rac1-dependent neuronal plasticity. Other possible mechanisms for Rac1 regulation by TRPM4 could be the regulation of local changes in membrane potential. For instance, voltage-gated Na^+ channel Nav1.5 has been proposed to promote Rac1 activity by producing membrane potential depolarization in breast cancer cells (Yang et al., 2020). Nav1.5-induced membrane potential depolarization causes Rac1 activation by promoting local redistribution of phosphatidylserine, an anionic phospholipid to which Rac1 binds, and known to be important for its activation (Finkielstein et al., 2006; Yang et al., 2020). Interestingly, TRPM4 facilitates cellular depolarization in bone marrow-derived mast cells (BMMC) (Vennekens et al., 2007), HeLa (Simon et al., 2010), and HEK293 cells (Fliegert et al., 2007). Thus, local changes in membrane potential mediated by TRPM4 might induce local phospholipid redistribution and Rac1 activation (**Figure 3**).

TRPM4 participates on damage caused by ischemic stroke (Leiva-Salcedo et al., 2017; Chen et al., 2019) and spinal cord injury (Gerzanich et al., 2009). Importantly, TRPM4 inhibition and silencing improves outcome of both spine cord injury (Gerzanich et al., 2009) and ischemic stroke (Loh et al., 2014; Chen et al., 2019). TRPM4 induces cellular swelling leading to neuronal cell death as a consequence of excessive Na^+ influx mediated by these channels (Gerzanich et al., 2009; Simon et al., 2010; Leiva-Salcedo et al., 2017). Interestingly, Rac1 participates in NADPH oxidase assembly (Acevedo and González-Billault, 2018). Moreover, increased activity of Rac1 induces ROS production by promoting the activation of the NADPH catalytic subunit, Nox2 (Stankiewicz and Linseman, 2014). This enzyme has been proposed as a potential ischemic stroke therapeutic target (Zhang et al., 2016). Consistently, Rac1 downregulation, similar to TRPM4 inhibition, leads to a protective effect in the hippocampal CA1 region and cortex in ischemia/stroke models (Raz et al., 2010) and after permanent middle cerebral artery occlusion (Karabiyik et al., 2018). Given that TRPM4 activity might lead to an increase in Rac1 activity, we hypothesize that an additional mechanism by which this channel could collaborate

with brain and vascular tissue-damaging, alongside oncotic cell death induction, is by promoting an increase in Nox2-dependent ROS production *via* Rac1 activation (**Figure 3**). Although this mechanism is only inferred, the suggested evidence makes it an interesting possible new approach to understanding TRPM4-mediated GTPase regulation's role in brain neuropathologies.

TRPM7

TRPM7 is a member of the TRP family that permeates both Ca^{2+} and Mg^{2+}. In addition, this channel has an active kinase domain in their carboxyl-terminal domain (Sun et al., 2015). TRPM7 is widely expressed in the human and mouse brain, with reports in animal models showing equivalent levels in neurons, astrocytes and microglia (Ratnam et al., 2018). TRPM7 localizes in the growth cone of hippocampal neurons, restricting the elongation of primary axons (Turlova et al., 2016). Interestingly, TRPM7 associates with several elements of cytoskeletal structures. For instance, mass spectrometry-based analyses revealed the interaction of TRPM7 with actin and α-actinin-1 (Turlova et al., 2016). Moreover, analysis of the TRPM7-associated interactome in neuroblastoma cells (**Figure 2**) identified proteins which were mainly related to the actin cytoskeleton, such as Myosin IIA, Drebrin and the Myosin phosphatase Rho-interacting protein (p116RIP) (Middelbeek et al., 2016). p116RIP is a modulator of the RhoA/ROCK axis (Koga and Ikebe, 2005), a key pathway that promotes invasion and metastasis of tumor cells (O'Connor and Chen, 2013). This suggests a role for TRPM7 in aggressive phenotypes in neuroblastoma cells *via* p116RIP-mediated RhoA/ROCK regulation and subsequent changes in cytoskeleton dynamics. Also, a functional coupling between TRPM7 and RhoA activation dependent on the TRPM7 kinase activity on hepatocellular carcinoma has been shown (Voringer et al., 2020). These data support a TRPM7-mediated RhoA activation involvement in diverse processes such as the inhibition of axonal growth cone and the migratory phenotype of brain tumor cells like neuroblastoma (**Figure 3**).

Several reports suggest that TRPM7 is a key mediator of neuronal death after ischemia/reperfusion episodes. TRPM7 knock-down in CA1 protected neurons from ischemia-induced cell death as well as maintained neuronal morphology and function (Sun et al., 2009). Moreover, TRPM7 blockage by carvacrol prevented brain damage in a mouse hypoxia-ischemia brain injury model (Chen et al., 2015). TRPM7 silencing leads to a decreased activity of RhoA (Su et al., 2011). This might be important in the context of ischemic damage since RhoA is upregulated in the brains of human stroke patients (Brabeck et al., 2003). The RhoA/ROCK pathway is key to determining neuronal cell death following ischemia/reperfusion events (Shin et al., 2008). This suggests that one of the mechanisms by which TRPM7 might be producing its pathological effect after ischemia/reperfusion is through the activation of RhoA. However, further studies into this association will help clarify this suggested relationship and find additional mediators.

Mg^{2+} permeability of TRPM7 is a particularity among the TRP family. A misbalance in the homeostasis of this ion has been involved in several pathological processes in the brain (Sun et al., 2015). TRPM7-mediated increases of Mg^{2+} intracellular concentration promotes endothelial cell proliferation and enhances the endothelial barrier integrity of the brain. This is due to induced cytoskeletal reorganization and expression of tight junction proteins such as VE-cadherin, occludin, and zonula occludens-1 (ZO-1) (Zhu et al., 2019). Interestingly, TRPM7 achieves this through the activation of S1P1, which in turn activates Rac1 (Zhu et al., 2019). This suggests that the TRPM7-mediated Mg^{2+} influx might play an important role in cerebral vasculature pathologies by regulating the S1P1-Rac1 pathway.

TRPM8

TRPM8 is a non-selective cation channel, which preferably permeates Ca^{2+} (McKemy et al., 2002). TRPM8 has a polymodal gating and is activated by cold and different cooling compounds, such as menthol and icilin (McKemy et al., 2002). Several single nucleotide polymorphisms (SNPs) related to the TRPM8 gene are associated with either the risk (Ling et al., 2019) or protection (Gavva et al., 2019) from migraines. Although the exact mechanisms are unknown, a possible mechanism for TRPM8-mediated risk of migraine headaches might be related to this channel's role in vasoconstriction regulation, which has been related to migraine development (Jacobs and Dussor, 2016). Interestingly, TRPM8 activation by menthol promoted vasodilation by inhibiting Ca^{2+} signaling–mediated RhoA/ROCK activation in mesenteric arteries (**Figure 3**) (Sun et al., 2014). In this regard, the RhoA/ROCK axis inhibition might lead to Myosin Light Chain Phosphatase (MLCP) activation and LIM Kinase 1 inhibition, resulting in an inhibition of the contraction of mesenteric arteries (Shimokawa et al., 2016). Furthermore, TRPM8 can function as an ER Ca^{2+} channel, regulating mitochondrial function and ROS production, which activates the RhoA/ROCK pathway (Xiong et al., 2017) (**Table 3**). Thus, TRPM8-dependent vascular tone modulation through the RhoA/ROCK axis regulation might play an important role. These mechanisms might not only be relevant in the development of migraines but also the progress of other cerebral vasogenic events such as reversible vasoconstriction syndromes.

Recent reports have associated alterations in TRPM8 expression with brain-related cancers. Moreover, TRPM8 was significantly overexpressed in glioblastoma tissue samples compared to normal tissue and its expression correlated with worse prognosis and survival in glioblastoma patients (Zeng et al., 2019). Also, TRPM8 was highly expressed in glioblastoma cell lines and its expression correlated with higher invasive and proliferative capacities (Zeng et al., 2019). In this context, TRPM8-mediated regulation of RhoA/ROCK might be responsible for the pro-tumoral effect of TRPM8, as the RhoA/ROCK pathway has been involved in the proliferation and migration of glioblastoma cells (Fortin Ensign et al., 2013). These data support a role for TRPM8 in diverse aspects of brain pathophysiology, including some that are more particular to this channel. Thus, more studies into the exact proteins mediating TRPM8-mediated RhoA/ROCK activation might be interesting to elucidate its precise role.

TRPML1

The Mucolipin-TRP (TRPML) subfamily are monovalent and divalent cation channels with predominant Ca^{2+} permeability (Peng et al., 2020) (see **Table 1**), although other permeabilities has been described to be important in their function (Dong et al., 2008). Contrary to other TRP subfamilies, these channels are particularly located in endolysosomal vesicles (Manzoni et al., 2004). Consistently, TRPML channels are associated with cellular processes such as vesicle trafficking and endolysosomal-dependent degradation pathways (Cheng et al., 2010). Structurally, these channels have full-length subunits of about 600 amino acids, thus being the smaller members of TRP channel subfamily. As to the other TRP channels, these channels have six transmembrane segments with pore-forming regions located between segments S5 and S6. In addition, amino- and carboxyl-terminal regions have cytoplasmatic orientation (Hellmich and Gaudet, 2014). Moreover, these channels have canonical EF-hand domains at their carboxyl-terminal domain, which allows direct Ca^{2+}-dependent activity modulation (Hellmich and Gaudet, 2014). TRPML activity is positively modulated by PIP_2 binding, which drives to channel activation (Dong et al., 2010). Interestingly, the resolved structure of TRPML1 indicates that its unique PIP_2-binding site is distinct to the reported in others TRP channels (Fine et al., 2018) (**Table 1**).

TRPML1 (also called Mucolipin-1) is an intracellular TRP channel located predominantly in endolysosomal membranes (Lee J.H. et al., 2015). Loss-of-function mutations reported in the TRPML1-encoding gene have been linked to a lysosomal storage disorder known as Mucolipidosis type IV (MLIV). This pathology is one of the first described neurological diseases caused by mutations in TRP channels (Bargal et al., 2000). MLIV is characterized by an early neurodevelopmental delay and a late neurodegenerative phenotype that leads to intellectual disability, delayed psychomotor abilities and retinal abnormalities (as described in OMIM #252650). Although the pathophysiological signs are well known, the cellular mechanisms involved in this disease remain unclear. TRPML1 modulates several aspects of membrane trafficking, particularly participating in trafficking of late endosome vesicles, and controlling the size (Cao et al., 2017), function and biogenesis of lysosomes (Wang et al., 2015). Moreover, TRPML1 regulates lysosome fusion with secretory vesicles, increasing exocytosis (Park et al., 2016). Interestingly, not only a disturbance in membrane trafficking but also lipid accumulation and loss of cell viability have been described as hallmarks of MLIV disorder (Wang et al., 2013), suggesting that TRPML1 loss-of-function might contribute in all these pathological alterations. However, detailed mechanisms by which TRPML1 regulates these processes are poorly understood. Rho GTPases are signaling nodes that coordinate membrane trafficking and lipid homeostasis (reviewed in Ridley, 2001; Olayioye et al., 2019). TRPML1 interactome reports (**Figure 2**) might reveal the mechanisms related to the coordination of cellular processes that lead to the neuropathological phenotype observed in MLIV (Krogsaeter et al., 2019). TRPML1 interacts and colocalizes with Rho GTPases, such as Rac1, Rac2, Cdc42 and RhoG (Spooner et al., 2013). Therefore, these data strongly suggest that TRPML1 might regulate the activity of Rho GTPases and, in this manner, control membrane trafficking (**Figure 3**). Thus, regulating TRPML1 activity might be an effective therapeutic approach for the treatment of other neurological disorders where low TRPML1 activity has been reported, such as ALS (Tedeschi et al., 2019), Parkinson disease (Tsunemi et al., 2019), Alzheimer's Disease (Zhang et al., 2017), and Niemann-Pick disease (Shen et al., 2012). Nevertheless, further studies are needed to determine the specific role of TRPML1-dependent Rho GTPases modulation in these neuropathological conditions and the proteins involved in this relationship.

OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Despite extensive evidence on the role of the canonical Rho proteins (RhoA/Rac1/Cdc42) in different neuronal processes and their possible modulation by TRP channels activity presented in this work, little is known about other Rho GTPase members. A limited number of studies have shown the participation of RhoF/RhoD (RhoF/Rif, RhoD) (Zanata et al., 2002; Fan et al., 2015) and atypical Rho GTPases (Aspenström et al., 2007) such as Rnd (Rnd1, Rnd2, and Rnd3/RhoE) (Ishikawa et al., 2003, 2006; Heng et al., 2008; Pacary et al., 2013), RhoU/RhoV (Wrch1, Chp/Wrch2) (Alan et al., 2018) and RhoBTB (RhoBTB3 and RhoBTB2) (Ramos et al., 2002) in neuronal processes. However, these proteins play an important role in developmental synaptogenic stage (Ishikawa et al., 2003), growth cone turning and collapse (Zanata et al., 2002), neurite formation (Tian et al., 2017) and retraction (Fan et al., 2015), axon guidance (Alan et al., 2018), cortical neuron migration (Heng et al., 2008; Tian et al., 2017), and neurogenesis (Pacary et al., 2013). Although no studies indicating that TRP channels regulate these Rho GTPase subfamilies, it has been reported that NFAT1c increases RND1 transcription (Suehiro et al., 2014) and rapid activation and translocation of NFAT to the nucleus is promoted by TRPV1 activity in sensory neurons (Kim and Usachev, 2009). Conversely, Rif has been established as a regulator of cytoskeletal rearrangements mediated by semaphorins (Tian et al., 2017). TRPC5 acts downstream of semaphorin signaling to cause neuronal growth cone morphology (Kaczmarek et al., 2012). These antecedents suggest that both TRP channels and atypical Rho GTPase activity could be involved in the different processes mentioned above. However, more studies need to be performed.

Regarding the discussion raised in this review, the TRP family of ion channels and Rho GTPases are functionally linked to each other due to; (1) mutual cellular functions whose deregulation leads to neuropathological phenomena and (2) Rho GTPases activity modulation by TRP channels. However, spatiotemporal features of TRP channel activity, and how these features could regulate Rho GTPase are still not clear.

TRP-associated interactome suggests that these channels associate with different Rho GTPases-related proteins (Shin et al., 2011) (**Figure 2**). This allows us to hypothesize a potential role for TRP channels as transduction hubs, implying local recruitment of Rho GTPases modulators and spatially delimited Rho GTPases activation/inhibition in subcellular structures. Moreover, Rho GTPases are mainly activated by Ca^{2+} signaling in neurons (Saneyoshi and Hayashi, 2012). Ca^{2+}-sensitive proteins such as the Ca^{2+}/CaM complex play an essential role in Rho GTPase activity modulation (Buchanan et al., 2000; Vidal-Quadras et al., 2011). Differential TRP-CaM binding affinities could lead to subtle changes in the local activity of Ca^{2+}/CaM-dependent proteins, such as CaMKs activation and subsequent Rho GTPases modulation (Wayman et al., 2011; Bossuyt and Bers, 2013). Conversely, TRP channels interaction with downstream targets of Rho GTPases suggests that these channels can be regulated by these proteins. Accordingly, Rho GTPases participate in the regulation of TRP channels trafficking and activity (Bezzerides et al., 2004; Puntambekar et al., 2005). Thus, TRP channels can regulate Rho GTPases and *viceversa*, suggesting a regulatory feedback loop between these proteins.

However, our research demonstrates that the relationship between TRP ion channels and Rho GTPases is not always positive (**Figure 3**). For instance, TRPM8 activation induces RhoA inhibition, while other members like TRPM7 and TRPC5 promote the activity of this Rho GTPase. We reasoned that multiple aspects contribute to establishing the relation between the respective TRP channel and Rho GTPase. Interactome and subcellular localization of both proteins and the biophysics properties of the channel might explain these differences. As we reviewed, most of the TRP channels that regulate Rac1 in neuronal context are localized in the same subcellular compartments (**Figure 3**).

Nevertheless, the proposed mechanisms for the regulation of Rac1 are different, which might be due to each TRP channel's differential interacting partners (**Figure 2**). A combination of complementary approaches to obtain a more global picture of the TRP channels-Rho GTPases network is needed to address this. The following section proposes some strategies to understand better the relationship between TRP ion channels and Rho GTPases.

Signaling mechanisms, such as those proposed above, often involve transient PPIs among regulatory components, which might represent a challenge for their identification due to the limited interaction time or low affinity of each interacting partners (Westermarck et al., 2013). Multiple proteomic approaches have emerged as alternatives to overcome these limitations (Bludau and Aebersold, 2020). These novel approaches could provide new possibilities to identify TRP/Rho GTPases PPIs pathways and give hints of the microdomains where these complexes localize.

Rho GTPases regulation by GEF/GAP/GDI is far more complex than a single GEF or GAP regulating only one Rho GTPase. Promiscuous activity has been reported for GEFs and GAPs, modulating more than one Rho GTPase (Bagci et al., 2020), increasing the complexity of the TRP-dependent modulation of these proteins. Proteomic studies may allow identification of GEFs/GAPs and GDIs implicated in Rho GTPases regulation by TRP channels. Complementing these approaches with assays for describing spatiotemporal dynamics will grant a more accurate characterization of the mechanisms involved in the TRP-dependent regulation of Rho GTPases and their role in neuropathological disorders. This will ultimately contribute to the designing of potential new interventions based on the TRP modulation of Rho GTPases. For instance, diverse strategies have been designed to monitor Rho GTPases activity through different means, such as biochemical approaches (Ren et al., 1999) and Förster Resonance Energy Transfer (FRET)-based sensors. The latter provides the possibility of performing *in vivo* tracking of GTPase activity (Kraynov et al., 2000; Pertz et al., 2006) and several authors have reviewed and corroborated its usefulness (Pertz, 2010; Schaefer et al., 2014). Recently, novel techniques to manipulate Rho GTPases have been designed. For instance, light-inducible sensors which promote RhoA (Wagner and Glotzer, 2016; Oakes et al., 2017) and Rac1 (Wu et al., 2009) translocation to the plasma membrane and subsequent activation. These tools might provide local and temporal inducible activation of Rho GTPases. Importantly, the application of these tools to the structures of TRP channels might also serve to define the role of these channels in the local and temporal regulation of Rho GTPases.

Another critical issue to study Rho GTPases regulation by TRP channels is the identification of Ca^{2+} as an intermediary in these pathways. Since local and broad Ca^{2+} waves have a differential effect on the modulation of Rho GTPases, the establishment of the spatiotemporal features of Ca^{2+} dynamics is necessary to strengthen what we know about these mechanisms. In this regard, fluorescent Ca^{2+} indicators derived from organic compounds provide information about the time-scale properties of Ca^{2+} waves but lack in providing accurate spatial information (Russell, 2011). Accordingly, genetically-encoded Ca^{2+} sensors provide an opportunity to study the time-scale and spatial distribution of Ca^{2+} signals, since they have been designed to monitor Ca^{2+} signals from intracellular organelles accurately (Bassett and Monteith, 2017). This approach has been used on neuronal models (Chen et al., 2012; O'Donnell et al., 2016), and even in the whole brain of living animals (Scott et al., 2018). These approaches, combined with biosensors to track the activity of Rho GTPases and its regulators, could provide novel and valuable information about the mechanisms involved in TRP channels-mediated regulation of Rho GTPases in physiological and pathophysiological brain conditions.

CONCLUDING REMARKS

The study of neurological and neuropsychiatric diseases has been linked to tumoral growth, cell death and loss of cellular structures, such as dendritic spines and axonal processes. In this context, ion channels are essential in cell physiology,

as the loss- or gain-of-function of these proteins trigger structural abnormalities and neuropathological conditions. TRP channels have emerged as novel candidates for the treatment of numerous CNS-affecting diseases. Thus, further knowledge of the mechanisms by which TRP channels exert their effects on cellular physiology constitutes an outstanding question. Herein, we exposed evidence and shared arguments and ideas that support general mechanisms by which TRP channels might modulate Rho GTPases in the brain. Although the evidence is strong enough to suggest several pathways, further studies are required to confirm the functional nature of these interactions. Future approaches considering quantitative proteomic analysis of TRP channels and Rho GTPases will reveal common partners that could mediate their functional relationship. Moreover, studies incorporating simultaneous analysis of localization, interaction, dynamic activity of Rho GTPases, TRP channels, and or GEFs/GAPs/GDIs will be required to determine the spatiotemporal features of these mechanisms. This new evidence would contribute to designing novel strategies based on the fine-tuning of transduction mechanisms involved in TRP-modulated processes, providing new therapeutic alternatives to overcome neuropathological conditions.

AUTHOR CONTRIBUTIONS

BL, PC, IS, OO-S, MPS, and OC wrote the manuscript. All the authors contributed to the article and approved the submitted version.

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Molecular and Functional Study of Transient Receptor Potential Vanilloid 1-4 at the Rat and Human Blood–Brain Barrier Reveals Interspecies Differences

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Transient receptor potential vanilloid 1-4 (TRPV1-4) expression and functionality were investigated in brain microvessel endothelial cells (BMEC) forming the blood–brain barrier (BBB) from rat and human origins. In rat, Trpv1-4 were detected by qRT-PCR in the brain cortex, brain microvessels, and in primary cultures of brain microvessel endothelial cells [rat brain microvessel endothelial cells (rPBMEC)]. A similar *Trpv1-4* expression profile in isolated brain microvessels and rPBMEC was found with the following order: *Trpv4* > *Trpv2* > *Trpv3* > *Trpv1*. In human, TRPV1-4 were detected in the BBB cell line human cerebral microvessel endothelial cells D3 cells (hCMEC/D3) and in primary cultures of BMEC isolated from human adult and children brain resections [human brain microvascular endothelial cells (hPBMEC)], showing a similar *TRPV1-4* expression profile in both hCMEC/D3 cells and hPBMECs as follow: *TRPV2* > > *TRPV4* > *TRPV1* > *TRPV3*. Western blotting and immunofluorescence experiments confirmed that TRPV2 and TRPV4 are the most expressed TRPV isoforms in hCMEC/D3 cells with a clear staining at the plasma membrane. A fluorescent dye Fluo-4 AM ester was applied to record intracellular Ca^{2+} levels. TRPV4 functional activity was demonstrated in mediating Ca^{2+} influx under stimulation with the specific agonist GSK1016790A (ranging from 3 to 1000 nM, EC_{50} of 16.2 ± 4.5 nM), which was inhibited by the specific TRPV4 antagonist, RN1734 (30 μM). In contrast, TRPV1 was slightly activated in hCMEC/D3 cells as shown by the weak Ca^{2+} influx induced by capsaicin at a high concentration (3 μM), a highly potent and specific TRPV1 agonist. Heat-induced Ca^{2+} influx was not altered by co-treatment with a selective potent TRPV1 antagonist capsazepine (20 μM), in agreement with the low expression of *TRPV1* as assessed by qRT-PCR. Our present study reveals an interspecies difference between Rat and

Human. Functional contributions of TRPV1-4 subtype expression were not identical in rat and human tissues reflective of BBB integrity. TRPV2 was predominant in the human whereas TRPV4 had a larger role in the rat. This interspecies difference from a gene expression point of view should be taken into consideration when modulators of TRPV2 or TRPV4 are investigated in rat models of brain disorders.

Keywords: TRPV1, TRPV2, TRPV4, interspecies differences, blood–brain barrier, brain endothelial cell

INTRODUCTION

During the last decade, the transient receptor potential (TRP) channels were identified as promising pharmacological targets and some drug candidates targeting TRP have even already entered clinical trials or been approved as drugs for treating neuropathic pain, epilepsia or heart failure (Moran, 2018). Once activated possibly by heat, mechanical/shear stress, various endogenous and exogenous molecules, some TRP channels expressed at the plasma membrane increase the influx of inorganic monovalent or divalent cations (Na^+, K^+, Ca^{2+}, Mg^{2+}), triggering several downstream cell functions depending on the cell type (Rodrigues et al., 2016). Indeed, the main knowledge of TRP functions and agonists come from their expression in sensory electrically excitable cells such as neurons and their role in the nociception and pain (Mickle et al., 2015). Recently, more studies have been done and evidenced the TRP expression and their ability in modulating the functionalities of non-excitable cells such as endothelial cells using various chemical agonists and antagonists, including a requirement in vasorelaxation and vascular permeability (Kassmann et al., 2013; Grace et al., 2014; Ambrus et al., 2017). TRP channels of the vanilloid subfamily (TRPV) are divided into 6 isoforms (TRPV1-6). TRPV5 and TRPV6 are mainly expressed in intestinal and renal epithelia (Peng et al., 2018) while transient receptor potential vanilloid 1-4 (TRPV1-4) are mostly expressed in diverse cell types of the CNS, exocrine organs, skin, eye, lung, heart, and blood vessels (Steinritz et al., 2018).

Transient receptor potential vanilloid 1-4 have been evidenced in peripheral endothelial cells where they modulate the vascular tone through the release of factors that induce relaxation of smooth muscle cells (Marrelli et al., 2007), play a role in modulating endothelial permeability (Villalta and Townsley, 2013), participate in angiogenesis of peripheral vascular (Smani et al., 2018), and sense hemodynamic and chemical changes (Kohler et al., 2006). Ca^{2+} modulation has been known to be an important factor in maintaining the function of brain microvessel endothelial cells (BMEC) forming blood–brain barrier (BBB) (Brown and Davis, 2002; De Bock et al., 2013). By modulating Ca^{2+} trafficking into BMEC, a growing evidence has been achieved that TRP channels may have a critical role on the BBB properties including its permeability, angiogenesis and inflammatory responses (Berrout et al., 2012; Huang et al., 2020). However, the expression or functionality of TRPV in BBB was much less studied than in non-cerebral endothelial cells. The expression or functionality of TRPV in BMEC forming the BBB was much less studied than in non-cerebral endothelial cells although there is growing evidence that TRP channels may have a critical role at the BBB by modulating Ca^{2+} trafficking into BMEC. Dysfunctions of the BBB are increasingly recognized as a cause or a consequence in the pathophysiology of diverse brain pathologies such as stroke, neurodegenerative and psychiatric diseases (Sweeney et al., 2019; Yang et al., 2019; Kealy et al., 2020; Yu et al., 2020). A dysfunctional BBB can thus be targeted with new drug candidates, which would be a promising way for treating brain pathologies. However, pre-clinical proof of concept studies of such drug candidates are usually developed with rodent models, thus raising the question of interspecies differences regarding the BBB equipment between rodents and human. Moreover, TRPV isoforms can be specifically activated by various physical and/or chemical stimuli such as heat, osmolality, and mechanical stress (Montell, 2001). Thus, the comparative expression levels of TRPV1-4 in the BBB of rodent pharmacological models and human are meaningful in clarifying the possible involvement of each isoform in physiological and pathological BBB. A whole transcriptomic analysis revealed a relative low Trpv2 expression in murine primary BMEC (Zhang et al., 2014) and rat Trpv2 was not immunolocalized in the brain vasculature (Nedungadi et al., 2012), whereas TRPV2 was shown highly expressed in the human cerebral microvessel endothelial cells D3 cells (hCMEC/D3) human cerebral microvessel endothelial cell line (Luo et al., 2019), suggesting interspecies differences in the expression of TRPV between rat and human BMEC.

The present work aims at determining TRPV1-4 expression in primary cultured rat BMEC, rat cortex and isolated cortex microvessels, as well as in primary cultured human BMEC isolated from patient biopsies and the hCMEC/D3 human cerebral microvessel endothelial cell line. Functional activity of human TRPV1-4 channels was subsequently studied in hCMEC/D3 cells.

MATERIALS AND METHODS

Reagents and Chemicals

Cannabidiol (CBD) (1 mg/mL in methanol), tranilast (TNL), capsaicin (CAP), capsazepine (CPZ), and GSK1016790A (GSK) were all analytical grade and purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). $CaCl_2$, $MgSO_4$, NaCl, NaH_2PO_4, $NaHCO_3$, KH_2PO_4, and KCl were purchased from Merck (Fontenay-sous-Bois, France).

RNA extraction kits were bought from Qiagen (Courtaboeuf, France). Primers, RT-PCR reagents and Lipofectamine® RNAi MAX transfection reagent were obtained from Invitrogen Life

Technologies (Cergy-Pontoise, France). The Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Applied Biosystems™, France). All other reagents and chemicals were purchased from Sigma.

Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 + Glutamax, Hank's buffered salt solution (HBSS), HEPES and Penicillin/Streptomycine were from Life Technologies (Cergy-Pontoise, France). fetal bovine serum (FBS) was from GE Healthcare Life Science. Liberase DL, bovine serum albumin (BSA) (cat. # A7906), Dextran (cat. # 31390), DNase I (cat. # DN25), Puromycine and b-FGF were from Sigma-Aldrich. plasma derived bovine serum (PDBS) was from First Link (Wolverhampton, United Kingdom).

Isolation of Rat Brain Microvessels

The isolation of rat brain cortex microvessels was done according to a protocol reported previously (Chaves et al., 2020). CO_2 anesthetized animals were decapitated, rat brains were removed and immediately soaked in ice-cold HBSS containing 10 mmol/L HEPES. Brain cortices were isolated, minced and homogenized, and subsequent brain homogenates were centrifuged (2000 × *g*, 10 min), the pellets were re-suspended in 17.5% 64–76 kDa dextran (Sigma Aldrich) and centrifuged one more time (4400 × *g*, 15 min). The resulting pellets were suspended in HBSS containing 1% BSA and were firstly filtered through a 100 μm nylon mesh, and then through a 20 μm nylon mesh. The retained cerebral cortex microvessels were immediately collected and stored at −80°C before analysis (diameter size around 4–6 μ m).

Cell Culture Conditions

Human Cerebral Microvessel Endothelial Cells D3 Cells (hCMEC/D3)

The hCMEC/D3 cell line kindly given by Cochin Institute (Paris, France). were used from passages 27 to 33 and cultured with the EndoGRO complete medium (Merck), supplemented with 5% FBS,1% streptomycin-penicillin (Gibco, Carlsbad, CA, United States), and 1 ng/mL b-FGF (Sigma) under 5% CO_2 and 37°C as previously described (Luo et al., 2019). Culture flasks and plates were pre-coated with 150 μg/mL rat tail collagen type I. Assessment of intracellular calcium kinetics was carried out on confluent cultures in 24-well plates seeded at 5×10^4 cells/cm^2.

HEK-293 Cells

Human embryonic kidney HEK293 cells were cultured with DMEM (Gibco) containing 10% FBS (Thermo) and 1% streptomycin-penicillin (Gibco) under 5% CO_2 and 37°C.

Primary Cultures of Rat Brain Microvessel Endothelial Cells (rPBMEC)

Adult male Sprague-Dawley rats weighing 300–350 g were purchased from Charles River laboratory (L'arbresle, France). They were housed in groups of four per cage under standard 12:12-h light/dark conditions (light from 08:00 to 20:00 h) in a temperature- and humidity-controlled room. They had access to food and water *ad libitum*. Rats were acclimated for 7 days before experimentation. The care and treatment of animals conformed to the standards and guidelines promulgated by the European Communities Council Directive (86/609/EEC). The protocol was approved by the ethics review committee of Paris Descartes University (approval n°12-185/12-2012). Rats were deeply anesthetized by intraperitoneal administration of diazepam (5 mg/mL) and ketamine (100 mg/mL). They were perfused transcardially with Buffer 1 (HBSS, 10 mmol/L HEPES) at room temperature (RT) for 3 min. All subsequent steps were done on ice except when indicated. The cortex was dissected, cleared from adhering white matter and meninges and gently crushed in a Petri dish using a glass slide. Tissue pieces from two cortices were pooled, rinsed in Buffer 1, then centrifuged (2 min, 600 g) and resuspended in 10 mL of DMEM containing 10 mM HEPES, 0.4 WU/mL Liberase DL and 100 U/mL DNase I. Digestion was performed for 40 min at 37°C with gentle mechanical trituration with a 10 mL pipette (at 10 min), then with a P1000 pipet tip (at 20 min) and finally with a roded glass Pasteur pipette (at 30, 35, and 40 min), to obtain an homogenate with a creamy texture and almost no visible remaining piece. Digestion was stopped by adding 30 mL of DMEM + 10% FBS and the homogenate was centrifuged (5 min, 1,000 × *g*). The supernatant was discarded and the pellet was resuspended in 25 mL of Buffer 1 + 18% BSA and centrifuged (15 min, 2,000 × *g*). The compact myelin disk was eliminated and the pellet resuspended in 50 mL of Buffer 1 + 1% BSA. This suspension was filtered on a 10 μm nylon mesh (cat. # NY1004700, Millipore). Cells from the filtrate were pelleted, resuspended in DMEM + 10% FBS and counted. Cells were then seeded in 12-wells plate (1.5×10^6/well) in complete medium (DMEM/F12 + Glutamax, 20% PDBS, 10 U/mL penicillin/streptomycin, 80 μg/mL heparin, 5 ng/mL b-FGF) containing 4 μg/mL puromycin. After 3 days, medium was changed for complete medium without puromycin and cells were grown until confluency.

Primary Cultures of Human Brain Microvascular Endothelial Cells (hPBMEC)

All human samples were provided by BrainPlotting (iPEPS, Institut du Cerveau et de la Moelle épinière, Hôpital Universitaire de la Pitié-Salpêtrière, Paris, France) in partnership with Sainte-Anne Hospital, Paris (neurosurgeon Dr. Johan Pallud) and harvested during tumor scheduled resection surgery with written informed consent from the patients (authorization number CODECOH DC-2014-2229). Human brain microvessels were obtained from surgical resections of three patients: a 70-years-old female suffering from Glioblastoma, a 2-year-old child suffering from glioma and a 8-year-old child suffering from astrocytoma. Microvessels were isolated from resections of tumor, peritumoral or healthy brain tissue using an enzymatic procedure (Chaves et al., 2020) adapting methods previously published for rats (Perriere et al., 2005, 2007). Briefly, tissue samples were carefully cleaned from meninges and excess of blood; then, an enzymatic mix was used to dissociate the tissues and microvessels were isolated by retention on a 10 μM mesh. Cells were cultured in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 20% serum and growth factors (Sigma) (Perriere et al., 2005, 2007). After seeding brain capillaries in petri dishes, brain primary microvascular endothelial cells were shortly amplified

and seeded (P1) on Transwell (Corning) with microporous membranes (pore size: 0.4 μm) in monoculture (CC205, CC206, and CC216) or in co-culture with the same patient's fresh primary human cultured astrocytes (CC216). Dry cell pellets were stored at −80°C before RT-qPCR experiments.

RNA Isolation, Reverse Transcription and Quantitative Real Time PCR (qPCR)

Total RNA was purified using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). The concentration and purity of the RNA samples were determined using the NanoDrop® ND-1000 instrument (NanoDrop Technologies, United States). Reverse transcription of total RNA was achieved in a thermocycler (PTC-100 programmable thermal controller, MJ research INC, United States) using the following conditions: 25°C for 10 min, then at 42°C for 30 min and at 99°C for 5 min, as previously reported (Dauchy et al., 2009). cDNAs were stored at −80°C. Gene expression was determined by SYBR Green fluorescence detection using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, United States) as previously reported (Luo et al., 2019). Samples were run in duplicate. Primers (**Table 1**) were designed using OLIGO 6.42 software (MedProbe, Norway). The lower the cycle threshold (Ct) value the higher the amount of mRNA, RT negative controls and no-template controls had Ct values > 40. RT negative controls and no-template controls had Ct values > 40. cDNAs from HEK293 cells were used to validate primers of human genes of interest. The relative expression of each gene of interest X as compared to the expression of the housekeeping gene encoding TATA box-binding protein (*TBP*) was calculated as $2^{-(Ct(X)-Ct(TBP))}$. Gene expression of genes having a Ct higher than 33 was not quantified.

Western Blot

Human cerebral microvessel endothelial cells D3 cells were washed twice with cold phosphate-buffered saline (PBS) on ice, then the protein lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.5% Triton X100, 0.5% sodium deoxycholate), and protease inhibitor cocktail (complete®, Sigma) was added. The Bradford assay was applied to quantify the protein concentration (BSA as a standard). Total proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad), blocked for 2 h with 5% non-fat dry milk. Membranes were incubated overnight with a mouse anti-human TRPV2 primary antibody (1/250, sc-390439, Santa Cruz Biotechnology, Dallas, TX, United States), a polyclonal rabbit anti-human TRPV4 primary antibody (1/200, ab94868, Abcam) or a monoclonal mouse anti-human β-actin primary antibody (1/3000, Millipore). A secondary anti-mouse or anti-rabbit IgG conjugated to HRP (1/2000, Santa Cruz Biotechnology) was then incubated for detection using an ECL plus Western Blot Detection System (GE Healthcare, Velizy, France).

Confocal Imaging

Human cerebral microvessel endothelial cells D3 cells were cultured on a 8-well ibidi μ-Slide (1.5 polymer coverslip, tissue culture treated from CliniSciences, Nanterre, France). At 80% confluence, cells were fixed with PBS containing 3.2% paraformaldehyde and permeabilized with 0.2% Triton-X-100 (Sigma) in PBS for 10 min. After 30 min in blocking solution (0.2% Triton-X-100, 1% BSA and 10% goat serum containing PBS) at RT, cells were incubated with the rabbit anti-human TRPV2 primary antibody (1:250, Thermo Fisher Scientific, Ref: PA1-18346), rabbit anti-human TRPV4 primary antibody (1:100, ab94868, Abcam), rabbit anti-human Claudin-5 primary antibody (1:250, Santa Cruz Biotechnology) or rabbit anti human VE-Cadherin primary antibody (1:500, Enzo Life Sciences) overnight at 4°C. After washing with PBS, μ-slides were incubated for 2 h at RT with a goat anti-rabbit-555 (1:500, Santa Cruz Biotechnology) and nuclei were stained with Hoechst 33342 (1:10000, Thermo Fisher Scientific, France). Negative control cells were incubated omitting primary antibodies. Visualization of the proteins was performed using a Zeiss LSM 510 Meta confocal microscope (Oberkochen, Germany).

Intracellular Ca^{2+} Signal Measurements

Fluorescence measurement of intracellular Ca^{2+} concentration was performed as described previously (Luo et al., 2019). hCMEC/D3 cells grown to confluency in 24-well plate were loaded with 2 μM of Fluo-4-AM (λ_{ex} = 496 nm, λ_{em} = 516 nm, F14201, Thermo Fisher Scientific) for 45 min at 37°C in Hank's buffer (5.37 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137.9 mM NaCl, 0.34 mM Na_2HPO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$, 5.56 mM D-Glucose at pH 7.4, all from Sigma,

TABLE 1 | Primers list for TRPVs channels and TBP applied in human and rat.

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)	Length (bp)
TBP or Tbp	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132
Human TRPV1	ACTGCCATCATCACTGTCATCT	CTTCACAGCCAACAGGTCTACCA	83
Human TRPV2	CGTGCAGATCCCCTTCGAGA	TGGGCAACTTGTGATACAGATGG	85
Human TRPV3	TACGTGGCTGAGGGAGAG	ATCGTGGCGTGAAGTCCA	92
Human TRPV4	TCAGGGAATCACAGTTGGC	AGCTCTTCATTGATGGATTCTT	82
Rat Trpv1	CTCGCTCTCCGCCATTCTTGC	TGTGCGTGACTTATGGGAGATGT	88
Rat Trpv2	ACCACCAAAAAGTGTCCCTTCTC	CGGCCTCTTTAGGAGTCTCACC	94
Rat Trpv3	GCTCCAGCCTGTGTTGAAAAT	CCTCCCAGTCCCTTGCTAAAG	90
Rat Trpv4	CCCCCTCAGCTCTTCACGG	CCCCACCCTGTTCCACTTTTTC	114

500 μL/well). Cells were then washed and replaced with HBSS (500 μL/well). After 10 min of incubation at 37°C, fluorescence in the 24-well plates were read using a Victor® X2 fluorescent microplate reader (PerkinElmer, Villebon, France).

Fluorescence was determined with diverse activators of TRP channels. Cells were also pre-treated with TRP antagonists (capsazepine 10 and 20 μM, tranilast 50 μM, RN1734 30 μM) for 5 min before adding TRP agonists (GSK1016790A ranging from 3 to 1000 nM, capsaicin 1 and 3 μM, cannabidiol 15 μM) and the fluorescent signals were then recorded in the presence of fixed concentration of TRP antagonists. All TRP activators and antagonists were dissolved with 100% DMSO or ethanol as stock solutions and the final solvent concentration in treated cells did not exceed 1%, a solvent concentration that had no effect on Ca^{2+} entry as compared to control cells without any solvent (data not shown). Data were normalized to the control group, which is expressed as F1/F0, where F0 is the average fluorescence of the control group (no activator of TRP) and F1 is the actual fluorescence at the corresponding time for the control group (in this case the average ratio for the control group is 1 or 100%) or the treated group.

To explore heating-induced Ca^{2+} influx, hCMEC/D3 cells were plated in 24-wells plate and loaded with Fluo-4-AM. The plate was then placed in the detecting room of Victor^{TM} X2 fluorescent microplate reader with automatically heating system. The plate was heating from RT to 50°C. The temperature and fluorescence were recorded at the same time by the Victor^{TM} X2 in the presence or not of the specific TRPV antagonist. Data was expressed as ratio normalized to the average of fluorescence intensity at RT in each well.

Statistical Analysis

Data are expressed as mean value ± SEM (standard error of the mean). Statistical analysis was performed using ANOVA with Dunnett *a posteriori* test to compare different groups with the control. An unpaired *t*-test was applied between two groups. p value < 0.05 was considered statistically significant. To calculate the EC_{50} of the agonist (GSK1016790A), the concentration-response data were fitted to a logistic function as follows: $Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1 + 10^{(logEC50-X)})$; where X is the log of the agonist concentration. Data fitting was performed in GraphPad Prism 5.01.

RESULTS

Thermo-Sensitive *Trpv1-4* Gene Expression in Rat Cortex, Brain Microvessels and rPBMEC

We measured the relative expression of *Trpv1-4* by qRT-PCR in primary cultured rat brain microvessel endothelial cells (rPBMEC) (**Figure 1**). *Trpv1* mRNA levels were barely detectable in whole cortex, brain microvessels and rPBMEC (**Figure 1A**). *Trpv2* expression was much higher in the whole cortex than in both brain microvessels and rPBMEC (7.99 ± 1.21, 1.19 ± 0.07, and 0.29 ± 0.04, respectively; $p < 0.05$ rat cortex vs. microvessels, $p < 0.01$ rat cortex vs. rPBMEC) and similar results were obtained for *Trpv3* (0.86 ± 0.13, 0.11 ± 0.07, 0.03 ± 0.02, respectively; $p < 0.05$ rat cortex vs. microvessels, $p < 0.05$ rat cortex vs. rPBMEC). Among *Trpv1-4*, *Trpv4* was the most highly expressed gene in brain microvessels and rPBMEC (3.13 ± 0.05 and 2.36 ± 0.16, respectively; $p < 0.001$ rat cortex vs. microvessels, $p < 0.01$ rat cortex vs. rPBMEC) while its expression in rat cortex

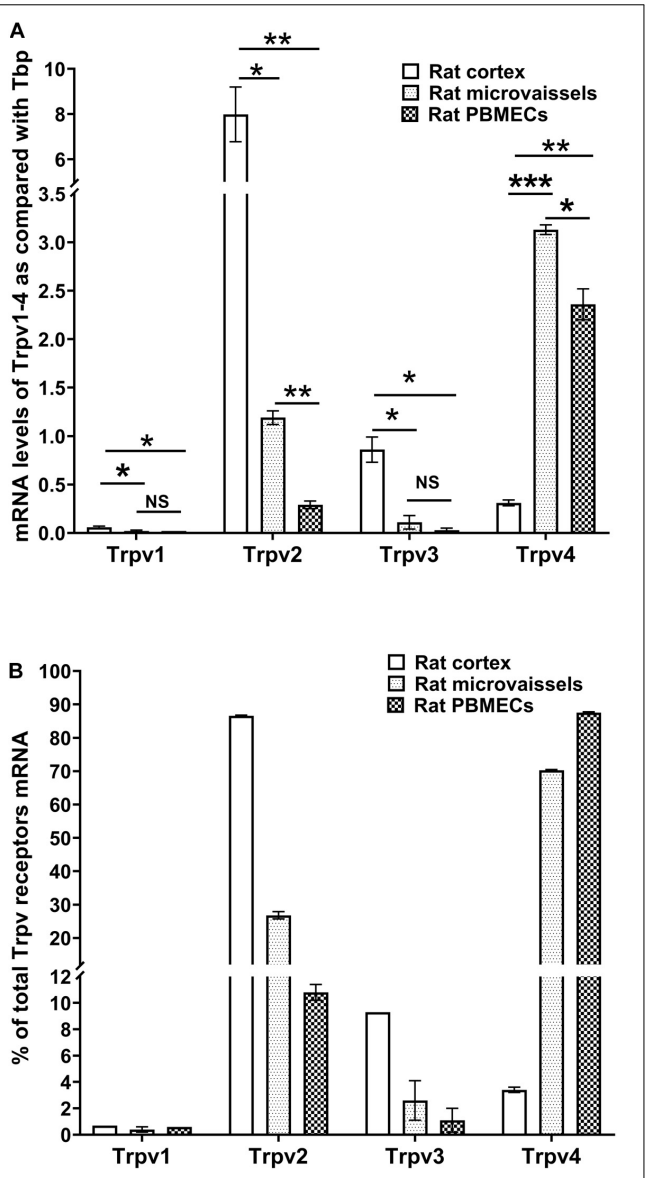

FIGURE 1 | Expression of thermo-sensitive Trpv in rat cortex, rat microvessels and BBB endothelial cells. **(A,B)** mRNA levels of *Trp1-4* were detected by qRT-PCR in rat brain cortex, rat brain isolated microvessels and primary cultures of rat brain microvessel endothelial cells (see section "Materials and Methods"). Data are expressed as ratio (mean ± SEM) of *Trpv* mRNA levels compared with those of the endogenous housekeeping gene *Tbp* in each sample from three different RNA extractions **(A)** or expressed as a percentage of total *Trpv1-4* expression set at 100% **(B)**. Inter-group comparisons were performed by ANOVA with Dunnett *a posteriori* test, NS not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

was far below that of *Trpv2*. Meanwhile, no significant difference was observed for the gene expression of *Trpv1* and *Trpv3* between brain microvessels and rPBMEC (**Figure 1A**), while a significant decrease of *Trpv2* and Trpv 4 gene expression was observed in rPBMEC compared with that in brain microvessels (**Figure 1A**, $p < 0.01$, $p < 0.05$ for *Trpv2* and *Trpv4*, respectively, rat microvessels vs. rPBMEC). The *Trpv1-4* gene expression profile expressed as a percentage of total *Trpv* genes expressed was similar in brain microvessels and rPBMEC with the following rank order: *Trpv4* > *Trpv2* > *Trpv3* > > *Trpv1* (**Figure 1B**). This is dramatically different from the *Trpv1-4* gene expression profile in the whole brain cortex with the following rank order: *Trpv2* > *Trpv3* > *Trpv4* > > *Trpv1*.

Thermo-Sensitive *TRPV1-4* Gene Expression in Human BMEC

We measured the relative expression of *TRPV1-4* by qRT-PCR in hCMEC/D3 cells and primary cultured human brain microvascular endothelial cells (hPBMEC) from three different brain tumor biopsies (**Figure 2A**). *TRPV1* mRNA levels were easily detected in hPBMEC of the adult patient culture CC216 and, albeit at a lower level, in hCMEC/D3 and hPBMEC from the two children cultures CC205 and CC206 (3.05 ± 0.26, 0.22 ± 0.01, 0.21 ± 0.01, and 0.76 ± 0.08, respectively, **Figure 2A**). It is noteworthy that *TRPV1* expression was increased significantly by 4.8-fold (from 3.05 ± 0.26 to 14.6 ± 2.0, $p < 0.01$) when cells were co-cultured with isolated primary astrocytes from the same patient, while no effect was found for the other 3 TRPV isoforms (**Figure 2A**). *TRPV2* was by far the most highly expressed gene in hCMEC/D3 and hPBMEC cells [*TRPV2* data for CC205 and CC206 was also shown in Luo et al. (2019)]. *TRPV4* was expressed at moderate levels in hPBMEC from the three patients (10.36 ± 2.23, 1.35 ± 0.04, and 1.79 ± 0.12) and hCMEC/D3 (1.38 ± 0.01) while *TRPV3* mRNA were barely detectable in all cells. A similar *TRPV1-4* gene expression profile expressed as a percentage of total *TRP* gene expressed was observed in hCMEC/D3 cells and hPBMEC with the following rank order: *TRPV2* > > *TRPV4* > *TRPV1* > > *TRPV3* (**Figure 2B**).

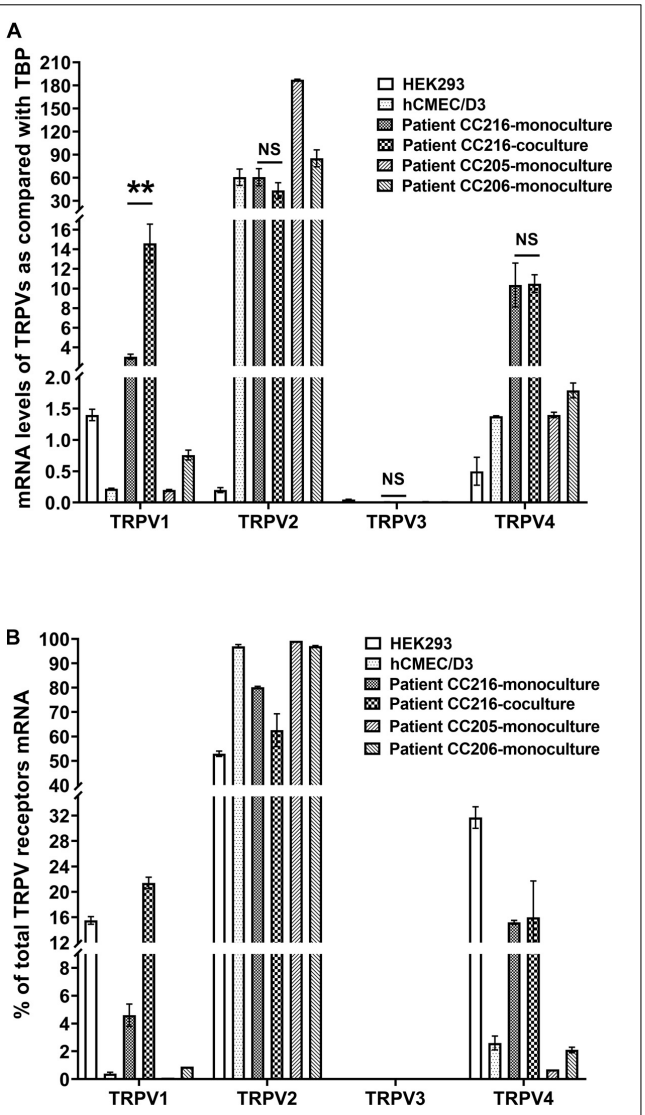

FIGURE 2 | Expression of thermo-sensitive Trpv in human BMEC. **(A,B)** mRNA levels of *TRPV1-4* were detected by qRT-PCR in primary cultures of brain microvessel endothelial cells obtained from patients (see section "Materials and Methods") and in hCMEC/D3 cells. Data are expressed as ratio (mean ± SEM) of TRPV1-4 mRNA levels compared with those of the endogenous housekeeping gene *TBP* in each sample from three different RNA extractions **(A)** or expressed as a percentage of total *TRPV1-4* expression set at 100% **(B)**. When comparing TRPV1-4 expression in human BMEC culturing alone or together with astrocytes isolated from the same patient, statistical significance was determined by an unpaired *t*-test, NS, not significant, $^{**}p < 0.01$.

Protein Expression of TRPV2 and TRPV4 in hCMEC/D3

The expression of TRPV2 and TRPV4, the two genes with the highest expression at the mRNA level in human brain endothelial cells, was then studied at the protein level. Western blot analysis showed that TRPV2 and TRPV4 proteins were expressed in hCMEC/D3 cells where they are detected in a single band at a molecular weight around 80–90 kDa, in agreement with the predicted value of TRPV2 and TRPV4 molecular weights (**Figure 3A**). This was confirmed by immunostaining and confocal microscopy which revealed a specific expression of both TRPV2 and TRPV4 at the plasma membrane of hCMEC/D3 cells as well as in intracellular compartments concentrated in the perinuclear region (**Figure 3B**). Omitting the primary antibody resulted in the absence of any detectable fluorescence signal (NEG in **Figure 3B**). The tight junction protein Claudine-5 was used as a positive control for brain endothelial cell phenotype as it was reported to be expressed in hCMEC/D3 cells (Weksler et al., 2005).

Functional Activity of TRPV in hCMEC/D3

Transient receptor potential vanilloid 1, TRPV2, and TRPV4 functional activity was then investigated in hCMEC/D3 cells.

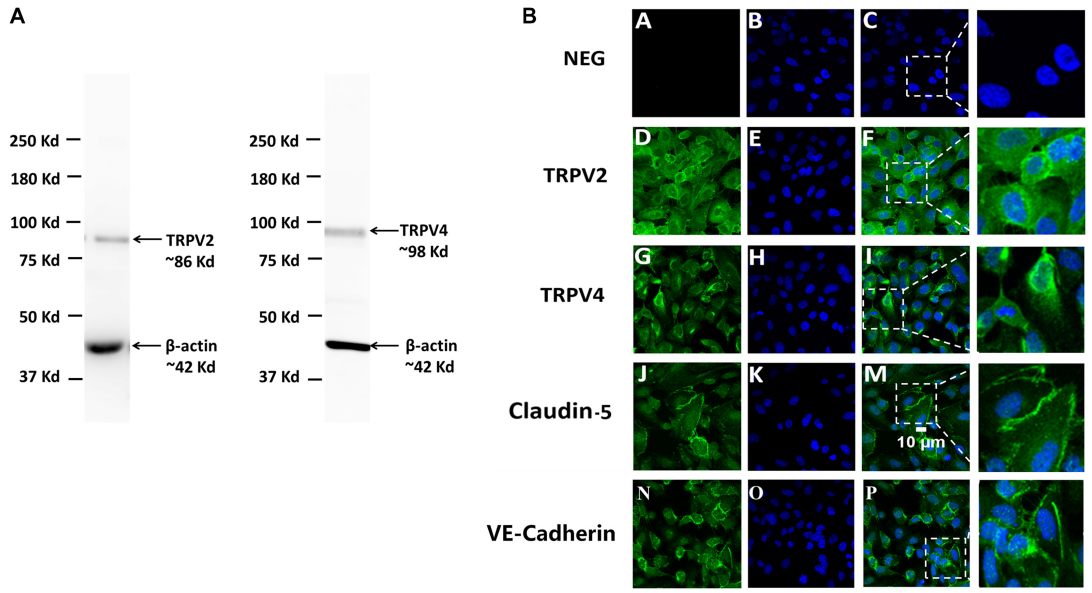

FIGURE 3 | Protein expression of TRPV2 and TRPV4 in hCMEC/D3. **(A)** Expression of TRPV2 and TRPV4 determined by Western blot of total crude proteins obtained from two different protein extractions of hCMEC/D3. β-actin served as a loading control housekeeping protein. **(B)** Confocal immunofluorescence localization of TRPV2 and TRPV4 in hCMEC/D3. TRPV2 or TRPV4 (green) and nuclei (blue) were stained in hCMEC/D3 as described in section "Materials and Methods" (second D, E, F and third G, H, I row panels). Claudin-5 (green) and nuclei (blue) were stained in hCMEC/D3 (fourth row panel J, K, M). VE-Cadherin (green) and nuclei (blue) were stained in hCMEC/D3 (fifth row panel N, O, P). Control cells (NEG) were stained omitting the primary antibodies (first row panel A, B, C).

Ionomycin, which triggers an immediate increase of the intracellular Ca^{2+} concentration, was used as a positive control (2 μM ionomycin) in all Ca^{2+} influx experiments.

The AUC of intracellular Ca^{2+} levels during 20 min were not significantly affected when cells were exposed for 20 min to 1 or 3 μM CAP, a highly specific and efficient agonist of TRPV1 (**Figures 4A,B**). By contrast, CAP, at lower concentrations than those used in our study, were high enough to fully activate human TRPV1 (Binshtok et al., 2007). In addition to compare the AUC of intracellular Ca^{2+} levels during 20 min, we further compared the intracellular Ca^{2+} levels at specific time (**Figures 4C,D**). Interestingly, a significant statistical difference between the control group and 3 μM CAP group at 7 min (p = 0.019), 11 min (p = 0.041), 12 min (p = 0.049), and 16 min (p = 0.045) were found (**Figures 4C,D**). However, no significant difference of Ca^{2+} influx at any specific times was found when cells exposed to 1 μM CAP (**Figures 4C,D**). Ca^{2+} flux levels were not significantly altered when cells exposed to CAP from 1 to 3 μM (**Figures 4C,D**). Meanwhile, a significant 3.3-fold increase of Ca^{2+} influx was observed in the positive control group treated with 2 μM ionomycin (**Figures 4A,B**, $p < 0.001$ compared with the control group). TRPV1 is a known thermosensitive channel (Mergler et al., 2010). Therefore, we asked whether CPZ, a well-known TRPV1 antagonist, was able to block the heat-induced increase of intracellular Ca^{2+} concentration. As shown in **Figures 4E,F**, exposing cells continuously from RT to 50°C for 40 min resulted in a significant increase in intracellular Ca^{2+} but this Ca^{2+} flux was not altered by co-treatment with CPZ (10 or 20 μM; **Figure 4E**), in agreement with the low expression of *TRPV1* assessed by qRT-PCR and the weak TRPV1 activation by CAP. Conversely, heat-induced Ca^{2+} influx could be blocked by TNL, a selective TRPV2 antagonist. Exposing the cells to CBD, a highly potent activator of TRPV2 and to a lesser extent of TRPV1, resulted in an increased Ca^{2+} influx in hCMEC/D3, an effect which was not reversed by CPZ (**Figures 4G,H**). Similar trends were demonstrated when comparing the Ca^{2+} influx at specific time (**Figures 4I,J**).

To assess TRPV4 activity in hCMEC/D3, we applied GSK1016790A (GSK), a highly selective and potent agonist of TRPV4. GSK induced a dramatic and rapid concentration-dependent increase in Ca^{2+} levels (**Figure 5A**). A maximal increase in intracellular Ca^{2+} levels (1.6-fold) was obtained with GSK ≥100 nM (**Figure 5B**). An apparent concentration-effect relationship was observed with an EC_{50} of 16.2 ± 4.5 nM (**Figure 5C**). The rise in intracellular Ca^{2+} concentration induced by 30 nM GSK1016790A was inhibited by RN1734 pretreatment (30 μM) a well-known TRPV4 antagonist (**Figures 5D,E**).

DISCUSSION

Brain microvessel endothelial cells, the main component of the BBB, are widely exposed to various stimuli from the blood and brain compartments. Among these, blood borne compounds and mechanical stress might modulate the activity of TRP channels, thus allowing Ca^{2+} entry into brain endothelial cells where Ca^{2+} concentrations need to be maintained within a certain range of concentrations through a dynamic equilibrium (Ma and Liu, 2019). Intracellular/cytosolic [Ca^{2+}] modulates

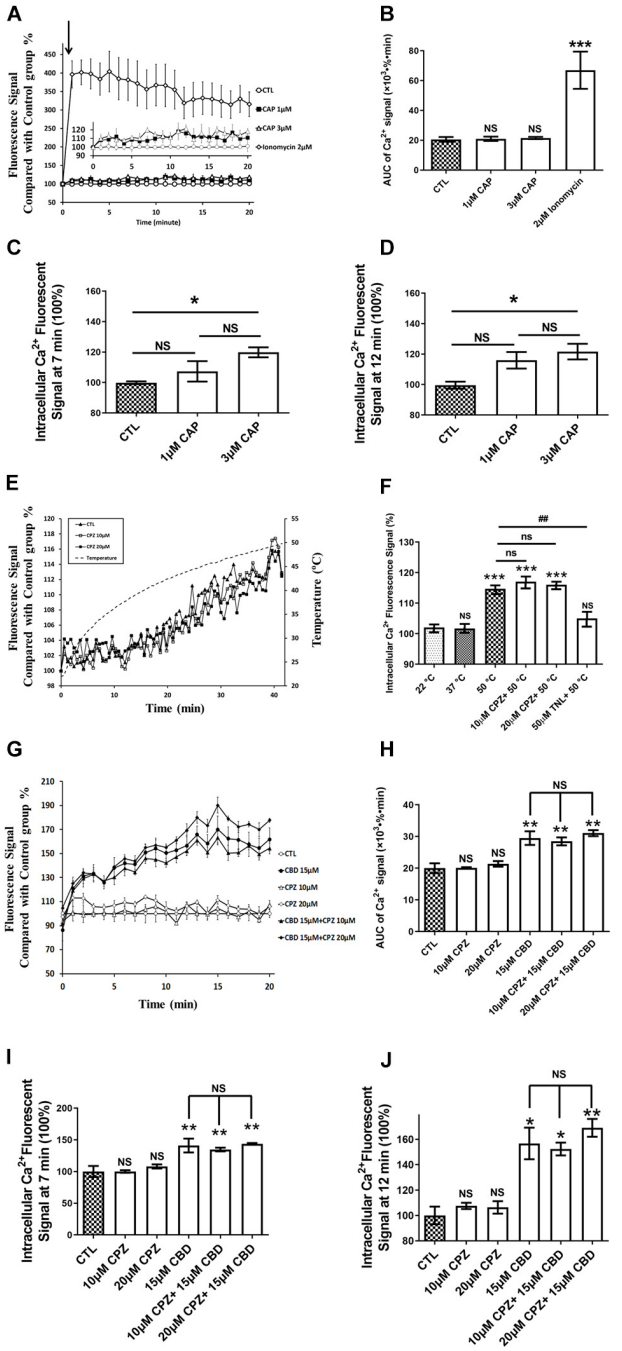

FIGURE 4 | TRPV1 is not functional in hCMEC/D3. **(A)** Representative time course of $[Ca^{2+}]_i$ in hCMEC/D3 under stimulation of TRPV1 by capsaicin (CAP). Cells were plated in 24-wells plate and loaded with Fluo-4-AM as described in section "Materials and Methods." CAP or ionomycin (as positive control of Ca^{2+} entry) was added and the fluorescence was recorded. **(B)** Change of the area under curve (AUC) over the 20 min obtained from the experiment shown in panel **(A)**. **(C)** Change in $[Ca^{2+}]_i$ at $T = 7$ min obtained from the experiment shown in panel **(A)**. **(D)** Change in $[Ca^{2+}]_i$ at $T = 12$ min obtained from the experiment shown in panel **(A)**. **(E)** Change of $[Ca^{2+}]_i$ upon heat exposure of hCMEC/D3 for 40 min from room temperature to 50°C. Cells were plated in 24-wells plate and loaded with Fluo-4-AM. The fluorescence was recorded in the presence or not of the specific TRPV1 antagonist

(Continued)

FIGURE 4 | Continued

capsazepine (CPZ; 10 or 20 μM). **(F)** Changes in $[Ca^{2+}]_i$ in hCMEC/D3 at 22, 37, or 50°C with or without CPZ or Tranilast (TNL, 50 μM) derived from panel **(E)**. **(G)** Representative time course of $[Ca^{2+}]_i$ in hCMEC/D3 stimulated by the TRPV1-2 agonist cannabidiol (CBD, 15 μM) and pre-treated or not with the TRPV1 specific antagonist CPZ. Cells were loaded with Fluo-4-AM. CBD was added and the fluorescence was recorded. When applying CPZ, cells were pre-treated with 10 or 20 μM CPZ for 5 min before incubation with 15 μM CBD and the fluorescence was recorded in the persistent presence of 10 or 20 μM CPZ. **(H)** Change of the area under curve (AUC) over the 20 min obtained from the experiment shown in panel **(G)**. **(I)** Change in $[Ca^{2+}]_i$ at $T = 7$ min obtained from the experiment shown in panel **(G)**. **(J)** Change in $[Ca^{2+}]_i$ at $T = 12$ min obtained from the experiment shown in panel **(G)**. Data are expressed as mean ± SEM and inter-group comparisons were performed by ANOVA with Dunnett *a posteriori* test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with CTL or 22°C groups or among indicated group.

various cellular processes such as excitability, proliferation, synaptic plasticity, resistance to oxidative stress, and cell death, depending on the concentration, timing and duration of the signal (Fliniaux et al., 2018). Modifications of intracellular $[Ca^{2+}]$ can trigger the proliferation of BMEC and increase the permeability of the BBB by altering tight junction proteins (Ma and Liu, 2019). Furthermore, BBB dysfunctions observed in several brain pathologies are usually associated with increasing intracellular Ca^{2+} concentrations, suggesting that TRP-mediated Ca^{2+} signaling may play crucial roles in maintaining and regulating BBB functions (De Bock et al., 2013). Interestingly, the highly potent TRPV2 activator CBD increased Ca^{2+} entry in hCMEC/D3 cells where it induces proliferation, tubulogenesis and migration (Luo et al., 2019). Moreover, CBD has anti-inflammatory activity and is able to reduce the disruption of the BBB observed in lipopolysaccharide (LPS) (Ruiz-Valdepenas et al., 2011) and multiple sclerosis mice models (Mecha et al., 2013). CBD was demonstrated as effective in preventing disruption of an *in vitro* BBB model under hypoxic and glucose deprivation conditions (Hind et al., 2016). Altogether, these results suggest that activating TRPV2 might be a relevant strategy to positively modulate the BBB in the context of brain disorders including ischemic stroke (Calapai et al., 2020). Here, we looked at interspecies differences in the expression and activity of TRPV in rat and human BBB.

Transient receptor potential vanilloid 1-4 were first to be known for their expression in sensory neurons and their function in sensing nociception and pain (Mickle et al., 2015). However, the expression of TRPV1-4 in the brain is quite region-specific and heterogeneous (Kauer and Gibson, 2009; Cavanaugh et al., 2011; Nedungadi et al., 2012). For TRPV1, previous studies have believed that it can be widely expressed throughout the brain (Kauer and Gibson, 2009). However, a recent study applied a highly sensitive method using gene editing to modify the Trpv1 genetic locus in two lines of reporter mice, showing that TRPV1 is highly limitedly expressed in several specific brain regions, such as the caudal hypothalamus, the dorsal motor nucleus of the vagus, mesencephalic trigeminal nucleus, and parabrachial nucleus (Cavanaugh et al., 2011). Litter expression was found for the other brain regions (Cavanaugh et al., 2011). TRPV2 was found to be intensively expressed mainly in several brain

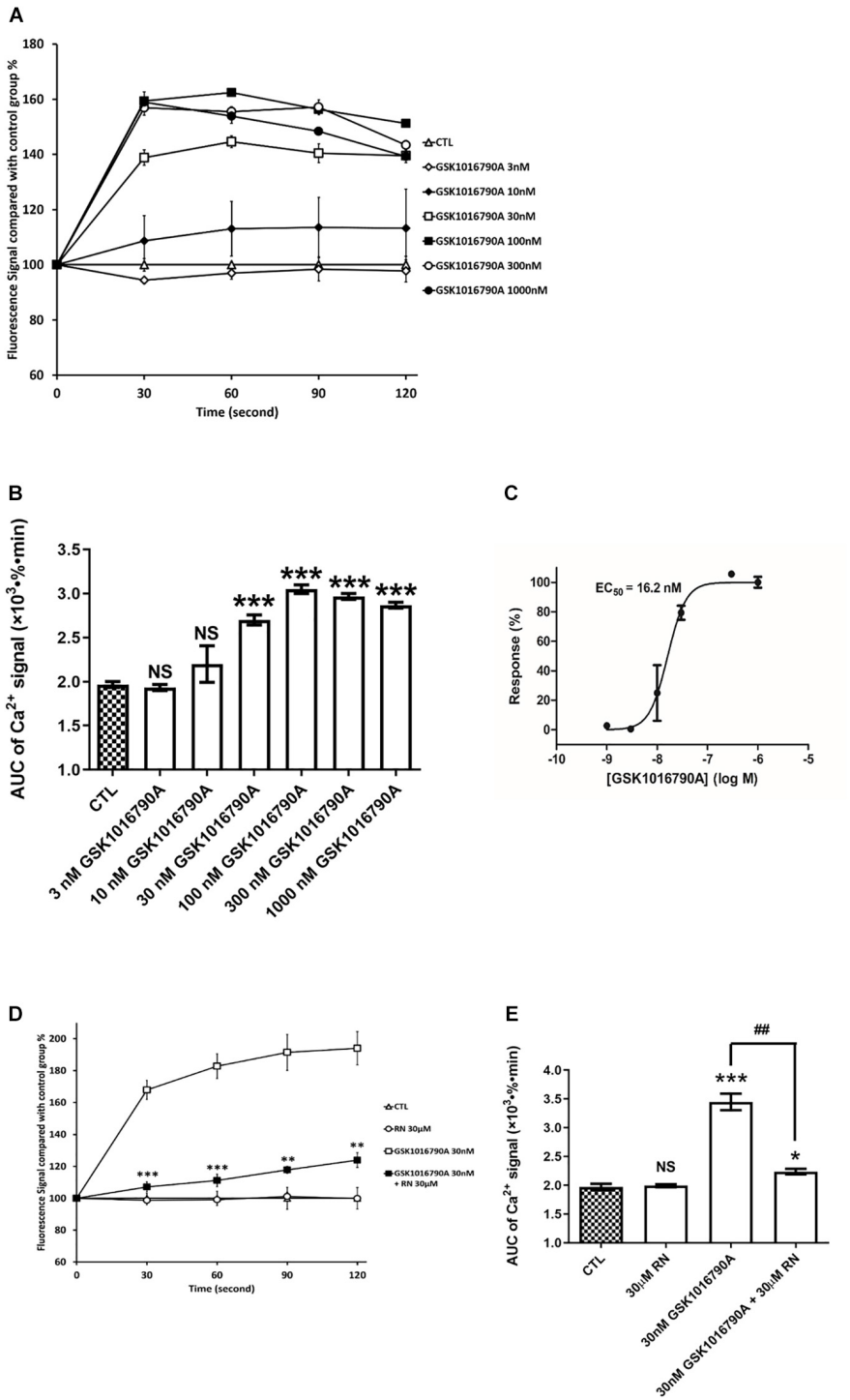

FIGURE 5 | TRPV4 is functional in hCMEC/D3. **(A)** Representative time course of $[Ca^{2+}]_i$ in hCMEC/D3 stimulated by the TRPV4 agonist GSK1016790A. Cells were plated in 24-wells plate and loaded with Fluo-4-AM as described. GSK1016790A was added and the fluorescence was recorded. **(B)** Change of the area under curve (AUC) over the 120 s obtained from the experiment shown in panel **(A)**. **(C)** Concentration-response relationship of GSK1016790A. Measurements were obtained from panel **(B)**. **(D)** Representative time course of $[Ca^{2+}]_i$ in hCMEC/D3 stimulated by GSK1016790A and pre-treated or not with the TRPV4 specific antagonist RN1734 (RN). When applying RN, cells were pre-treated with 30 μM RN for 5 min and the fluorescence was recorded in the persistent presence of 30 μM GSK1016790A. **(D)** Change of the area under curve (AUC) over the 120 s obtained from the experiment shown in panel **(C)**. Data are expressed as mean ± SEM. For panels **(B,E)**, inter-group comparisons were performed by ANOVA with Dunnett *a posteriori* test, NS, not significant, $^*p < 0.05$, $^{***}p < 0.001$ compared with CTL group, $\#\#p < 0.01$ compared with 30 nMGSK1016790A group, $n = 3$ in triplicate. For panel **(D)**, inter-group comparisons were performed by ANOVA with Dunnett *a posteriori* test, $^*p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with GSK1016790A group.

areas including: hypothalamus, the nucleus of the solitary tract, hypoglossal nucleus, and the rostral division of the ventrolateral (Nedungadi et al., 2012). TRPV3 protein expression has not been detected in the mouse brain, although TRPV3 mRNA transcripts has been detected in the brain at low levels (Luo and Hu, 2014). TRPV3 is also evidenced to be absent in the mouse bEnd.3 brain endothelial cell line and in the freshly isolated brain capillaries and primary cultured BMECs from rat (Mickle et al., 2015). This is in accordance with the whole transcriptomic analysis demonstrating the absence of Trpv3 in murine primary BMECs (Zhang et al., 2014) and by brain vascular single-cell transcriptomics study (Vanlandewijck et al., 2018). TRPV3 is most abundantly expressed in skin keratinocytes and in cells surrounding hair follicles, where it plays an essential role in cutaneous sensation. RT-PCR or q-PCR analysis showing the absence of TRPV3 in human BMECs (Golech et al., 2004; Hatano et al., 2013) and gain-of-function mutations in human TRPV3 are associated with Olmsted syndrome, which is characterized by severe palmoplantar and periorificial keratoderma (Greco et al., 2020; Zhang et al., 2020). TRPV4 has been reported to be widely expressed throughout the brain including hippocampus, hypothalamus, cerebellum, lamina terminalis and optic chiasm, as well as a special enhanced expression in the olfactory placodes (Mangos et al., 2007; Kauer and Gibson, 2009; Shibasaki et al., 2015). Unfortunately, it is still remained largely unknown which types of cells were responsible for their expression in a specific brain region (Kauer and Gibson, 2009). Our results showed significant mRNA levels of some TRPVs in human brain endothelial cells which follow this rank of expression: *TRPV2* > *TRPV4* > *TRPV1*, while *TRPV3* was barely detected. It should be noted that this rank order is based on mRNA levels and functional studies since the absolute amount of each TRPV at protein level was not investigated in this study. These observations are supported by a study showing the qualitative expression of TRPV1, 2, and 4 in human primary cultured brain endothelial cells by RT-PCR (Hatano et al., 2013). TRPV2, a thermo-sensitive TRPV channel, was by far the most highly expressed isoform in hPBMEC and hCMEC/D3 while its relative expression was much lower than that of Trpv4 in rat microvessels and rPBMEC. TRPV1 and TRPV4 were also expressed both in rPBMEC, hPBMEC and hCMEC/D3 cells, and TRPV2 was even more expressed in hPBMEC than in hCMEC/D3 cells. TRPV4 expression was further evidenced by Western blot and immunofluorescence analysis in hCMEC/D3 cells supporting previous RT-PCR and/or immunofluorescence results in human BMEC (Sullivan et al., 2012; Hatano et al., 2013). However, using specific peptides of TRPV2 and TRPV4 targeted by the primary anti-TRPV2 and antiT-RPV4 antibodies used in our study would have increased the specificity of the immunofluorescence staining. Interestingly, we detected a 4.0- and 5.8-fold higher expression of, respectively, *TRPV1* and *TRPV4* mRNA, in the brain endothelial cells derived from the adult patient (CC216) as compared with the two children (CC205 and CC206), suggesting that TRPV1 and TRPV4 expression might increase with age while that of TRPV2 might decrease. This is in agreement with the reported age-dependent expression and function of Trpv1 in the rodent forebrain (Koles et al., 2013), although we cannot exclude, in our case, that these differences might result from the limited numbers of patients and/or reflect the underlying pathology or the exposure of the patients to medication. Interestingly, *TRPV* expression in hPBMEC was modulated by co-culture with astrocytes as shown by the 4.8-fold TRPV1 increase in hPBMECs co-culture with isolated primary astrocytes obtained from the same patient. In contrast, this effect was absent for *TRPV2,3,4* isoforms. Recently, Mannari et al. (2013) have reported that the astrocytic *TRPV1* could directly sense the blood-borne signals in the sensory circumventricular organs in adult mouse brains. Trpv1 is regarded as a target in regulating BBB permeability (Hu et al., 2005; Beggs et al., 2010) and might be also a downstream target in the modulated BBB permeability mediated by astrocytes (Abbott, 2002; Abbott et al., 2006).

A similar expression profile was found between hPBMEC isolated from patients and the human BBB cell line hCMEC/D3, confirming previous data (Dauchy et al., 2009; Ohtsuki et al., 2013) and suggesting that hCMEC/D3 is a proper *in vitro* surrogate in exploring TRPV properties at the human BBB. *Trpv2* gene expression was 25.8-fold more expressed in brain cortex than in rPBMEC, in accordance with a more pronounced Trpv2 immunostaining in the rat brain (Nedungadi et al., 2012). Notably, we evidenced a remarkable interspecies difference regarding the expression profile of *TRPV1-4* between rPBMEC and the two human *in vitro* BBB models (hPBMEC and hCMEC/D3). Although *TRPV2* and *TRPV4* were expressed both in rat and human BMEC, *Trpv4* and *TRPV2* were the most expressed isoform in rPBMEC and hBMEC, respectively. Brown et al. (2008) have demonstrated *Trpv1-4* expression by RT-PCR in the bEnd.3 murine BBB cell line and in isolated mouse brain microvessels, showing a higher *Trpv2* expression than *Trpv4*, suggesting TRPV expression profile could be similar in murine and human BMEC. A whole transcriptomic analysis revealed a similar expression of *Trpv2* and *Trpv4* in murine primary BMEC, while *Trpv1* and *Trpv3* transcripts were undetectable (Zhang et al., 2014). Except for TRPV1-4, many other transporters and enzymes expressed at the BBB also demonstrated interspecies differences in expression and function levels (Deo et al., 2013). PET studies have demonstrated a significant interspecies differences for the expression and functionality of ABCB1 (i.e., P-gp), an important efflux transporter at the BBB (Syvanen et al., 2009). The brain-to-plasma concentration ratio of [^{11}C]GR205171, a substrate of P-gp, was almost 9-times higher in humans compared with rats (Syvanen et al., 2009; Ball et al., 2013). In fact, interspecies differences in expression and/or function levels were also verified for ABCG2 (BCRP) (Ito et al., 2011; Uchida et al., 2011; Agarwal et al., 2012), MRPs (Agarwal et al., 2012), some OATPs (Ito et al., 2011; Uchida et al., 2011) and some SLCs (Ito et al., 2011; Agarwal et al., 2012).

Our current work did not indicate whether TRPV receptors are expressed at BBB in a polarized way or not. Many receptors/transporters are localized specifically at luminal or abluminal side of the brain endothelial cells in charge of different functions (Dragoni and Turowski, 2018). Very poor data were reported regarding the subcellular localization of TRPV1-3 in brain microvessels. In the rat middle cerebral arteries, TRPV4 was found to be expressed preferentially on the abluminal membrane

of endothelial cells (Marrelli et al., 2007) but the observation that a selective P2Y2 receptor agonist could induce TRPV4-mediated Ca^{2+} entry across the luminal and abluminal face of an endothelial monolayer (Guerra et al., 2018), suggests that TRPV4 could be also expressed on the luminal face of endothelial cells. Benfenati et al. (2007) confirmed that expression of TRPV4 is abundantly localized on astrocytic endfeet area abutting pial and parenchyma blood vessels. Also, TRPV2 has been shown to be prominently localized to the umbrella apical membrane of bladder epithelium cells, while TRPV4 was identified on their abluminal surfaces (Yu et al., 2011).

Transient receptor potential vanilloid-mediated modulation of the intracellular Ca^{2+} concentration was further investigated using specific agonist and antagonists. The highly potent TRPV1 agonist CAP, usually effective at nanomolar levels (Flockerzi, 2007), did not increase the AUC of intracellular Ca^{2+} during 20 min in hCMEC/D3 at a concentration of 1 or 3 μM. Further statistical tests revealed that 3 μM CAP can stimulate the Ca^{2+} influx at specific time (7, 11, and 12 min post CAP stimulation), indicating a possible weak function of TRPV1 in the BBB. This in agreement with the low gene expression of *TRPV1* in hCMEC/D3 and also with the recent observation that no functional activity with CAP up to 1 mM could be detected in human renal podocytes although these cells expressed TRPV1 (Ambrus et al., 2017). Notably, TRPV1-induced Ca^{2+} influx under CAP stimulation at specific times is not quite dose-dependently as similar Ca^{2+} influx levels were found in 1 or 3 μM CAP, from the view of either the AUC of Ca^{2+} during 20 min or the Ca^{2+} influx at any specific time, further identifying the quite lower TRPV1 expression at the membrane of hCMEC/D3 cells. In fact, the much lower expression of TRPV1 in BBB endothelial cells were also demonstrated by Golech et al. (2004) by RT-PCR and polyclonal antibodies. TRPV1 is also functional in responding to heat stimulation, however the heat-induced Ca^{2+} influx was not significantly inhibited by CPZ (10 or 20 μM), a widely used TRPV1 antagonist, suggesting that TRPV1 might not be the main functional isoform in hCMED/D3 cells. In addition, it was reported that TRPV1 could be also activated by CBD, although its agonist potency was lower than toward TRPV2 (Qin et al., 2008). Yet, no significant alteration in intracellular Ca^{2+} was observed when co-treating cells with CBD and CPZ, a TRPV1 antagonist, further suggesting the lack of TRPV1 activity or weak TRPV1 activity in hCMEC/D3 cells. However, we cannot exclude a functional activity of TRPV1 in hPBMECs since TRPV1 gene expression was much higher in those cells than in hCMEC/D3 cells, especially when they were co-cultured with astrocytes from the same patient. Moreover, CPZ is not a very potent antagonist of TRPV1 (Rigoni et al., 2003), that could only produce an insufficient/partial blocking of TRPV1. Therefore, the absence of functional activity of TRPV1 in hCMEC/D3 cells remains questionable.

The specific and potent TRPV4 agonist GSK triggered a rapid elevation of intracellular Ca^{2+} with an E_{max} at 100 nM (EC_{50} ∼16.2 nM), which can be totally inhibited by the specific TRPV4 antagonist RN1734. The EC_{50} for GSK in hCMEC/D3 cells was close to that reported for stimulating Ca^{2+} influx in another study (26.9 nM) (Sullivan et al., 2012). Previous studies have identified the expression of TRPV4 in commercial human BMECs at gene and protein levels (Hatano et al., 2013). The expression and function of TRPV4 are also verified using patch-clamp electrophysiology techniques in freshly BMECs isolated from brain capillaries of C57BL/6J mouse (Harraz et al., 2018). The specific TRPV4 agonist GSK-stimulated currents was completely abolished in TRPV4-knockout mice or when co-applying the specific TRPV4 antagonist HC-067047 in murine BMECs (Harraz et al., 2018). It is also reported that TRPV4 might play a vital role in regulating the function of blood-retina barrier, similarly to BBB, via modulating Ca^{2+} homeostasis in human retinal microvascular endothelial cells (Phuong et al., 2017). Due to their diverse cellular and subcellular localizations, TRP channels could mediate Ca^{2+} influx and generate distinct cytosolic/local intracellular Ca^{2+} waves that could regulate specific subcellular downstream pathways leading to different cell responses including death or survival (Fliniaux et al., 2018). In this regard, mitochondrial expression of TRPV1-2-3 has been evidenced in human odontoblasts (Wen et al., 2017), which is consistent with the intracellular staining we observed in hCMEC/D3 cells for TRPV2 and TRPV4.

CONCLUSION

In conclusion, our present study shows that TRPV1, TRPV2 and TRPV4 are expressed in hPBMECs. Even less expressed than TRPV2, TRPV4 is functionally expressed in the human BBB. Recently, TRPV4 has been shown to be highly expressed and functional in retinal endothelial cells (Phuong et al., 2017). The authors nicely showed by different electrophysiological and Ca^{2+} imaging techniques and using an *in vitro* retinal barrier model that TRPV4 highly contributed to Ca^{2+} homeostasis and barrier function. The role of TRPV4 in BBB barrier function and physiology should be evaluated in further experiments before claiming whether or not TRPV4 could be a target to be investigated for modulating the BBB. TRPV1 is much less expressed than TRPV2 and TRPV4 in the human BBB but still shows weak or limited functionality when activated by relative high concentration of selective chemical agonist. An interspecies difference between rat and human TRPV2,4 was evidenced in the BBB using a gene expression approach which may be taken into consideration for rat to human translational pharmacological approaches targeting TRPV in the BBB. This interspecies difference between rat and human TRPV should be further confirmed from a functional point of view by using for example electrophysiological techniques like patch-clamp and measuring whole-cell currents of TRPV2 and TRPV4 between human and rat BMEC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by French Ministry of Higher Education and Research (CODECOH DC-2014-2229). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Ethics Review Committee of Paris Descartes University.

AUTHOR CONTRIBUTIONS

HL, BS, SCh, VC, MS, FG, and CC: experiments. HL and XD: writing – original draft preparation. BS, SCi, and XD: writing – review and editing. SCi and XD: supervision. All authors have read and agreed to the submitted version of the manuscript.

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REVIEW
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Transient Receptor Potential Channels as an Emerging Target for the Treatment of Parkinson's Disease: An Insight Into Role of Pharmacological Interventions

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by the symptoms of motor deficits and cognitive decline. There are a number of therapeutics available for the treatment of PD, but most of them suffer from serious side effects such as bradykinesia, dyskinesia and on-off effect. Therefore, despite the availability of these pharmacological agents, PD patients continue to have an inferior quality of life. This has warranted a need to look for alternate strategies and molecular targets. Recent evidence suggests the Transient Receptor Potential (TRP) channels could be a potential target for the management of motor and non-motor symptoms of PD. Though still in the preclinical stages, agents targeting these channels have shown immense potential in the attenuation of behavioral deficits and signaling pathways. In addition, these channels are known to be involved in the regulation of ionic homeostasis, which is disrupted in PD. Moreover, activation or inhibition of many of the TRP channels by calcium and oxidative stress has also raised the possibility of their paramount involvement in affecting the other molecular mechanisms associated with PD pathology. However, due to the paucity of information available and lack of specificity, none of these agents have gone into clinical trials for PD treatment. Considering their interaction with oxidative stress, apoptosis and excitotoxicity, TRP channels could be considered as a potential future target for the treatment of PD.

Keywords: Parkinson's disease, TRP channels, TRPM2, TRPV1, TRPC1

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative condition characterized mainly by the loss of dopaminergic neurons in regions of the basal ganglia. It is the second most prevalent form of neurodegenerative disorder and stands next to Alzheimer's disease (AD) in terms of number of individuals affected across the globe (Parkinson's News Today, 2020). There are a number of therapeutics (Levodopa, Dopamine (D2) receptor agonists, monoamine oxidase B inhibitors, catechol-*o*-methyl transferase inhibitors, Adenosine 2A antagonists) approved for the treatment of PD. However, most of them provide only symptomatic relief. Also, currently available drugs show severe side effects and require frequent dosing resulting in low patient compliance of these medications. This has warranted a search for new mechanisms as well as molecular targets which

could be utilized for the development of drug candidates in PD (Kumar et al., 2009; Uppalapati et al., 2014; Das and Sharma, 2015; Zhen and Chu, 2020). There are a number of *in vitro* and *in vivo* models which have been used for the therapeutic screening of pharmacological agents targeting these new molecular targets in PD. PD models include administration of neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP^+), rotenone, 6-hydroxydopmaine (6-OHDA) and alpha-synuclein pro-fibrils to induce dopaminergic neuronal death in the substantia nigra and the striatum. In addition, transgenic mice (knock-in and knock-out) are available for induction of PD. Pathophysiology of PD is complex and multi-factorial. Studies done using these preclinical models as well as PD patients have pointed out at the involvement of Ca^{2+} overload and oxidative stress in PD pathology. Cumulative evidence in recent times has demonstrated a major contribution of Transient Receptor Potential (TRP) channels toward Ca^{2+} mediated excitotoxicity and oxidative stress-induced dopaminergic neuronal death in PD. In total, 28 members of the TRP family have been discovered in mammals which are activated in response to diverse physiological stimuli (Zheng, 2013; Samanta et al., 2018). However, most of these members are non-selective cation channels which are responsible for the influx of monovalent and divalent ions, including Ca^{2+} into the cell. Oxidative stress and reactive oxygen species (ROS) have also been postulated to play a role in mechanisms involving activation and inhibition of these TRP channels (Adhya and Sharma, 2019; Akyuva and Naziroglu, 2020). These properties make them an attractive target for the treatment of neurodegenerative conditions, including PD (Thapak et al., 2020c). As a result, last few decades have seen enormous progress in the field of TRP research, and many of the TRP channel modulators like AMG571, MK-2295 and AZD1386 (TRPV1 antagonists) have even managed to progress to the clinical trials for the treatment of pain disorders (Kaneko and Szallasi, 2014). However, further analysis of these TRP channel modulators has exposed their dark side with side effects raging form hyperthermia and to reduced noxious perception to heat (Gavva, 2009; Kaneko and Szallasi, 2014). Thus, most of these studies had to be terminated owing to the risk of scalding injuries to the patients. Keeping in mind the crucial role of these channels, it is now expected that detailed structure-activity relationships and pharmacophore studies would help to improve their safety and efficacy and ensure the translational success of TRP channel modulators in PD.

In this review, we have provided a detailed account of preclinical evidence suggesting the vital involvement of TRP channels in PD and how their knowledge may help researchers in this field gain in-depth insight about these channels. Additionally, a number of upcoming drug candidates targeting TRP channels have also been discussed.

TRP FAMILY

TRP family of ion channels was first discovered in 1969 when some of the drosophila fly mutants turned blind in response to bright light (Cosens and Manning, 1969). This identification was followed by a series of biochemical, electrophysiological and molecular studies which led to the characterization of the TRP channel family. It is now well established that these channels are evolutionarily conserved across species and are found in most cells, tissues, and organisms. Subsequently, a total of 28 members belonging to 6 subfamilies were identified in mammals which include TRPV (Vanilloid), TRPM (Melastatin), TRPC (Canonical), TRPML (Mucolipin), TRPA (Ankyrin), and TRPP (Polycystic) (Li, 2017). Besides, other families, such as TRPY and TRPN were also identified in the non-mammalian species. Amongst these, TRPY is the most distant relative which is only present in yeast. It plays a role in the release of Ca^{2+} from the vacuoles when exposed to hyperosmotic conditions (Denis and Cyert, 2002; Venkatachalam and Montell, 2007). TRPN has also been reported but only in a limited number of vertebrates such as xenopus and zebrafish, where it serves as the mechanosensitive channel for performing the normal physiological function (Hardie, 2007). However, these channels bear little relevance to human disease and hence have not been discussed in greater detail. Structurally all the TRP channels form six transmembrane helixes with both the N and C terminal located within the cytoplasm. These channels are activated in response to a diverse range of physiological and sensory stimuli which include mechanical, thermal, electrical and chemical cues (Steinberg et al., 2014). Therefore, these TRP channels are involved in the sensation of touch, vision, olfaction, temperature and nociception (Voets et al., 2005). Moreover, these TRPs exhibit a widespread expression in the brain areas involved in pathophysiology of PD, making them a very relevant molecular target for the discovery of new drugs to treat PD (**Table 1**). The detailed role of TRP channels has been discussed in the further sections.

CONTRIBUTION OF TRP CHANNELS TO PD

TRPV Involvement in PD

TRPV channels are abundantly expressed in several areas of the brain. Although different members of the TRPV subfamily perform a very diverse physiological role, all of these have modest permeability toward Ca^{2+}. It makes them important for various central nervous system (CNS) functions, including learning and memory (Thapak et al., 2020a). It has therefore been reported that any change in the expression of TRPV channels contributes toward CNS disease pathologies such as AD, PD, cerebral ischemia, depression and anxiety (Bashiri et al., 2018; Thapak et al., 2020c).

Amongst the members of the TRPV family, TRPV1/4/5 have been studied for their involvement in PD pathology. TRPV1 is expressed in the regions, striatum and substantia nigra, which are known to be affected in PD (Musella et al., 2009; Nam et al., 2015). However, its role remains controversial pertaining to modulation of different downstream signaling pathways by it. Though an upregulation of TRPV1 has been reported by several authors in different PD models, both agonists and antagonists targeting

TABLE 1 | Expression of the different TRP channels in the regions involved in the pathophysiology of PD.

S No.	TRP channel	Substantia nigra pars compacta	Striatum	Subthalamic nuclei	Substantia nigra pars reticulata	References
1	TRPV1^{c}	+	+	+	nd	Roberts et al., 2004; Musella et al., 2009; Nam et al., 2015
2	TRPV2^{*nk}	nd	+	nd	nd	Kunert-Keil et al., 2006; Shibasaki et al., 2013
3	TRPV3^{nk}	+	+	nd	nd	Xu et al., 2002; Guatteo et al., 2005
4	TRPV4rd	+	+	nd	nd	Guatteo et al., 2005; Tjaša et al., 2016
5	TRPV5^{*nk}	nd	nd	nd	+	Kumar et al., 2017
6	TRPV6^{nk}	+	+	nd	+	Lehen'kyi et al., 2012
7	TRPM1^{nk}	nd	nd	nd	nd	Fonfria et al., 2006
8	TRPM2^{id}	+	+	+	+	Fonfria et al., 2005; Lee et al., 2013; Yu et al., 2014
9	TRPM3^{*nk}	nd	nd	nd	nd	Lee et al., 2003
10	TRPM4^{nk}	+	+	nd	nd	Mrejeru et al., 2011; Chen et al., 2019
11	TRPM5^{*nk}	nd	nd	nd	nd	Kunert-Keil et al., 2006
12	TRPM6^{*nk}	nd	nd	nd	nd	Kunert-Keil et al., 2006
13	TRPM7^{id}	+	+	nd	nd	Fonfria et al., 2005; Yu et al., 2014
14	TRPM8^{nk}	+	nd	nd	nd	Ordás et al., 2019
15	TRPC1rd	+	+	+	+	Martorana et al., 2006; Sun et al., 2017
16	TRPC2^{nk}	nd	nd	nd	nd	
17	TRPC3rd	+	+	+	+	Chung et al., 2007
18	TRPC4^{nc}	+	+	+	+	Chung et al., 2007
19	TRPC5^{nc}	+	+	+	+	Chung et al., 2007
20	TRPC6^{nc}	+	+	+	+	Chung et al., 2007
21	TRPC7^{nk}	+	+	+	+	Chung et al., 2007
22	TRPML1^{*nk}	nd	nd	nd	nd	Anglade et al., 1997; Dong et al., 2008; Grimm et al., 2012
23	TRPML2^{nk}	nd	nd	nd	nd	
24	TRPML3^{nk}	nd	nd	nd	nd	
25	TRPA1^{nk}	nd	+	nd	nd	Bodkin et al., 2014
26	TRPP1^{nk}	nd	nd	nd	nd	
27	TRPP2^{nk}	nd	nd	nd	nd	
28	TRPP3^{nk}	nd	nd	nd	nd	

*$^{+}$Expression is this region has been determined. ndExpression in this region has not yet been determined. *Expression in basal ganglia has been characterized but individual distribution within basal ganglia has not been reported in much detail. ^{c}There is a controversy related to whether the expression is increased or reduced in PD. ^{nk}Change of expression in PD has not yet been studied. ^{id}Expression is known to be increased in PD. rdExpression is known to be reduced in PD. ^{nc}No change of expression in PD.*

these have been successful in alleviating symptoms of disease in the preclinical stages (**Figure 1**) (Nam et al., 2015; Li et al., 2019).

Studies related to other TRPV channels like TRPV4/5 have also been carried out but remain rather limited. Expression of TRPV4 was downregulated in SNpc and striatum of rats after 6-OHDA administration suggesting a possible correlation between TRPV4 expression and neuronal death (Tjaša et al., 2016). Similarly, there is not much data to support the involvement of TRPV5 channels in PD. However, a transcriptome analysis suggested its possible role in the regulation of the pathophysiological hallmarks of PD in 6-OHDA treated rats (Li et al., 2019). Nevertheless, the lack of enough data and the absence of any pharmacological study targeting these channels have denied them a concrete claim as a possible drug target for PD.

TRPM Involvement in PD

TRPM channel subfamily has been extensively explored for its potential involvement in pathophysiology of several neurodegenerative disorders including PD (Ostapchenko et al., 2015; Sun et al., 2018b; Thapak et al., 2020b). It has been reported that the members of the TRPM subfamily lead to an influx of monovalent and divalent cations inside the cell which might serve as a contributing factor for the excitotoxicity mediated neuronal death (Adhya and Sharma, 2019).

Amongst these, TRPM2 channels have gained importance for their involvement in several mechanisms related to neuronal death. These channels are activated in response to oxidative stress and adenosine diphosphate ribose (ADPR), both of which are increased in PD. Though the permeability ratio of TRPM2 for Ca^{2+} to Na^{+} (PCa:PNa) is nearly 0.7 indicating a higher permeability for Na^{+}, a substantial Ca^{2+} influx also takes place because of the longer opening time of TRPM2 channels (Belrose and Jackson, 2018). The increased Ca^{2+} levels as a result of TRPM2 activation lead to excitotoxicity and contribute toward the aggravation of the underlying PD pathology.

Apart from TRPM2, TRPM7 has also been investigated for its involvement in PD. Genetic variants of TRPM7 have been

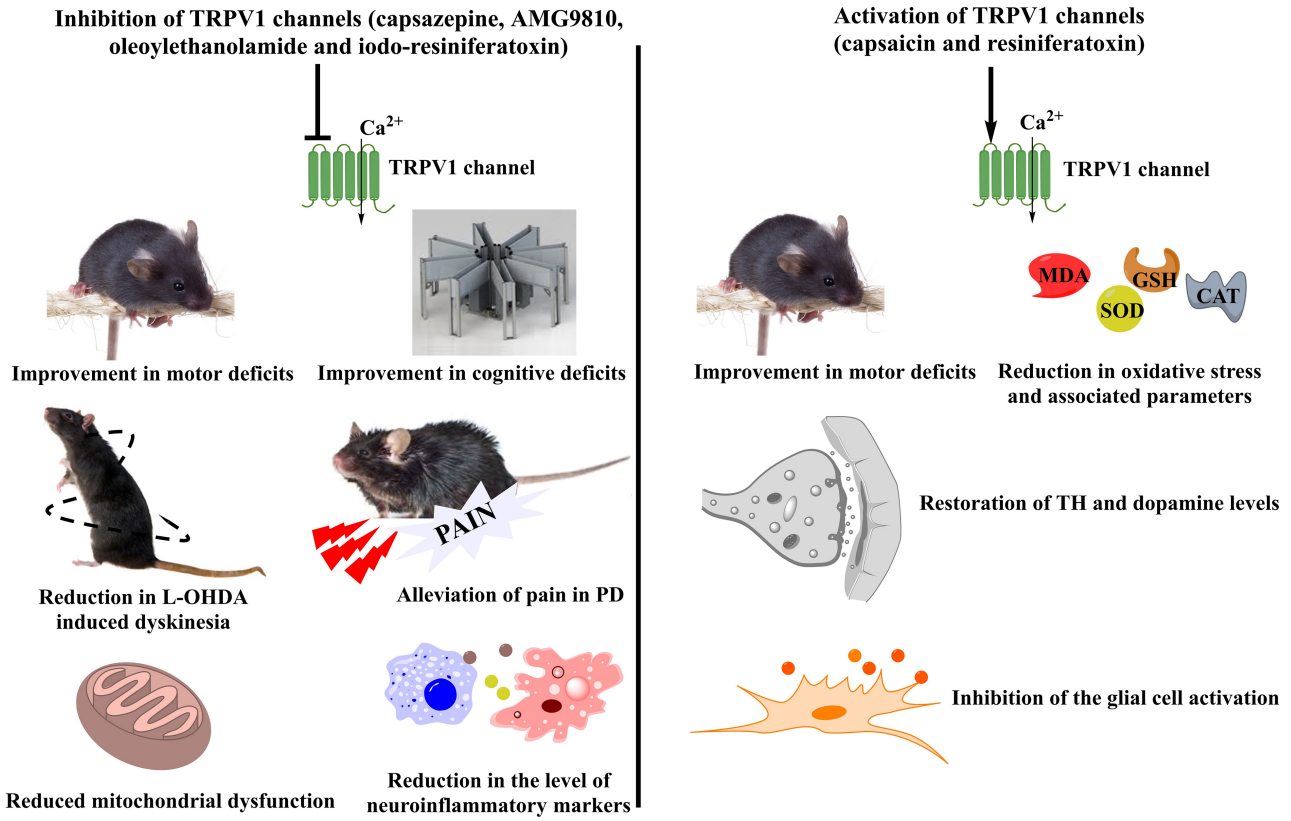

FIGURE 1 | Controversial role of TRPV1 channels in PD. A number of TRPV1 modulators have been tested for their beneficial effects in PD. Different agents working either via TRPV1 inhibition or TRPV1 activation have been studied for the mitigation of PD symptoms in preclinical models. Inhibition of TRPV1 led to an improvement in the motor and cognitive deficits of PD accompanied by reduced mitochondrial dysfunction and decreased levels of inflammatory mediators. Moreover, TRPV1 inhibitors also showed a reduction in the 6-OHDA induced dyskinesias and provided relief from pain which is one of the major non-motor symptoms of PD. On the other hand, TRPV1 activation had similar effects on improvement of motor coordination. It also reduced the levels of oxidative stress and led to the restoration of the tyrosine hydroxylase and dopamine levels. Furthermore, a significant reduction in the microglial activation was also observed after the administration of TRPV1 activators. TRPV1, Transient Receptor Potential Vanilloid 1; PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; TH, Tyrosine hydroxylase; MDA, Malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; CAT, catalase.

linked to the ionic dyshomeostasis, mitochondrial dysfunction, inflammation and increased oxidative stress, all of which led to its identification as a candidate susceptibility gene for familial PD (Hermosura et al., 2005; Hermosura and Garruto, 2007). In addition, PC-12 cells exposed to 6-OHDA also showed an increased expression of TRPM7, which was reduced after overexpression of miR-22. Furthermore, miR-22 overexpression also protected from the effects of TRPM7 upregulation by inhibition of apoptosis, reduction of ROS and improved cell viability (Yang et al., 2016). Despite these findings, there are other controversial reports which have observed neuroprotective effects of the TRPM7 mediated Mg^{2+} influx in the MPP^{+} based *in vitro* model (Shindo et al., 2015). Hence, more studies with selective TRPM7 modulators are required to conclusively elucidate the role of these channels in PD.

TRPM8 is another candidate belonging to the TRPM subfamily which has been identified as a risk factor for pain in PD (Williams et al., 2020). Pain is a frequently occurring non-motor symptom, which has been reported in about 86% of the PD patients (Beiske et al., 2009; Skogar and Lokk, 2016). Therefore, strategies targeting TRPM8 channels could be utilized in future for the management of pain in PD patients.

In totality, these findings suggest that TRPM channels are critical molecular players for neuronal death in PD (**Figure 2**). However, the dearth of information related to their signaling pathways has largely limited the development of selective modulators for these channels in the PD models.

TRPC Involvement in PD

TRPC subfamily has a widespread distribution in the CNS. It was also shown to exhibit high expression in the areas of SNpc and striatum, both of which are involved in the pathophysiology of PD (Sukumaran et al., 2017). As a result, different members of the TRPC subfamily have been investigated for their potential involvement in PD. TRPC1 was the first member of the TRPC subfamily to be identified in mammals. It is localized to the dendritic processes and co-expressed with tyrosine hydroxylase (TH) in the dopaminergic neurons of the SNpc (Martorana et al., 2006). This pointed out at the physiological relevance of TRPC1 channels in the dopaminergic

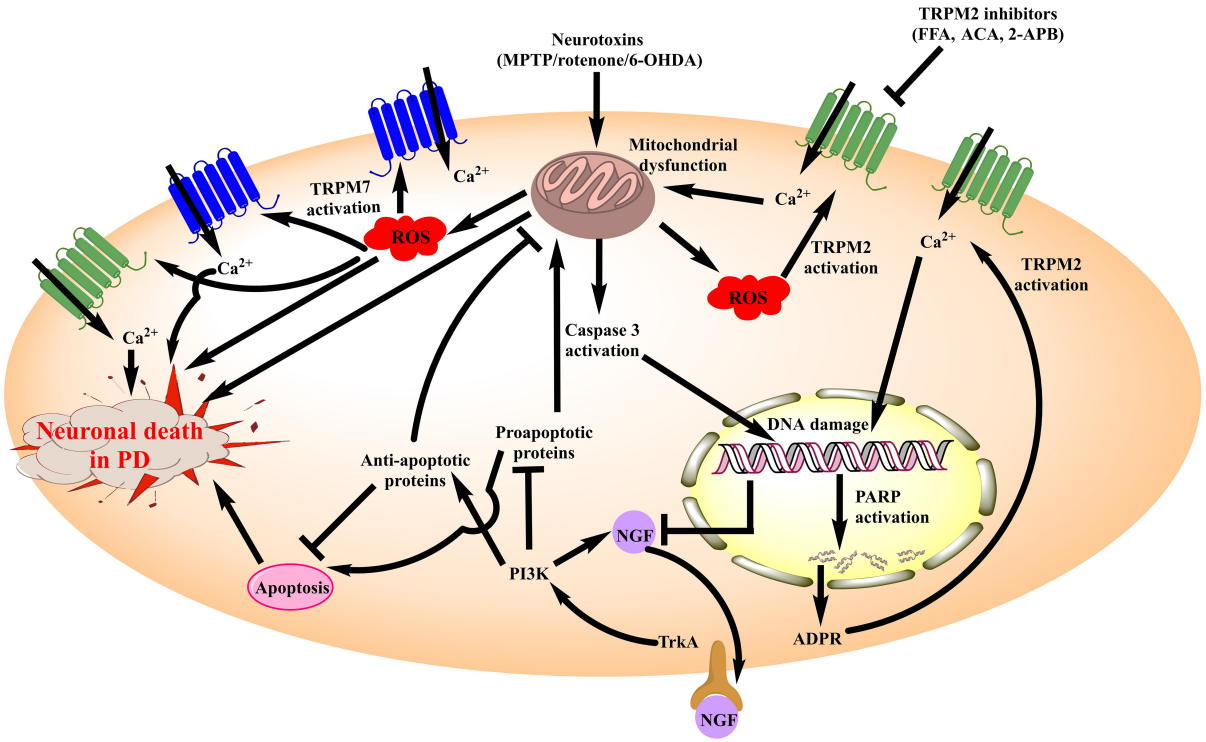

FIGURE 2 | Schematic diagram illustrating the role of TRPM channels in neuronal death associated with PD. Amongst the TRPM channels, TRPM2 and TRPM7 have been elucidated for their involvement in PD. Neurotoxins such as MPTP, 6-OHDA and rotenone inhibit the mitochondrial complexes producing mitochondrial dysfunction. It generates ROS which acts as an activator of both TRPM2 and TRPM7 leading to the increased Ca^{2+} influx within neurons. These events in totality increase the neuronal death in PD besides aggravating mitochondrial dysfunction. Moreover, cytochrome c (Cyt c) is released from the mitochondria in this process which promotes pro-apoptotic signaling and damages DNA. Damage to the DNA leads to poly ADP ribose polymerase (PARP) mediated ADPR formation which is also an activator of TRPM2 channel. Therefore, the cascade of events exhibits a positive feedback loop which aggravates neuronal death. Besides, there is a reduced expression of NGF and associated TrkA/PI3K signaling, which intensifies the pro-apoptotic response and plays its part in the TRPM2 and TRPM7 mediated neuronal death in PD. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxy dopamine; ROS, reactive oxygen species; FFA, Flufenamic acid; ACA, *N*-(*p*-amylcinnamoyl) anthranilic acid; 2-APB, 2-Aminoethoxydiphenyl borate; TRPM, Transient receptor potential melastatin 2; NGF, Nerve growth factor; PARP, poly ADP ribose polymerase; PI3K, phosphoinositide 3-kinase; ADPR, Adenosine di-phosphate ribose; TrkA, Tropomyosin receptor kinase A.

system and raised the possibility that these channels may contribute toward the pathogenesis of PD. Studies carried out using the MPP^+/MPTP model showed that the expression of TRPC1 was reduced in both the SNpc as well as PC12 neuronal cells in PD. Besides, $TRPC1^{-/-}$ mice also reported motor deficits and increased Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the basal ganglia suggesting the critical role of TRPC1 in regulating apoptotic signaling within the cell (He et al., 2016). Similar results were observed in other studies where TRPC1 overexpression protected from the effects of 6-OHDA, MPP^+ and α-synuclein toxicity in the neuronal cells (Bollimuntha et al., 2005; Sukumaran et al., 2018).

Several groups then looked at the downstream signaling associated with the neuroprotection offered by TRPC1 channels in PD models (Selvaraj et al., 2012). One of the possible mechanisms for this was the reduced endoplasmic reticulum (ER) stress which is otherwise increased in PD (Deng and Bai, 2012). It was shown that overexpression of TRPC1 channels in the SH-SY5Y cells treated with MPP^+ led to the restoration of phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (Akt/mTOR) signaling and increased dopaminergic neuronal survival. Interestingly, similar findings were reported in the brains of the PD patients and $TRPC1^{-/-}$ mice which further showed an increased unfolded protein response (UPR) and reduced number of dopamine neurons in the brain (Selvaraj et al., 2012). On the other hand, TRPC1 deletion mediated ER stress was found to be associated with increased ROS, dysregulation of the glucose-regulated protein 78 (GRP78) and alteration in protein kinase RNA-like ER kinase (PERK) signaling pathways (Wang et al., 2018). In addition, potential crosstalk between the TRPC1-Stromal interaction molecule (STIM) and L-type calcium channels was also established using an electrophysiological study in the SNpc of the $TRPC1^{-/-}$ mice (Sun et al., 2017). As L-type calcium channel blockers like isradipine have already gone into clinical trials for PD, TRPC1 performing the same physiological function in dopaminergic neurons could be looked at as a molecular target in PD (Simuni et al., 2010; Sun et al., 2017). Consistent with these results are the findings of Sun et al. (2018a), where MPP^+ was found to reduce TRPC1 expression and store-operated calcium entry in the mesenchymal stem cell-derived dopaminergic neurons.

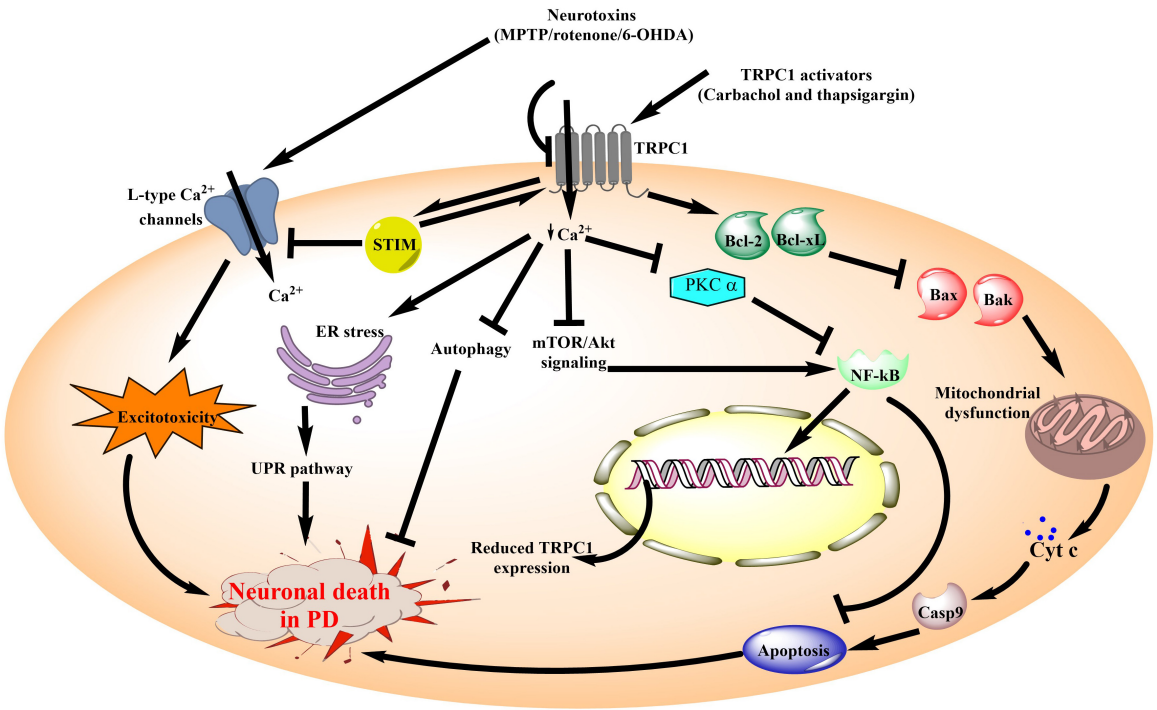

FIGURE 3 | Schematic diagram illustrating the role of TRPC1 channels in neuronal death associated with PD. TRPC1 channel is the most widely studied member of the TRPC subfamily for its involvement in PD. Ca^{2+} influx through TRPC1 channels is known to be neuroprotective in the PD models. Additionally, review of literature suggests that inhibition of Ca^{2+} influx from TRPC1 by the administration of neurotoxins causes aggravation of the PD pathology. It leads to inhibition of mTOR/Akt signaling as well as reduced levels of PKC α. These events reduce NF-κB expression which in turn increases the DNA damage and pro-apoptotic signaling to compromise neuronal survival. On the other hand, TRPC1 activation directly promotes anti-apoptotic signaling to reduce mitochondrial dysfunction and associated cell death in PD. In addition, diminished Ca^{2+} influx from the TRPC1 channels results in impaired autophagy and ER stress. Moreover, in association with STIM, TRPC1 channels inhibit the Ca^{2+} influx via L-type calcium channels which are one of the major contributors toward excitotoxicity mediated neuronal death in PD. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxy dopamine; TRPC1, Transient Receptor Potential Canonical 1; ER, endoplasmic reticulum; STIM, Stromal interaction molecule; UPR, unfolded protein response; mTOR, mammalian target of rapamycin; Akt, Protein kinase B; PKC, Protein kinase C; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Cyt c, cytochrome c; Casp9, caspase 9; Bcl2, B-cell lymphoma 2; Bcl-xl, B-cell lymphoma-extra large; Bax, Bcl2-associated X protein; Bak, Bcl-2 homologous antagonist/killer.

Other pathways that were affected in PD due to reduced TRPC1 expression include attenuation of autophagy as well as impairment of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Tropomyosin receptor kinase A (TrkA) signaling (Yu et al., 2013; Sukumaran et al., 2018). In contrast to these findings, downregulation of STIM, a TRPC1 activator was shown to prevent oxidative stress and apoptosis mediated neuronal death in 6-OHDA treated PC-12 cells (Li et al., 2014; Sukumaran et al., 2017). However, despite this discrepancy, most of the other studies have suggested a beneficial role of TRPC1 channels in the pathophysiology of PD are shown in **Figure 3**.

Other TRPC channels such as TRPC3 are also expressed in the SNpc and striatal regions of the brain. These are important for the regulation of firing intensity and neuronal depolarization in these brain regions (Zhou et al., 2008; Xie and Zhou, 2014). Additionally, these channels are also required for synaptic transmission and motor coordination, making them an important target for investigation in the context of PD (Hartmann et al., 2008). Initial observation though suggested little to no change in the TRPC3 expression in PD, studies carried out much later have suggested otherwise (Selvaraj et al., 2009, 2012; Yu et al., 2013; Streifel et al., 2014). In the 6-OHDA model, data from RT-PCR studies showed reduced expression of TRPC3 in the SNpc (Yu et al., 2013). Similar results were reported by other groups which revealed that the inhibitory effects of neurotoxins, MPP^{+} and 6-OHDA on TRPC3 was mediated through alteration in the purinergic calcium signaling (Lei et al., 2008; Streifel et al., 2014). However, drugs targeting TRPC3 have shown neuroprotective effects further challenging the role of molecular mechanisms related to TRPC3 in PD.

Besides the channels discussed in detail, most studies have invariably found no change in the expression of other TRPC channels including TRPC4/5/6 in different PD models as well as patients (Selvaraj et al., 2012; Lortet et al., 2013; Sukumaran et al., 2018). But, their ability to regulate the influx of calcium and other cations into the cell makes these channels an attractive candidate for studies in future.

TRPML Involvement in PD

TRPML channels are non-selective cation channels localized intracellularly on the endosomes and lysosomes. These are

responsible for the intracellular calcium influx after activation by different cellular pathways (Grimm et al., 2012). Amongst the members of TRPML subfamily, mutations in TRPML1 have also been linked to the lysosomal storage disorder, mucolipidosis type IV (MLIV) which is also a form of neurodegeneration. It is known to produce motor and cognitive deficits, similar to those seen in PD patients (Dong et al., 2008). Moreover, the role of TRPML1 in the autophagy-related signaling has also been elucidated, making it an important channel for cell survival. Although there is no direct evidence as of now for its involvement, it has been hypothesized that TRPML1 activation could restore the impaired autophagy seen in PD patients (Anglade et al., 1997). Furthermore, mutations in GBA gene, which codes for lysosomal enzyme glucocerebrosidase is one of the potential risk factors for PD and has also been known to cause lysosomal dysfunction (DePaolo et al., 2009). Therefore, there are several studies currently underway to investigate the possible role of TRPML channels in PD (The Michael J. Fox Foundation for Parkinson's Research, 2019[1]; The Michael J. Fox Foundation for Parkinson's Research, 2020[2]). These studies are expected to provide deeper insights into the pathophysiology of PD and help in the identification of new molecules which could be useful for PD management.

[1]https://www.michaeljfox.org/grant/assessing-trpml1-agonists-gba-parkinsons-disease

[2]https://www.michaeljfox.org/grant/development-novel-trpml1-activator-parkinsons-disease

TRPA Involvement in PD

TRPA channel subfamily remains relatively unexplored in the context of PD. Though there is no study pertaining to its direct involvement, agents acting on these channels have provided important clues related to the development of side effects associated with current PD therapy. These have been discussed ahead in much more detail.

POTENTIAL PHARMACOLOGICAL INTERVENTIONS TARGETING TRP CHANNELS IN PD

A number of pharmacological agents have shown protective effects across different *in vitro* and *in vivo* studies. Detailed investigations pertaining to the beneficial effect of these agents has been discussed ahead and in **Table 2**.

TRPV1

A number of TRPV1 modulators have been studied by different groups in PD models. These include TRPV1 agonists (capsaicin and resiniferatoxin) as well as TRPV1 antagonists (capsazepine, AMG9810, oleoylethanolamide and iodo-resiniferatoxin). These agents exerted beneficial effects in the PD models. Capsaicin is an active phytochemical present in several *Capsicum* spp. which is highly selective toward TRPV1 channels (EC_{50} = 0.71 μM). It has been studied extensively for its disease-modifying effects

TABLE 2 | TRP channel modulators which accorded neuroprotection in the different PD models.

S No	TRP channel	Model	Pharmacological interventions	Major outcome	References
1	TRPM2	MPP^+	Flufenamic acid and *N*-(*p*-amylcinnamoyl) anthranilic acid	• Inhibition of ROS production and apoptosis • Increased neuronal cell viability by TRPM2 inhibition *in vitro*	Sun et al., 2018b
2	TRPM2	Rotenone	Flufenamic acid and 2-Aminoethoxydiphenyl borate	Inhibition of oxidative stress	Nazıroğlu et al., 2011
3	TRPV1	MPTP and 6-OHDA	Capsaicin	• Reduction in microglial activation, inflammation and oxidative stress • Improvement in neurobehavioral parameters and restoration of TH levels in the dopaminergic neurons	Chung et al., 2017; Zhao et al., 2017
4	TRPV1	Drosophila fly mutants expressing human α-synuclein	Capsaicin	• Reduction in oxidative stress and restoration of dopamine levels • Improvement in climbing ability	Siddique et al., 2012, 2018
5	TRPV1	6-OHDA	AMG9810	• Reduced neuronal death • Improved performance in the rotarod test	Razavinasab et al., 2013
6	TRPV1	6-OHDA	Capsazepine and Oleoylethanolamide	• Reduction in L-DOPA induced dyskinesias through crosstalk with endocannabinoid signaling	Morgese et al., 2007; González-Aparicio and Moratalla, 2014
7	TRPV1	AAV-A53T knock in mouse	Resiniferatoxin	• Improvement in motor function • No change in the dopamine levels	Sorrento Therapeutics Inc, 2020
8	TRPC1	MPP^+/MPTP	Carbachol and thapsigargin	• Accorded neuroprotection by increased AKT1 phosphorylation	Selvaraj et al., 2012
9	TRP3/6/7	MPP^+	SKF-96365	• Reduction of intracellular Ca^{2+} overload by reducing the overexpression of post synaptic scaffolding protein Homer1	Chen et al., 2013

in PD (Caterina et al., 1997; Musfiroh et al., 2013). It accorded neuroprotection in both the *in vitro* and *in vivo* rodent systems as well as in the drosophila model of PD. Capsaicin was further shown to improve rotarod performance and led to the restoration of dopaminergic signaling in the MPTP treated mice. Additionally, it also prevented glial cell activation and reduced oxidative stress in the astrocytes of MPTP induced PD mice. These effects were reversed after treatment with TRPV1 antagonists (capsazepine and iodo-resiniferatoxin) which confirmed that the beneficial effects were indeed attributed to the activation of TRPV1 channels (Chung et al., 2017). Similar observations were made in a study by Nam et al., which demonstrated that neuroprotective effects of capsaicin on TRPV1 were mediated through endogenous ciliary neurotrophic factor (CNTF) levels and CNTF-α receptors (Nam et al., 2015). It has been shown that the symptoms of PD first appear after 60–70% of the dopaminergic neuronal death has already taken place (Cheng et al., 2010). In a study, capsaicin was found to be capable of restoring the dopaminergic function even after the complete injury to the substantia nigra pars compacta (SNpc) and striatum (Kim et al., 2019). These results suggest the possibility for capsaicin to be effective in the later stages of PD when traditional medications fail to work. Besides, capsaicin also exerted a positive modulatory effect against oxidative insults and reversed the behavioral deficits induced by MPTP and 6-OHDA in the rodent models (Fong et al., 2016; Zhao et al., 2017). Capsaicin treated animals showed an increase in distance traveled in the open field and a significant reduction in the amphetamine-induced rotations after induction of PD. It was accompanied by a significant reduction in the malondialdehyde (MDA) levels and restoration of the superoxide dismutase (SOD) and catalase activity in the SNpc (Nam et al., 2015; Zhao et al., 2017).

The neuroprotective effects of capsaicin were further replicated in the drosophila fly mutants, which expressed a gene for human α-synuclein. These PD flies displayed a progressive form of neurodegeneration characterized by loss of dopamine function, oxidative stress and impaired performance in the climbing assay. Reversal of the pathological changes occurred in a dose-dependent manner which confirmed the therapeutic potential of capsaicin through TRPV1 activation for PD treatment (Siddique et al., 2012, 2018).

In contrast to these studies, other groups have shown that blockade of TRPV1 channels is also helpful in relieving some of the PD symptoms. A study involving the use of AMG9810, a selective TRPV1 blocker demonstrated a positive attenuation of the motor deficits after 6-OHDA administration which was evident from the rotarod test. Additionally, cresyl violet staining also showed that there was reduced neuronal death in the SNpc of the AMG9810 treated animals (Razavinasab et al., 2013). In line with the same, another TRPV1 blocker, capsazepine also exhibited neuroprotective effects in a number of PD models. Moreover, it was also found to reduce the occurrence of levodopa (L-DOPA) induced dyskinesia which is a prominent side effect seen with L-DOPA therapy in PD (Morgese et al., 2007). The most probable explanation for these effects came from a study where it was observed that TRPV1 blockade positively regulates the crosstalk between endocannabinoid receptors and TRPV1 downstream signaling which helps prevent L-DOPA induced dyskinesias (Lee et al., 2006; Giuffrida and Morgese, 2007; Morgese et al., 2007; Dos-Santos-Pereira et al., 2016). It is also supported by the findings in which oleoylethanolamide, another TRPV1 blocker showed protection in the 6-OHDA induced hemiparkinsonian model (González-Aparicio and Moratalla, 2014). Furthermore, blockade of TRPV1 has also been elucidated to play a crucial role in the modulation of pain pathways in PD (Li et al., 2020). TRPV1 expression was upregulated in the trigeminal subnucleus caudalis (Vc) which plays a role in central terminal sensitization of primary nociceptive neurons. In addition, its co-expression with 5-HT3A receptors in the rat dorsal root ganglion (DRG) and ability of TRPV1 blockers to inhibit the pain induced by 5-HT3A receptor agonists makes it a useful target for pain modulation in PD (Zeitz et al., 2002; Li et al., 2020).

Despite these promising results, the therapeutic window for the beneficial effects of TRPV1 modulators is very narrow and higher doses of TRPV1 blockers such as AMG9810 have been associated with learning and memory impairments in the preclinical studies (Razavinasab et al., 2013). Moreover, its interaction with endocannabinoids raised concern over the possible addictive potential and development of tolerance for these agents. However, when investigated, no such association was observed in studies involving TRPV1 blockers which makes them a viable option for studies in future (González-Aparicio and Moratalla, 2014).

In totality, these shreds of evidence suggested that targeting TRPV1 channels could be of therapeutic relevance for combating the motor and non-motor symptoms of PD. Therefore, to provide more proof to the speculation, Capsaicin is being investigated in patients for the elucidation of mechanisms related to swallowing and cough dysfunction in PD[3]. Another TRPV1 agonist, resiniferatoxin was shown to be effective in controlling the motor symptoms in the AAV-A53T knock-in PD mouse model (Sorrento Therapeutics Inc, 2020). According to the recent reports, it is soon expected to enter clinical trials which could open up new avenues of research for the management of PD.

Together, these studies capture a basic insight into the pharmacological modulation of TRPV channels which could be targeted in future for the treatment of PD. But, as the doubts over the clear molecular mechanisms, clinical efficacy and specificity persist, more detailed investigations are still necessary for the development of TRPV channel modulators as drug candidates in future.

TRPM2

Studies conducted in preclinical models as well as PD patients, showed an increase in TRPM2 expression, suggesting its possible involvement in PD (Sun et al., 2018b; An et al., 2019). It was also demonstrated that MPP^+ exposure leads to increased levels of TRPM2 accompanied by elevated oxidative stress and increased apoptosis in the SH-SY5Y neurons. It further resulted in reduced cell viability and caspase 3 activation in these neuronal cells (An et al., 2019; Ding et al., 2019). These effects were reversed by treatment with TRPM2 blockers, flufenamic acid (FFA) and *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA). Moreover, similar results were obtained when knockdown of

[3]https://clinicaltrials.gov/ct2/show/NCT03321019

TRPM2 was carried out using a siRNA which resulted in reduced neuronal death and inhibition of apoptosis in the SH-SY5Y cells (Sun et al., 2018b). The detailed mechanism of this neuroprotection was delineated later in another study which attributed it to the working network of long non-coding RNA, p21 and microRNA, miR-625 present inside the neurons. It was also reported that long non-coding RNA, p21 is a positive modulator of TRPM2 expression and its knockdown could rescue from the toxicity induced by MPP^+ treatment (Ding et al., 2019).

Other authors have also confirmed the involvement of TRPM2 in PD with the help of different toxin-based models *in vitro* and *in vivo*. In a study by Yu et al. (2014) it was shown that TRPM2 expression was significantly increased in the SNpc of 6-OHDA treated rats. This increased expression was attenuated by treatment with nerve growth factor, which accorded neuroprotection through phosphatidylinositol 3-kinase (PI3K) signaling pathway (Yu et al., 2014). Furthermore, it was demonstrated that TRPM2 mediated increased ROS production and caspase 3 activity was suppressed by increased expression of transcription factor GATA3 binding protein (GATA3) (Zhou and Han, 2017). This suggests the involvement of multiple downstream pathways affected by TRPM2 associated neuronal death in PD. In line with these shreds of evidence, rotenone also led to increased TRPM2 activation by ROS dependent mechanisms (Freestone, 2009). These effects were reversed after treatment with TRPM2 blockers FFA and 2-aminoethoxydiphenyl borate (2-APB), which confirmed the potential of TRPM2 blockade in the treatment of PD (Nazıroğlu et al., 2011). Furthermore, electrophysiological findings have revealed that increased spontaneous firing of the substantia nigra pars reticulata (SNr) GABAergic neurons seen in PD is modulated by increased activation of the TRPM2 channels (Lee et al., 2013). Conclusively, the close association of TRPM2 and intracellular ionic homeostasis has been established and any change in it makes the individual more susceptible toward PD (Hermosura and Garruto, 2007). Though there is ample evidence available for its involvement, there is no report which has investigated the effect of pharmacological interventions targeting TRPM2 *in vivo*. This has largely hindered the development of more potent analogs which could be tried for their translational potential of TRPM2 in PD. Further, the neuroprotective potential of TRPM2 antagonists in neurodegenerative disorders like AD strengthens the claim of TRPM2 antagonists as neuroprotective agents (Thapak et al., 2020b).

TRPC1

Silencing of TRPC1 as well as its functional blockade was also associated with mitochondrial dysfunction and decrease in the TH levels after sub-chronic administration of MPTP to the C57BL/6 mice. These pathological changes were reversed after treatment with TRPC1 activators carbachol and thapsigargin or after TRPC1 overexpression which led to an increase in anti-apoptotic signaling (Selvaraj et al., 2009). Similar results were observed in other studies involving transgenic or toxin model-based systems (Bollimuntha et al., 2005; Sukumaran et al., 2018).

TRPC3/6/7

Studies have either reported reduced or no change in the expression of TRPC3/6/7 channels in PD (Selvaraj et al., 2009, 2012; Yu et al., 2013; Streifel et al., 2014). However, the neuroprotective effects of SKF-96365, a non-selective TRPC3/6/7 blocker in the MPP^+ model suggests a need to validate these findings (Chen et al., 2013). Therefore, challenging the reproducibility of these observations in other model systems could help resolve these concerns in the near future.

TRPA1

Expression of TRPA channels has not been directly correlated to the pathophysiology of PD. However, it was observed that apomorphine which is an approved medication for PD is a bimodal modulator of TRPA1 channels, the only known member of the TRPA subfamily (Schulze et al., 2013). Apomorphine is often used as an add-on therapy for the management of "off" episodes in PD but exhibits adverse effects like nausea and local reactions at the injection site (Carbone et al., 2019). Schulze et al. (2013) demonstrated with the help of cultured dorsal route ganglion (DRG) neurons and in the enterochromaffin model cell line QGP-1, that these adverse effects might be attributed to the activation of TRPA1 channels by apomorphine. Therefore, strategies targeting TRPA1 channels could be useful for the management of side-effects associated with apomorphine and other currently available medications for PD.

CHALLENGES IN THE DEVELOPMENT OF TRP CHANNEL MODULATORS AS DRUG CANDIDATES

TRP channels are involved in the regulation of a number of physiological processes in different organs of the body. Though alteration of their functions in some of the brain areas may be involved in the pathophysiology of PD, targeting these alterations in a selective and specific manner poses a greater challenge. This was evident during the studies of TRPV1 modulators in other conditions like pain where a number of clinical trials were terminated because of the off-target effects (Krarup et al., 2011; Rowbotham et al., 2011). Encouraging results obtained in rodent studies with these agents couldn't be replicated in patients due to a number of side effects like a diminished response to damaging heat, alteration in body temperature and reduced perception of taste were observed (Gavva et al., 2008). Therefore, management of safety and toxicity still remain the biggest hurdle for the translational success of TRP channel modulators. Another largely neglected area of research is the organellar distribution of TRP channels where they are coupled together to perform a plethora of unknown functions (Zhang et al., 2018). Besides, a report has also highlighted that the neuroprotection accorded by these agents is sex-specific which adds further complexity to the clinical development of these agents (Jia et al., 2011). Additionally, a number of agonists and antagonists of the same TRPs like TRPV1 and TRPM8 are together being investigated in the clinical trials which tell us that there is a lot more to the story than what we understand at present (Moran et al., 2011;

De Caro et al., 2019; Garami et al., 2020). This further raises the possibility of these modulators to be working through other unknown mechanisms that have not been investigated till date. To develop the understanding, several transgenic mice (TRP knock in and knock out) were made, but high embryonic or postnatal lethality has largely limited their use. This has further pushed back the progress of this field and necessitated the use of alternative approaches (Park et al., 2011; Woudenberg-Vrenken et al., 2011). Another pitfall in TRP research is the lack of selective agents as activators or inhibitors for any of the ion channel. As the structure and function of TRP channels are conserved across species, preclinical and animal data using selective agents could be highly useful for the further development of these agents (Hof et al., 2019). Therefore, increased knowledge of the TRP structure is also required and needs to be studied with the help of new approaches.

Despite these limitations, there are a number of clinically approved agents like probenecid and tranilast (TRPV2), glibenclamide (TRPM4), flufenamic acid and clotrimazole (TRPM2) and menthol (TRPM8) which have also been reported to modulate TRP channel activity. There are proof of concept studies which have found them to be effective in different CNS disorders (Thapak et al., 2020a; Zubov et al., 2020). Hence, it is possible that TRP channels may be indistinctly important for brain functions, and these molecules may pave the way for a completely new class of drugs for brain-related disorders including PD.

CONCLUSION

TRP channels perform a plethora of physiological functions in the CNS. The increased understanding of their role has led several researchers to investigate their likely contribution in the pathophysiology of neurodegenerative disorders, including PD. Moreover, their regulatory function in ionic homeostasis has made TRP channels an attractive pharmacological target where Ca^{2+} dyshomeostasis, oxidative stress and excitotoxicity mediated neuronal death are reported. The current review has presented a brief overview of the reports which have studied the pathophysiological involvement of TRP channels in PD. Additionally, beneficial effects of many of the pharmacological agents targeting TRP channels have also been highlighted, which suggests the possibility of a novel class of therapeutics for PD treatment in future. Amongst the agents known so far, ones targeting TRPV1, TRPM2 and TRPC1 have been most promising for PD treatment. Design of novel and selective analogs targeting these TRPs is expected to open up new avenues of research in future. Though these findings pertaining to the involvement of TRP channels in PD are encouraging, more studies highlighting the molecular mechanisms at length are required to ensure the translational success of these compounds in the clinical trials.

AUTHOR CONTRIBUTIONS

BV and SSS conceived the idea of the manuscript. BV wrote all the sections. SSS corrected the manuscript. Both authors approved the final proof for submission.

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REVIEW
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Resolvins: Potent Pain Inhibiting Lipid Mediators via Transient Receptor Potential Regulation

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Chronic pain is a serious condition that occurs in the peripheral nervous system (PNS) and the central nervous system (CNS). It is caused by inflammation or nerve damage that induces the release of inflammatory mediators from immune cells and/or protein kinase activation in neuronal cells. Both nervous systems are closely linked; therefore, inflammation or nerve damage in the PNS can affect the CNS (central sensitization). In this process, nociceptive transient receptor potential (TRP) channel activation and expression are increased. As a result, nociceptive neurons are activated, and pain signals to the brain are amplified and prolonged. In other words, suppressing the onset of pain signals in the PNS can suppress pain signals to the CNS. Resolvins, endogenous lipid mediators generated during the resolution phase of acute inflammation, inhibit nociceptive TRP ion channels and alleviate chronic pain. This paper summarizes the effect of resolvins in chronic pain control and discusses future scientific perspectives. Further study on the effect of resolvins on neuropathic pain will expand the scope of pain research.

Keywords: resolvins, TRP channel, pain, inflammatory, neuropathy

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INTRODUCTION

The International Association for the Study of Pain defines pain as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Raja et al., 2020). Pain is classified into three types according to its cause. First, the pain generated when individuals are exposed to noxious stimuli such as extreme temperatures or sharp objects, is called nociceptive pain. Generally, pain is considered to be a negative feeling that needs to be eliminated, but nociceptive pain is protective and essential. It protects us from being injured by a hostile environment (Scholz and Woolf, 2002; Woolf, 2010). Second, inflammatory pain is caused by activation of the immune system, and it is also protective. Inflammation following tissue injury or infection is characterized by releasing proinflammatory mediators including bradykinin, prostaglandins, nerve growth factors (NGF), proinflammatory cytokines, and chemokines from immune cells. These mediators bind to pain receptors expressed in nociceptors (Woolf, 2010; Lim et al., 2015). Pathological pain is not protective and results from abnormal functioning of the nervous system. This can be categorized into two types depending on the presence of damage; one

is neuropathic pain, which occurs after nerve damage; the other is dysfunctional pain, which can occur in the absence of inflammation or nerve damage (Woolf, 2010).

Inflammation and nerve damage can induce neural plasticity that results from changes in the nervous system and reduces the pain threshold. Therefore, inflammatory pain and neuropathic pain are characterized by peripheral nociceptor sensitization, arising from spontaneous nociceptor activity, hyperalgesia, and allodynia. Transient receptor potential (TRP) ion channels that are expressed in nociceptors are persistently activated by inflammatory mediators during the pathological state. TRPV1 and TRPA1 are notable nociceptive TRP ion channels, and activation of these channels can stimulate hyperexcitability of the peripheral nociceptors that transmit pain signaling to the central nervous system (CNS) (Scholz and Woolf, 2002; Patapoutian et al., 2009; Lim et al., 2015; Jardin et al., 2017; Mourot et al., 2018). Inflammatory pain includes pain from a surgical wound or an inflamed joint, while the etiology of neuropathic pain includes diabetes and chemotherapy-related peripheral neuropathy.

Resolvins (RVs) are lipid mediators that are biosynthesized from omega-3 polyunsaturated fatty acids (PUFAs) that are abundant in marine oil. Aspirin/cyclooxygenase-2 (COX-2) and other enzymes convert omega-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to resolvin D and E through multiple mechanisms (Serhan et al., 2002). These lipid mediators are generated during the resolution phase of acute inflammation and not only terminate inflammation but also alleviate pain (Ariel and Serhan, 2007; Xu et al., 2010). The Ji lab found that the resolvin series has an analgesic effect on various types of inflammatory pain models through regulation of TRP channels (Xu et al., 2010; Park et al., 2011b).

This review will highlight TRP channels, with an emphasis on TRPV1 and TRPA1, as therapeutic targets for the resolution of both inflammatory and neuropathic pain using resolvins. To address this, pain and TRP are first described prior to resolvins. At the end, the importance of further study on the role of resolvins in neuropathic pain via TRP regulation is also suggested.

CONNECTIVITY OF PERIPHERAL AND CENTRAL NERVOUS SYSTEMS IN PAIN PATHWAYS

Anatomical Pain Pathway

Primary afferent neurons are pseudounipolar neurons that have one axon from the soma and bidirectional branches at the axon terminals (Amir and Devor, 2003; Gibson and Ma, 2011). The branches project to the peripheral (target organ) and central projection (spinal cord), respectively (Gibson and Ma, 2011), and transduce sensory information (mechanical, thermal, and chemical) from the peripheral to the central nervous system (Hasegawa et al., 2007).

Primary afferent nerve fibers are classified by fiber diameter or myelination and project to distinct laminae in the dorsal horn of the spinal cord. Fiber diameter and myelination are related to conduction velocity. Myelinated Aα and Aβ fibers have a large diameter and fast conduction velocity. Rapidly conducting Aβ fibers conduct proprioception and touch sensations and project to deeper laminae: III, IV, and V. However, studies have revealed that some Aβ fibers also are related to pain regulation (Xu et al., 2015; Tashima et al., 2018; Nagi et al., 2019). While thinly myelinated Aδ fibers and unmyelinated C fibers both have relatively small diameters, the former have faster conduction velocities than do the latter. Aδ fibers conduct touch, pressure, and "first" pain signals to laminae I and V. Slowly conducting C fibers respond to heat and mechanical stimuli and conduct "second" pain related signals to lamina I (peptidergic C fibers) and lamina II (non-peptidergic C fibers) (Julius and Basbaum, 2001; Furue et al., 2004; Basbaum et al., 2009). Unmyelinated C fibers and thinly myelinated Aδ fibers are activated by high-intensity stimuli (Ji and Woolf, 2001). The activation of Aδ fibers evokes sharp and pricking pain, whereas the activation of C fibers evokes a burning sensation (Bishop, 1980).

Different pathways to the brain carry different types of sensory information. Pain and heat signals from C and Aδ fibers in the superficial dorsal horn cross the midline and ascend the spinal cord. Then, sensory information is carried to the brain through the lateral spinothalamic tract (Melzack and Wall, 1962; Basbaum et al., 2009). On the other hand, proprioception and touch signals do not cross the spinal cord but ascend to the brain through the anterior spinothalamic tract (Basbaum et al., 2009).

Central Sensitization

Central sensitization involves the increased responsiveness of nociceptive neurons in the CNS that can be generated from intense and repeated peripheral inputs. Sensitized C-fibers lead to the increased release of neurotransmitters such as glutamate, substance P (SP), calcitonin gene-related peptide (CGRP), and brain-derived neurotrophic factor (BDNF) into central terminals of the spinal cord dorsal horn (postsynaptic). Released neurotransmitters bind to their receptors, including *N*-methyl-D-aspartate receptors (NMDAR), α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), metabotropic glutamate receptors (mGluR), neurokinin-1 receptors (NK-1R), and tropomyosin receptor kinase (Trk) receptors, on the postsynaptic neurons. These series of processes induce hyperactive/hyperexcited states in the postsynaptic neurons and result in hyperalgesia (an abnormal increase in pain sensitivity from painful stimuli), allodynia (pain caused by non-painful stimuli), and the expansion of the receptive fields of nociceptors (Hylden et al., 1989; Latremoliere and Woolf, 2009; Woolf, 2011). Additionally, peripheral injury can redistribute the central terminals of myelinated afferents and induce pain by non-painful afferents (Woolf et al., 1992; Tashima et al., 2018; Nagi et al., 2019). This process seems similar to peripheral sensitization in so far as it also results in hyperalgesia and allodynia but differs in the range of sites that cause the resulting pain. Peripheral sensitization occurs when nociceptors are exposed to inflammatory mediators and damaged tissue. Consequently, the abnormal pain sensation

prompted by peripheral sensitization is limited to inflamed or damaged sites. Hence, this phenomenon can function to protect sites of injury. By contrast, central sensitization can induce pain either in sites where inflammation or injury has already been resolved or without no obvious inflammation or injury.

Intracellular Ca^{2+} is important to initiating central sensitization. Under normal conditions, the pores of NMDARs are blocked by Mg^{2+}. However, glutamates released from presynaptic buds dislodge Mg^{2+} and allow Ca^{2+} to enter the cytosol. Further, activation of the group I mGluRs (mGluR1 and 5) opens the Ca^{2+} channels on the endoplasmic reticulum (ER). Intracellular Ca^{2+} activates protein kinase C (PKC), PKA, and calmodulin-dependent protein kinase II (CaMKII). These kinases phosphorylate ion channels and glutamate receptors, and their properties are rapidly changed. PKC phosphorylates Ser831 on the GluR1 of AMPAR, Ser880 on GluR2, and Ser896 on NR1 of the NMDAR subunit. PKA phosphorylates Ser890 and Ser897 on NR1 and Ser845 on GluR1. CaMKII phosphorylates Ser1303 on NR2B and Ser831 on GluR1 (Suo et al., 2013; Ji et al., 2018). These processes correspond to the early, phosphorylation-dependent phase of central sensitization in which extracellular signal-regulated protein kinase (ERK) is important to sustain central sensitization. The activation of ERK stimulates increases in the transcription of several genes, such as *c-Fos*, NK1, TrkB, and Cox-2, the phosphorylation of NMDAR and Ser616 on Kv4.2, and the insertion of AMPAR into the membrane. Together, these events increase neuronal excitability in the spinal cord (Ji and Woolf, 2001; Pace et al., 2018).

TRP CHANNEL AND PAIN

Unpleasant and painful sensations signal organisms to subsequently avoid these situations, allowing the organisms to survive noxious insults and injuries. Such noxious stimuli are detected by nociceptors, specialized peripheral sensory neurons that send the threatening signals to the spinal cord and the brain. If a stimulus is intense enough to reach the noxious range, ion channels expressed in the nociceptors are activated and generate action potentials. Ion channels called TRP channels are considered to play an important role in transducing noxious thermal, chemical, and mechanical stimuli.

The TRP channels were first discovered in the eyes of a TRP-mutant *Drosophila melanogaster*, where the mutant fly displayed a transient abnormal response to a bright light, while wild type flies showed a sustained response (Cosens and Manning, 1969). Later, TRP channels in mammalian were introduced with an extended protein family comprising more than 30 distinct subtypes (Ramsey et al., 2006). The TRP family can be distinguished into 6 members: TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPC (canonical), TRPP (polycystin), and TRPML (mucolipin) (Chung and Caterina, 2007). These TRP families are similar in architecture, having six putative transmembrane domains with cytoplasmic amino and carboxyl termini, although they differ in amino-acid conservation. Among these members of TRP family, at least six subtypes, including TRPV1-4, TRPA1, and TRPM8, are closely related to painful thermal, chemical, and mechanical stimuli evoking nociceptive pain (Patapoutian et al., 2009; Dai, 2016). Intracellular Ca^{2+} concentration increases in response to stimuli that are specific to certain TRP channels, such as Ca^{2+} entry channels (Zheng, 2013). TRP channels also stimulate intracellular signaling pathways (e.g., PKC activity) and cause transcriptional changes, all of which promote the expression and release of pro-inflammatory peptides such as SP and CGRP (Veldhuis et al., 2015). Moreover, G protein-coupled receptors (GPCRs) are located in the plasma membrane along with TRP channels and are responsible for the central transduction of painful signals from the periphery nociceptors. These GPCRs are activated by noxious stimuli from the extracellular environment and control TRP channel activity through several mechanisms, wherein the G-protein subunits stimulate second messenger kinases [e.g., cyclic adenosine monophosphate (cAMP)-dependent protein kinase A, phospholipase C (PLC), PKC] (Veldhuis et al., 2015).

TRPV1 as Thermal Sensor

Transient receptor potential channels specifically related to pain and thermosensation were first suggested when the capsaicin receptor, TRPV1, was cloned from a rodent dorsal root ganglia (DRG) in 1997 and was reported as a non-selective cation channel exhibiting high calcium permeability (Caterina, 2007). TRPV1 is mainly expressed in small diameter neurons in the DRG and trigeminal ganglion (TG). TRPV1 is activated by both noxious heat ($\geq$43°C) and capsaicin, the pungent ingredient of chili peppers, triggering a sensation of burning pain. In addition to capsaicin and noxious heat, TRPV1 is also activated by spider toxin (Siemens et al., 2006) and low pH (protons) (Caterina et al., 1997). Therefore, TRPV1 is defined as a polymodal receptor (Clapham, 2003). Moreover, certain studies revealed that TRPV1-deficient mice had a complete loss of physiological and behavioral responses to capsaicin, along with significant impairment in responses to noxious heat and mechanical stimuli (Caterina et al., 2000; Davis et al., 2000; Bolcskei et al., 2005). However, other studies reported that TRPV1 null mice had no differences in sensitivity to acute noxious heat compared to wild type mice (Davis et al., 2000; Woodbury et al., 2004). This suggests that TRPV1 is not solely responsible for the sensation of noxious heat, indicating the presence of other possible compensatory mechanisms.

TRPA1 as a Chemical and Thermal Sensor

Another non-selective cation channel of the TRP family subtypes, TRPA1, is also found to be critical in nociception. TRPA1 is highly co-expressed with TRPV1 in small-diameter nociceptors in the DRG and TG and can be influenced by TRPV1 (Salas et al., 2009; Staruschenko et al., 2010). TRPA1 is a polymodal receptor, like TRPV1 (Story et al., 2003; Dai et al., 2007). TRPA1 is activated by a variety of noxious stimuli that induce acute painful burning or stinging sensations including chemicals,

such as mustard oil (isothiocyanates), garlic (allicin), and cinnamon oil (cinnamaldehyde) (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005). These chemicals are all electrophilic and activate TRPA1 by covalent modification of cysteine residues in the channel, suggesting that the reactivity of such chemicals is not necessarily dependent on its structure (Hinman et al., 2006; Macpherson et al., 2007). In TRPA1 knockout mice studies, those chemicals showed reduced nociceptive pain behavior (Macpherson et al., 2007; McNamara et al., 2007), indicating TRPA1 as a key detector of chemical damage. Additionally, TRPA1 has also been suggested to detect noxious cold stimuli (<15°C) (Story et al., 2003; Sawada et al., 2008), even though studies of TRPA1-deficient mice showed discordant results (Bandell et al., 2004; Jordt et al., 2004; Zurborg et al., 2007; Karashima et al., 2009).

TRP CHANNELS IN INFLAMMATORY PAIN CONDITIONS

Depending on pathological conditions such as inflammation (inflammatory pain) or nerve injury (neuropathic pain), changes occur in the expression of TRP channels and their function, including transcriptional and translational regulation and post-translational alteration (Patapoutian et al., 2009). As mentioned above, inflammatory pain is generated from the activation and sensitization of nociceptive pain signaling in the form of a reduced threshold and an increased responsiveness of nociceptors. Three cellular and molecular mechanisms have been suggested in the development of inflammatory pain involved in TRP channels. First, TRP channel expression increases in the sensory neurons either transcriptionally or post-translationally during inflammation. For instance, the activation of C-C chemokine receptor type 2 (CCR2) by the macrophage inflammatory protein-1α (MIP-1α/CCL3) increases the transcription of TRPV1 and TRPA1 (Jung et al., 2008); i.e., TRPV1 levels in peripheral terminal or nociceptors are increased in order to maintain the inflammatory heat hypersensitivity (Ji et al., 2002). TRPA1 in DRG neurons is also upregulated by peripheral inflammation transcriptionally, leading to inflammatory cold hyperalgesia (Obata et al., 2005). In other words, the increase in expression and the oxidative products from tissue damage and inflammation led to a drastic decrease in the activation threshold, thereby increasing sensitivity to noxious stimuli (via peripheral sensitization) (Hucho and Levine, 2007). Second, translocation of functional TRP channels from the cytoplasm to the plasma membrane is induced following the activation of second-messenger pathways and subsequent post-translational modification (e.g., channel phosphorylation or glycosylation) (Morenilla-Palao et al., 2004; Nguyen et al., 2005; Zhang et al., 2005). Third, channel phosphorylation as a disinhibition mechanism with inflammatory mediators might cause channel structure alteration and functionally enhance the channel sensitivity (Dai et al., 2007). Tumor necrosis factor (TNF)-α increases the frequency, not the amplitude, of spontaneous excitatory postsynaptic currents (sEPSCs) in wild type mice but not in TRPV1 KO mice (Park et al., 2011a). Importantly, studies conducted using knockout mice lacking TRPV1 showed that the development of inflammatory thermal hyperalgesia became defective (Keeble et al., 2005; Barton et al., 2006). This suggests that TRPV1 is a key component of the mechanism in which inflammation causes thermal hyperalgesia and pain hypersensitivity (Caterina et al., 2000; Davis et al., 2000; Bolcskei et al., 2005; Basbaum et al., 2009). In addition to TRPV1, TRPA1-deficient mice studies also showed markedly reduced development of hyperalgesia in response to inflammation-related chemicals that were injected such as formalin and bradykinin (Bautista et al., 2006; McNamara et al., 2007; Andersson et al., 2008).

TRP CHANNELS IN NEUROPATHIC PAIN CONDITIONS

Physiological changes such as increasing the local Ca^{2+} ion concentration in primary sensory neurons or in the spinal cord owing to nerve injury consequently affect signal processing in the CNS, evoking neuropathic pain (Fernyhough and Calcutt, 2010). When neuropathic pain impulses are transmitted from the periphery to the CNS, nociceptive transmitters such as SP are released by exocytosis from the primary sensory terminals in the spinal dorsal horn, where TRP channels are mediated by voltage-dependent Ca^{2+} channels (Verkhratsky and Fernyhough, 2008).

After nerve damage, expression in TRP channel changes dynamically in sensory neurons, and different TRP channels are involved in the management of neuropathic pain. In the spinal nerve section model, the expression of TRPV1 mRNA is reduced in the axotomized ganglia, suggesting loss of trophic support after the injury (Michael and Priestley, 1999). In L5 spinal nerve ligation model, TRPV1, TRPA1, and TRPM8 are upregulated in uninjured L4 somata (Hudson et al., 2001; Obata et al., 2005). It has been suggested that the injured neurons might release growth factors and neurotransmitters into the surrounding region, causing an increase in the excitability of nearby uninjured spared neurons (Fukuoka et al., 2001; Sexton et al., 2014). Vilceanu et al. (2010) revealed that the proportion of TRPV1-expressing DRG neurons was increased and its TRPV1 function was improved after nerve injury induced by spinal nerve ligation (SNL). Moreover, sciatic nerve transection in rats results in up-regulation of TRPV1 at the central terminals of the primary afferent neurons in the spinal cord, increased release of inflammatory neuropeptides such as calcitonin gene-related peptide and SP from the presynaptic central terminals, and enhanced glutamatergic neurotransmission (Kanai et al., 2005; Lappin et al., 2006; Lee and Kim, 2007; Spicarova et al., 2011). Both TRPV1 and TRPA1 are involved in peripheral neuropathy and neuropathic pain induced by chemotherapeutic agents such as cisplatin, oxaliplatin, and paclitaxel. Inhibition of TRPA1 function eliminates mechanical as well as cold allodynia induced by cisplatin and oxaliplatin, which are most commonly used for chemotherapy (Baron, 2009; Nassini et al., 2011; Zhao et al., 2012). Paclitaxel chemotherapy mediates

neuropathic pain behaviors by the release of mast cell tryptase to activate the protease-activated receptor 2 (PAR 2), which then sensitizes TRPV1, TRPV4, and TRPA1 via PLC, PKC, and PKA signaling (Chen et al., 2011); it also enhances the TRPV1 mRNA transcripts and TRPV1 protein in small-to-medium diameter DRG neurons (Hara et al., 2013). Additionally, in neuropathic pain induced by a chronic constriction injury model, inhibition of TRPA1 function also diminishes cold allodynia (Hara et al., 2013).

Certain pro-inflammatory lipid mediators [e.g., leukotrienes (LTs) and prostaglandins (PGs)] in the spinal cord reportedly contribute to neuropathic pain. Of interest, a growing evidence has established that microglial activation has emerged as key players in maintaining pain hypersensitivity in the dorsal horn. Together, the activation of microglia induces dramatic changes including an intracellular signaling molecules [e.g., mitogen-activated protein kinases (MAPKs)], leading to increase the production of pro-inflammatory lipid mediators in the microglia. Following peripheral nerve injury, adenosine triphosphate (ATP) is known to be released from the primary afferent central terminals in the spinal cord, and studies have shown that expression of the purinergic P2 (P2X) receptors was upregulated in microglia to be able to detect extracellular ATP in the spinal cord (Tsuda et al., 2003; Ulmann et al., 2008). LTs are a group of lipid mediators derived from arachidonic acid (AA) which is converted into leukotriene A4 (LTA4) via the 5-lipoxygenase (5-LO) pathway in microglia. LTA4 is then enzymatically converted into LTB4 (Noguchi and Okubo, 2011). In the rat model of spared nerve injury (SNI), LTB4 was expressed in both the spinal neurons and microglia along with LTB4 receptor 1 (BLT1), and the BLT1 was expressed in the spinal neurons. The effect of LTs on neuropathic pain behaviors was identified by intrathecal administration of a 5-LO inhibitor that attenuated the mechanical hypersensitivity caused by SNI surgery (Okubo et al., 2010). Okubo and researchers also found that the increase of 5-LO in spinal microglia was reduced on treatment with an p38 MAPK inhibitor, but not mitogen-activated protein kinase (MEK) inhibitor, indicating that the p38 MAPK pathway is crucial in the generation of neuropathic pain (**Figure 1**). While BLT1 binds LTB4 as its agonist, resolvin E1 and E2 are considered as endogenous receptor antagonists for BLT1. A previous study showed that intrathecal pre-treatment of RvE1 attenuated neuropathic pain by modulating microglial activation in the spinal cord (Xu et al., 2013); thus, there may be an association of BLT1 with RvE1 and E2. In the context of AA metabolites, it is also known to be metabolized into the epoxygenase metabolite, 5,6-EET, which directly activates TRPV4 channels (Vriens et al., 2004). However, not all AA-derived mediators have pro-inflammatory action. Lipoxin A4 (LXA4) is an eicosanoid derived from AA through sequential actions of lipoxygenases, 15-LO and 5-LO; it is known to be an agonist for ALX/FPR2, acting as an endogenous "stop" signal in acute inflammation to switch into the resolution phase (Levy et al., 2001). Martini et al. showed that treatment with LXA4 modulated microglial activation and TNF-α release via ALX/FPR2 receptors, thereby reducing neuropathic pain in rodents after spinal cord hemisection (Martini et al., 2016). PG, a pro-inflammatory mediator synthesized from AA by the COX enzyme in microglia, has been identified as another factor in mechanical allodynia in an SNI model. PGH2 is synthesized from AA by the action of COX and serves as a substrate of the prostaglandin synthase enzymes for producing the bioactive prostaglandins, PGE2 and PGD2, which bind to the EP and DP receptors, respectively (Kanda et al., 2013). Like LTB4, p38 activation in spinal microglia induces the release of PGE2 (Ji and Suter, 2007). Nakayama et al. reported an increase in PGE2 concentration and activation of EP1 receptors in the spinal dorsal horn in the late phase after carrageenan-induced mechanical hyperalgesia in rats (Nakayama et al., 2002). Moreover, following peripheral injury, an increase in PGD2 was noted, which was subsequent to the upregulation of COX-1, and the intrathecal injection of DP2 receptor antagonists attenuated the mechanical allodynia (Kanda et al., 2013). The activation of both EP and DP receptor initiate G-protein coupling to increase intracellular concentration of Ca^{2+} or cAMP in the dorsal horn (Jang et al., 2020). Although evidence of the mechanisms of prostaglandin and its receptors have been accumulated, their role in neuropathic pain and the resolution strategy remain unknown.

Moreover, these pathways of pro-inflammatory lipid mediators contributing to neuropathic pathways has not been studied in the context of TRP regulation. Moreover, even though these findings do not involve TRP regulation, LTs and PGs might be implicated in neuropathic pain by acting like G-protein-mediated signal transducers to initiate second messenger systems, which in turn activate TRP channels (**Figure 1**). Interestingly, TRPV1 is found to be upregulated in the spinal cord dorsal horn in the chronic constriction injury (CCI) neuropathic pain model, indicating possible interaction between the receptors mentioned above (Kanai et al., 2005). Further research will contribute identifying the analgesic effect caused by those pro-inflammatory mediators on neuropathic pain.

Transient receptor potential channels are upregulated in order to manage hypersensitivity of both inflammatory and neuropathic pain at the site of inflammation and the remaining intact neurons close to the damaged nerve, respectively. However, different mediators and intracellular mechanisms of TRP channels are involved, depending on what type of pain is occurring. Hence, it is important to elaborate the role of TRP channels in the resolution of different types of pathological pain using resolvin, which will be discussed in detail next.

RESOLVINS (ENDOGENOUS LIPID MEDIATORS) INHIBIT TRP CHANNELS

Lipid Mediators in Resolution of Acute Inflammation

Acute inflammation is a host defense system, and its resolution may serve as a gateway to chronic inflammation. In health status, inflammatory responses are self-limited, with many cell types and tissues involved in initiation and termination of acute inflammation (Serhan, 2007). Tissue injury or infection

triggers the release of a train of signals, including damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), lipid mediators and chemokines, such as formylmethionyl-leucyl-phenylalanine (fMLP), ATP, high mobility group box-1 (HMGB1), F-actin, N-glycan, LTB4, CCR1 ligands, chemokine (C-X-C motif) ligand (CXCL) 2, and CXCL8, from injury sites (McDonald and Kubes, 2010; Selders et al., 2017; Peiseler and Kubes, 2019; Gong et al., 2020). Neutrophils move through the chemoattractant gradient, while the integrin molecules on neutrophil surface bind to integrin receptors on endothelial cells enabling their infiltration into the tissues. The infiltrated neutrophils remove pathogens via phagocytosis and release reactive oxygen species (ROS), degradative enzymes, microbicidal agents, and neutrophil extracellular traps (Metzemaekers et al., 2020). Neutrophils are the first defender against hazardous invaders, but their high numbers can induce excessive inflammation (Freire and Van Dyke, 2013). Since excessive inflammation is considered to be a component in many chronic diseases, such as vascular diseases, metabolic syndrome, neurological diseases, and many others, proper resolution of acute inflammation is important. (Serhan et al., 2015b). Resolution of acute inflammation was introduced as a passive process following disappearance of the chemoattractant and other chemical mediators, but Dr. Serhan's group uncovered endogenous lipid mediators in inflammatory exudate: resolvins, protectins, and maresins (Serhan et al., 2002; Serhan, 2014). These pro-resolving lipid mediators are often referred to as specialized pro-resolving mediators (SPMs) because they stimulate efferocytosis of polymorphonuclear leukocytes (PMNs) to facilitate complete removal of pathogens, recruit macrophages, attenuate activated neutrophil through NF-κB inhibition, and initiate the resolution phase of acute inflammation (Flower and Perretti, 2005; Lee et al., 2013; Francos-Quijorna et al., 2017; Yurdagul et al., 2017; Rymut et al., 2020).

Biosynthesis of Resolvins

Notable omega-3 fatty acids are alpha-linolenic acid (ALA), EPA, and DHA. In humans, ALA converts to EPA and DHA, but the conversion rate of ALA to EPA is 8–20%, and that to DHA is 0.5–9%, which are very low (Stark et al., 2008).

In vascular endothelial cells, EPA is converted into 18R-hydroperoxy-EPE (18-HpEPE) with aspirin/COX-2 or microbial p450. 18-HpEPE is converted to 5S(6)-epoxy-18-hydroxy-HEPE

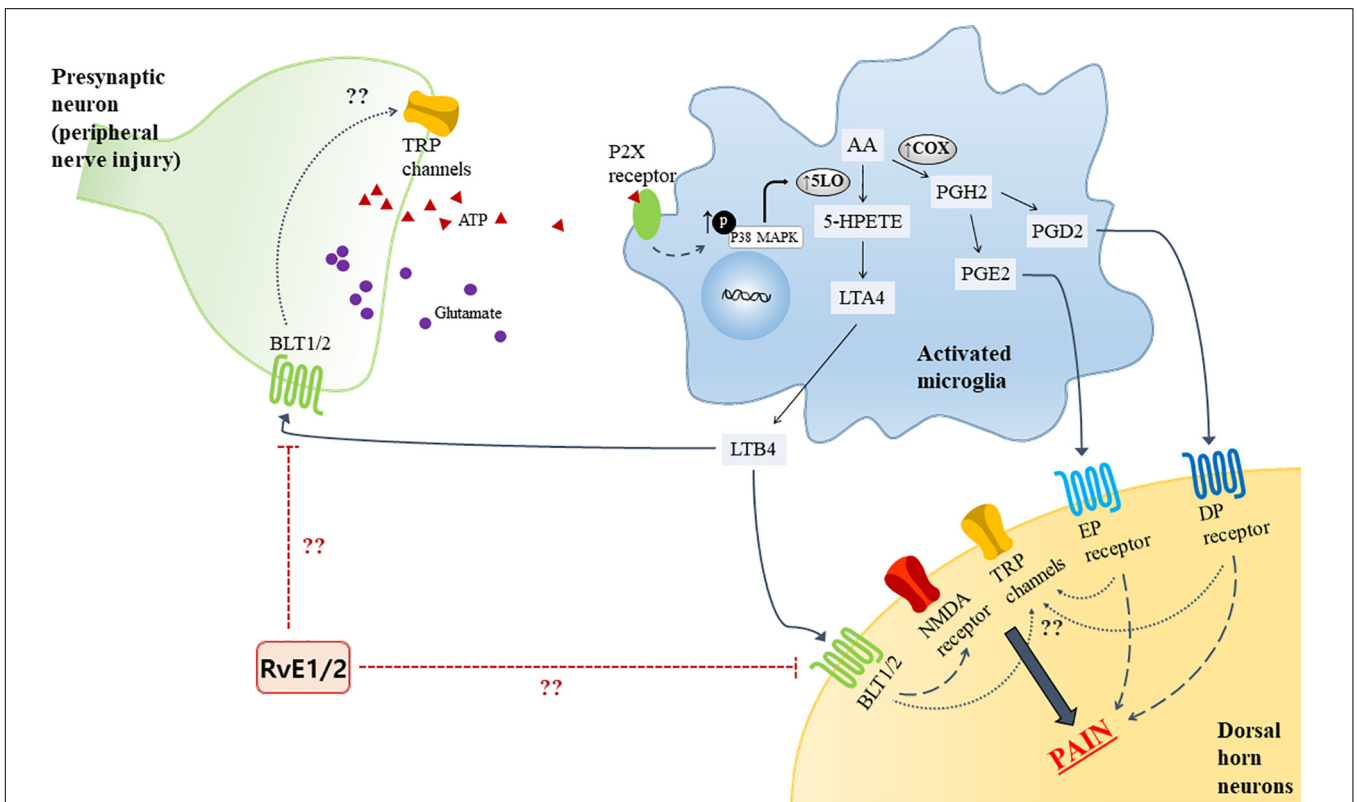

FIGURE 1 | Proposed model for LTs and PGs synthase mechanism from AA in activated microglia by ATP due to peripheral nerve injury, and the receptors in the spinal cord. The activated microglia phosphorylate p38 MAPK, upregulating 5-LO expression. Bioactive LTB4, PGE2, and PGD2 bind to their receptors (BLT1/2, EP and DP receptors, respectively) and evoke pain. The possible analgesic role of RvE1 and E2, which is known to be antagonists of BLT1, and the relationship TRP channel in the presynaptic and spinal dorsal horn neuron are depicted. AA, Arachidonic acid; ATP, adenosine triphosphate; BLT, leukotriene B4 receptor; DP, D–type prostanoid; EP, E-type prostanoid; MAPK, mitogen-activated protein kinases; PG, prostaglandin; PGD, prostaglandin D; PGE, prostaglandin E; RvE1, resolvin E1; RvE2, resolving E2; TRP, transient receptor potential; 5-LO, 5-lipoxygenase.

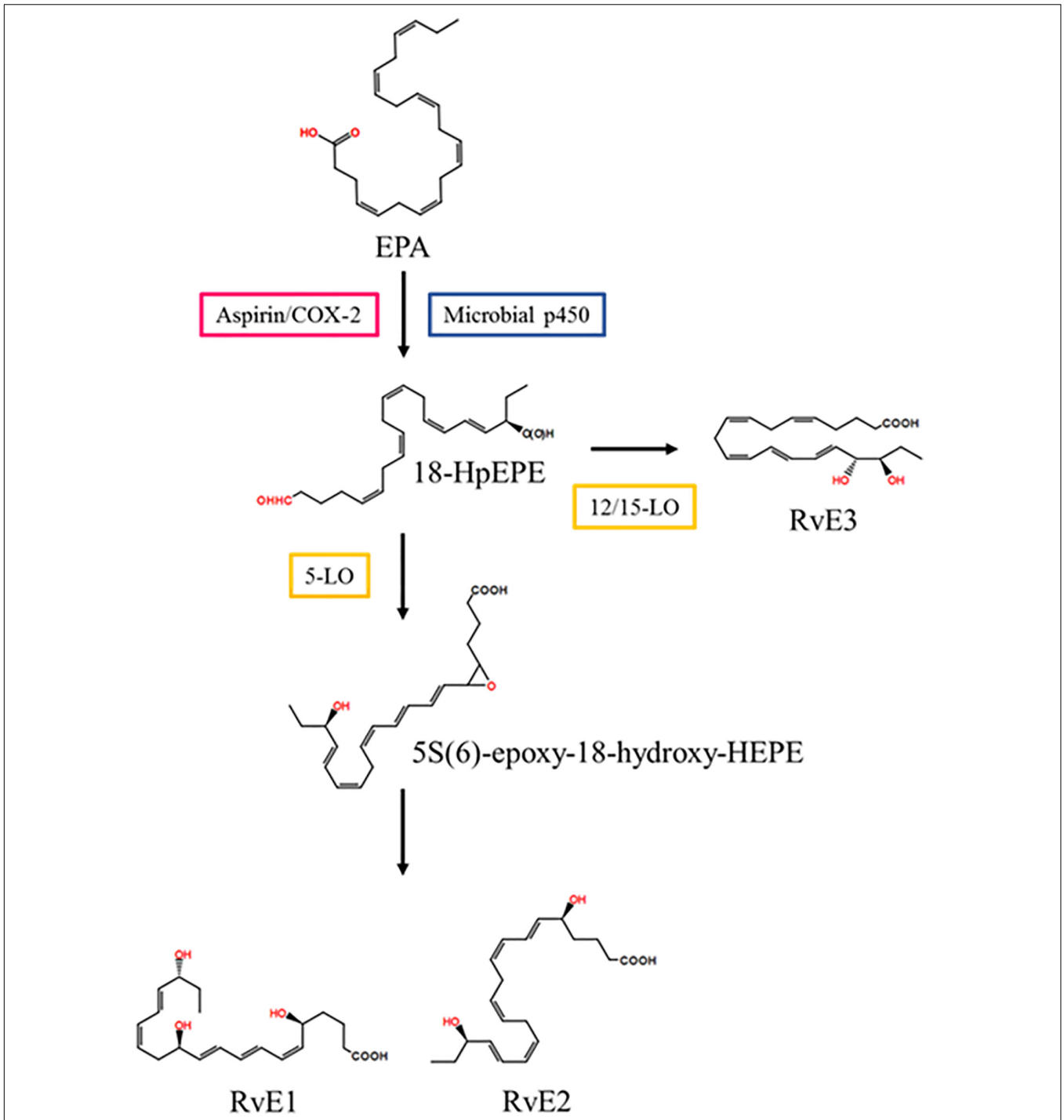

FIGURE 2 | Resolvin E series biosynthetic pathway. EPA is converted into 18-HpEPE. 5S(6)-epoxy-18-hydroxy-HEPE is synthesized from 18-HpEPE with 5-LO and is transformed into RvD1 and RvD2. 18-HpEPE is also transformed into RvD3 by 12/15-LO. EPA, eicosapentaenoic acid; LO, lipoxygenase; RvD, resolvin D; 18-HpEPE, 18R-hydroperoxy-EPE.

with 5- LO in leukocytes and then converted to resolvin E1 via enzymatic hydrolysis or to resolvin E2 via reduction (Serhan et al., 2015a). In eosinophils, 18-HpEPE is also converted to resolvin E3 via 12/15-LO pathways (Isobe et al., 2012) (**Figure 2**).

In exudates, resolvin D series are biosynthesized by PMNs or macrophages. DHA is metabolized to 17-hydroxydocosahexaenoic acid (17-HDHA) via 17-hydroperoxydocosahexaenoic acid through 15-LO (Gleissman et al., 2010). 17-HDHA is converted to resolvin D1, D2, D3, D4,

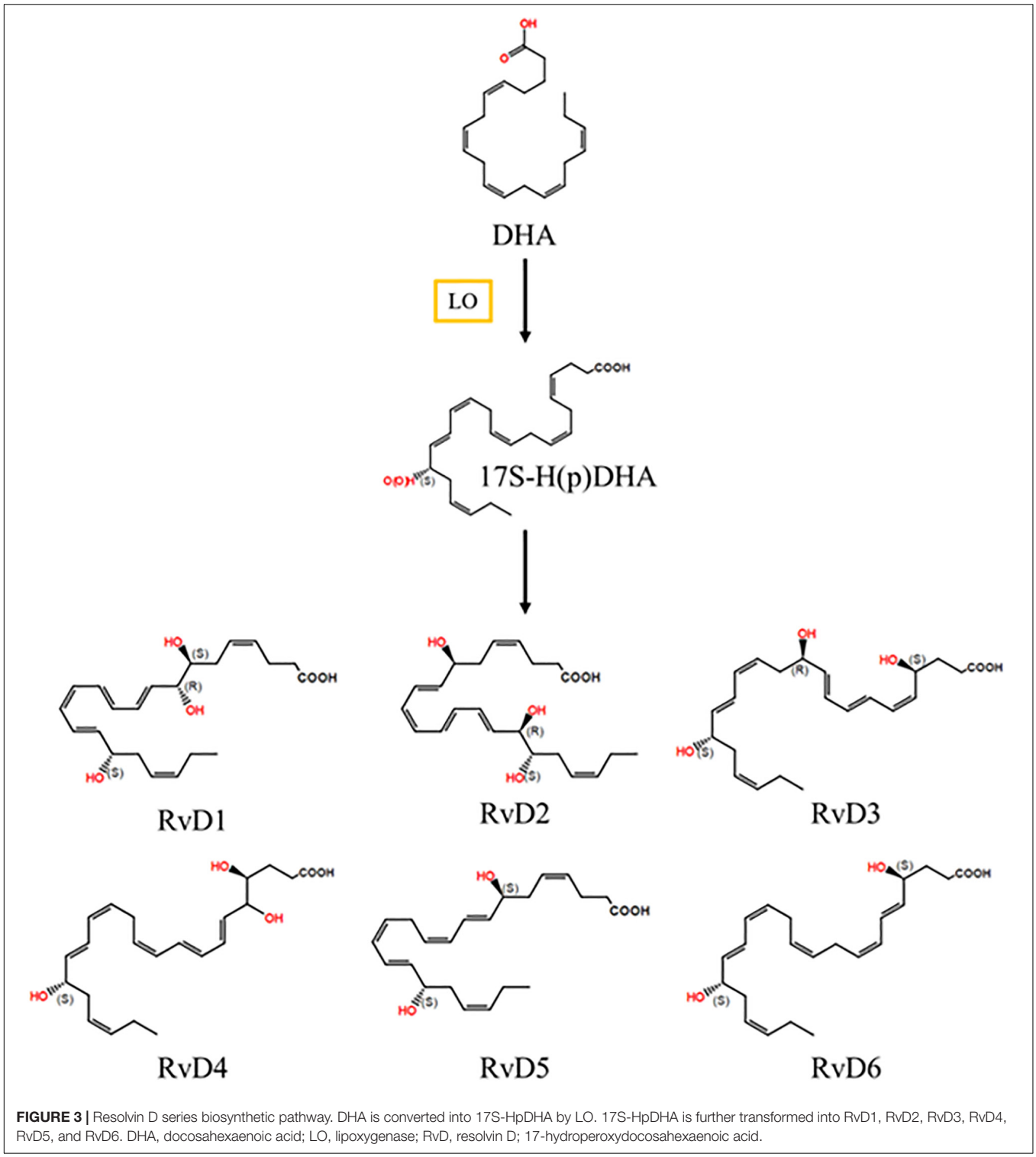

FIGURE 3 | Resolvin D series biosynthetic pathway. DHA is converted into 17S-HpDHA by LO. 17S-HpDHA is further transformed into RvD1, RvD2, RvD3, RvD4, RvD5, and RvD6. DHA, docosahexaenoic acid; LO, lipoxygenase; RvD, resolvin D; 17-hydroperoxydocosahexaenoic acid.

D5, and D6 through epoxidation and 5-LO (**Figure 3**). DHA is also metabolized with aspirin/COX-2, and then the aspirin-triggered resolvin D (AT-RvD) series is formed (Lim et al., 2015; Serhan et al., 2015a; Serhan and Levy, 2018). AT-RvD3 is more potent than resolvin D3 in phagocytosis of 10 and 100 pM zymosan (Dalli et al., 2013).

Since TRP channels are expressed in nociceptors where pain is generated, the roles of TRP channels in mediating pathological pain make them potential therapeutic targets for resolution. Until now, in addition to RvE1 and RvD1/AT-RvD1, more resolving family members have been biosynthesized: RvD2, RvD3, RvD4, and RvD5. RvE1, RvD1, and AT-TvD1 are already well-known

agonists that resolve inflammation and inflammation-associated pain by mediating specific GPCRs [e.g., chemerin receptor23 (ChemR23) and GPR32], which are expressed by immune cells, glia cells, and neurons. This, in turn, reduces inflammation, glial activation, and spinal cord synaptic plasticity (Ji et al., 2011). Mounting evidence on resolvins as potent inhibitors for both inflammatory and neuropathic pain will be discussed with recent findings.

Resolvin Receptors

Resolvins can directly act on nociceptors but little is known about how resolvins inhibit TRP channels in neurons. Five GPCRs – ERV/ChemR23, BLT1, ALX/FPR2, DRV1/GPR32, and DRV2/GPR18 – are receptors for resolvin D and E series that play an important role in the modulation of the TRP channels (Pirault and Back, 2018) (**Figure 4**).

ChemR23, resolvin E1 and chemerin receptors (Herova et al., 2015) are co-expressed with TRPV1 in small sized-DRG neurons and in the spinal cord dorsal horn. Activation of chemR23 by resolvin E1 or chemerin blocked capsaicin-evoked spontaneous excitatory postsynaptic current frequency increases via the ERK pathway in the spinal cord dorsal horn, and resolvin E1 also blocked phosphorylation of ERK by capsaicin treatment in DRG neurons (Xu et al., 2010; Park et al., 2011b; Jo et al., 2016). ERK is a member of the mitogen-activated protein kinase superfamily of signaling pathways, an important kinase in painful conditions. In addition to capsaicin, noxious heat, cold, and prick upregulate the pERK intensity in neurons (Ji et al., 1999). In particular, pERK is upregulated in DRG and in the spinal cord in neuropathic pain models of partial sciatic nerve ligation (PSNL), SNL, CCI and diabetes (Seltzer et al., 1990; Song et al., 2005; Liu et al., 2012; Xu et al., 2014; Guo et al., 2019; Wang et al., 2020).

BLT1, LTB4 (agonist), and resolvin E1 (antagonist) receptor are co-expressed with TRPV1 in DRG neurons (Andoh and Kuraishi, 2005; Serhan and Chiang, 2013). LTB4 functions as an agonist and sensitizes TRPV1-mediated Ca^{2+} increase. Although pain behavior in the second phase of the formalin pain model is significantly attenuated in BLT1-KO mice relative to WT mice, no analgesic effect has been observed in the first phase. This is because the first phase is mainly mediated by *TRPA1* and *TRPV1* gene expression, which do not change in the DRG and spinal cords of BLT1-KO mice. Hence, BLT1 may not be involved in mediating the properties of TRPA1 (McNamara et al., 2007; Asahara et al., 2015).

Resolvin D1 inhibits TRPA1, TRPV3, and TRPV4 in heterogeneous systems. FPR2/ALX is a resolvin D1 receptor, but treatment with FPR2/ALX agonists, cathelicidin LL-37 and Trp-Lys-Tyr-Met-Val-Met (WKYMVM), do not affect TRPA1, TPRV3, and TRPV4 inhibition (Bang et al., 2010). However, there is another resolvin D1 receptor, GPR32, that is also activated

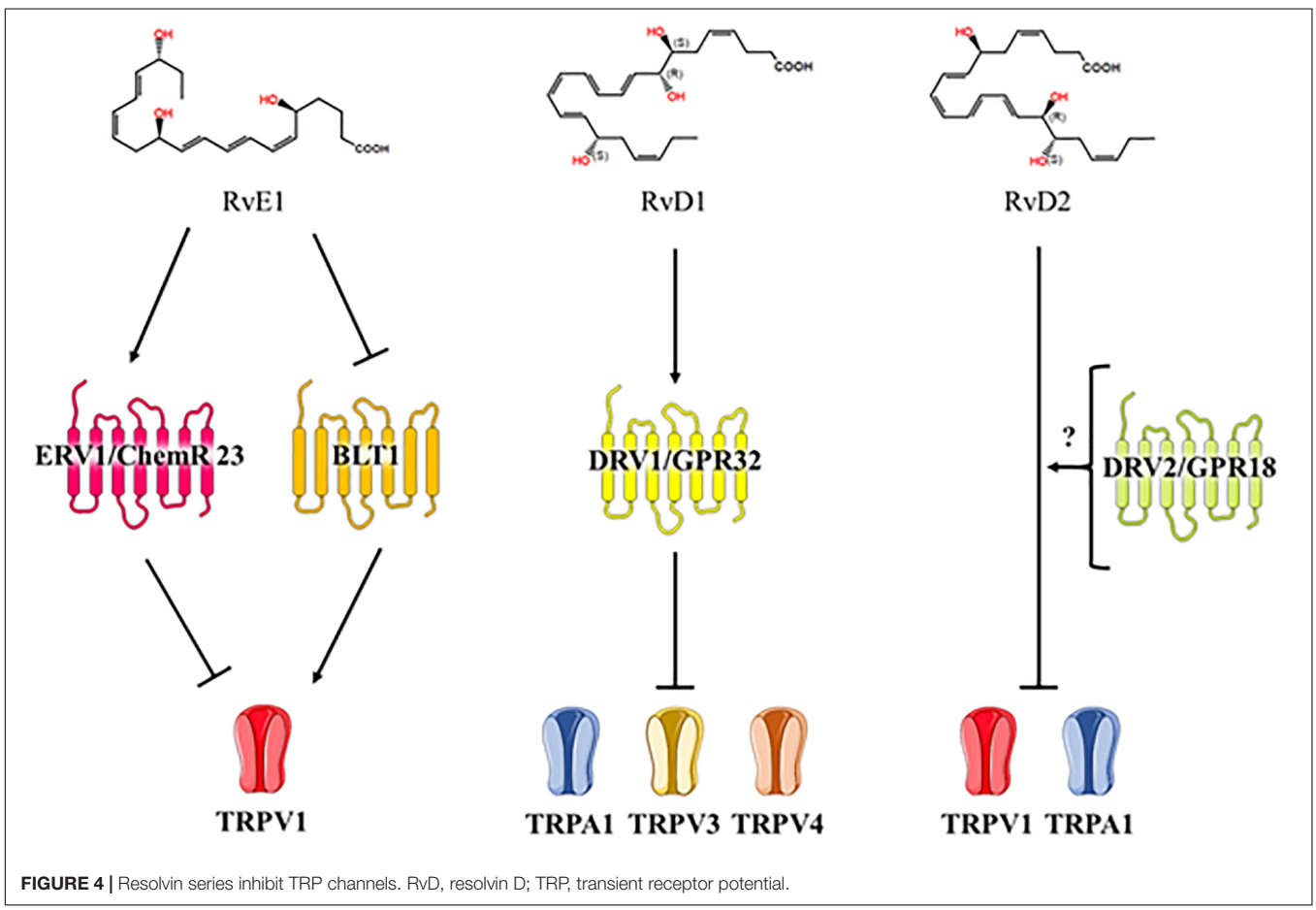

FIGURE 4 | Resolvin series inhibit TRP channels. RvD, resolvin D; TRP, transient receptor potential.

by AT-RvD1. FPR2/ALX and GPR32 are GPCRs that regulate specific microRNAs and their target genes that promote the resolution of acute inflammation (Krishnamoorthy et al., 2012).

Resolvin D2 is known to activate a cell surface GPCR, GPR18/DRV2 (Chiang et al., 2015, 2017). GPR18 is expressed in lumbar DRG and in the spinal cord at a genetic level and under neuropathic conditions; it is also upregulated in the spinal cord (Malek et al., 2016). Resolvin D2 inhibits TRPV1 and TRPA1 activation dose-dependently. Resolvin D2 is ten times more potent than resolvin E1 on TRPV1 inhibition ($IC_{50} = 0.04 \pm 0.01$ and 0.4 ± 0.05, respectively), and it is four times more potent than resolvin D1 on TRPA1 inhibition ($IC_{50} = 0.8 \pm 0.2$ and 3.2 ± 0.05, respectively) (Park et al., 2011b).

RESOLVINS ALLEVIATE CHRONIC PAIN

Resolvins in the Resolution of Inflammatory Pain

Studies have shown that both peripheral (intraplanar) and central (intrathecal) administration of RvE1 and RvD1 reduce inflammatory pain (Bang et al., 2010; Xu et al., 2010; Lima-Garcia et al., 2011; Park et al., 2011b) and postoperative pain (Huang et al., 2011; Wang and Strichartz, 2017) by modulating the activation of TRP channels. Bang et al. reported that pretreatment with RvD1 (20 ng) suppressed pain behaviors induced by intraplanar formalin injection (Bang et al., 2010) and attempted to determine whether activators of the RvD1 receptor (FPR2/ALX) inhibits the effect of TRP channels (TRPV3/4 and A1), however, no such effect was found (Krishnamoorthy et al., 2010). Xu et al. (2010) found that both RvD1 and RvE1 (1–20 ng) suppressed inflammatory pain behaviors induced by formalin, carrageenan, or complete Freund's adjuvant (CFA); they also indicated that the possible mechanism where RvE1 inhibits ERK signaling pathways to block TNF-α induced increased EPSC frequency and NMDAR hyperactivity in spinal dorsal horn neurons. Additionally, an agonist of ChemR23, which was considered to be an RvE1 receptor, also abolished capsaicin-induced increase in sEPSC frequency, implicating the role of RvE1 in mediating TRPV1 activity via ChemR23. Moreover, Park et al. examined RvD2 at very low doses (10 ng) injected intrathecally and found that it prevented spontaneous pain induced by formalin as well as reversed inflammatory pain induced by CFA (Park et al., 2011b). They identified RvD2 as endogenous inhibitors both for TRPV1 and TRPA1, by which RvD2 acts on specific GPCRs. **Table 1** shows a list of the published findings of the resolution effect of resolvins specifically on TRP channels for inflammatory pain.

Resolvins in the Resolution of Neuropathic Pain

While there is mounting research on the analgesic effect of resolvins in inflammatory pain, the role of resolvins in TRP channels to alleviate neuropathic pain is not well known. One study evaluated the analgesic actions of resolvin D-series (D1 to D5) in a TRPA1 and TRPV1 knockout mouse model of chemotherapy-induced peripheral neuropathy induced by paclitaxel (Luo et al., 2019). They found that RvD1 and RvD2 reduced mechanical allodynia in both sexes of TRPV1 and TRPA1 deficient mice, whereas RvD3 and RvD4 had no effects on either sex. Interestingly, RvD5 reduced mechanical allodynia only in male mice but not in female mice, indicating there is a sex dimorphism in pain regulation. Since RvD5 affected the TRPV1 and TRPA1 knockout male mice only, this might implicate the TRP channel in the role of RvD5 to attenuate neuropathic pain in female mice.

TABLE 1 | Analgesic effects of different resolvins in distinctive TRP channels for inflammatory and neuropathic pain.

Research article	Pain type	Resolvin	TRP channel	Results	Potential mechanisms
Bang et al., 2010	Inflammatory	RvD1	TRPA1 TRPV3 TRPV4	↓activities the three TRP channels at nanomolar and micromolar levels ***No effects of FPR2/ALX agonists on the three TRP channels*	
Xu et al., 2010	Inflammatory	RvE1 RvD1	TRPV1	↓EPSC frequency increases induced by capsaicin, with the ChemR23 agonist ↓EPSC frequency increase by TNF-α and NMDAR hyperactivity with RvE1	RvE1 modulates the ERK signaling pathway to abolish TNF-α-evoked NMDA receptor hyperactivity in dorsal horn neurons
Park et al., 2011b	Inflammatory	RvD1 RvD2 RvE1	TRPV1 TRPA1	↓sEPSC increases both TRP channels with RvD2 at extremely low doses (compared to RvD1/E1) ***No effect of RvD2 when GPCRs are blocked*	Distinct mechanisms of the resolvins in regulating TRP channels RvD2 involves specific GPCRs
Luo et al., 2019	Neuropathic (CIPN induced) Inflammatory (**RvD5 only)	RvD1 RvD2 RvD5	*TRPA1* *TRPV1* *(**Knock-out mice)*	↓ neuropathic pain behaviors, with RvD1/D2 *** only in male mice, with RvD5* ↓inflammatory pain behaviors in male mice only with RvD5	Sex dimorphism of RvD5's analgesia in both pain models

*CFA, complete Freund's adjuvant; CIPN, chemotherapy-induced peripheral neuropathy; EPSC, excitatory postsynaptic current; ERK, extracellular signal-regulated kinase; FPR2/ALX, formyl peptide receptor 2; GPCRs, G-protein coupled receptors; NMDAR, N-methyl-D-aspartic acid receptor; RvE, resolvin E; RvD, resolvin D; TNF-α, tumor necrosis factor-α; TRP, transient receptor potential. ** Indicates supplementary information.*

CONCLUSION: IMPORTANCE OF PAIN RELIEF USING RESOLVINS VIA TRP MODULATION

Opioid and non-opioid analgesic drugs for the treatment acute and chronic pain. Opioids are a type of medications that are used to relieve severe chronic pain. However, as opioid receptors are expressed throughout the entire nervous system and gastrointestinal tract and do not operate on specific pain pathways, their abuse and misuse can induce unexpected physiological and psychological side effects that devastate the lives of the affected individuals and their families. Morphine, one of the most widely prescribed opioid analgesics, increases pain hypersensitivity at low doses (Ghelardini et al., 2015). Morphine-6-glucuronide (M6G), a metabolite of morphine, has more potent analgesic effect than does morphine. However, M6G is dangerous to patients with renal failure and relate to respiratory depression (Lotsch, 2005). For these reasons, several nations and international organizations have thoroughly controlled the prescription and use of opioids (Rosenblum et al., 2008; Rogers et al., 2013). Further, the consequences of opioid use underscore the need to develop non-opioid analgesics and thus minimize the prescription of opioid analgesics.

Non-opioid analgesics include both selective and non-selective cyclooxygenase (COX) inhibitors, as well as well-known non-steroidal anti-inflammatory drugs and acetaminophen. COX contributes to the synthesis of PG, which causes inflammation, pain and fever. Because of this, COX inhibitors are used for antipyretics and antiphlogistics, as well as analgesics (Qureshi and Dua, 2020). However, the administration of COX-2 inhibitors can induce cardiovascular complications (Nussmeier et al., 2005).

Therefore, it is necessary to develop new analgesic targets that are specific to nociceptive neurons. One of the targets is TRP channels that mediate nociception, especially TRPV1. Many models have shown that TRPV1 inhibition reduces chronic pain including inflammatory and neuropathic pain. TRP channels in nerve systems have been emerging targets for pain management, and several TRPV1 antagonists (ABT-102, AMG-517, AZD-1386, DWP-05195, JTS-653, MK-2295, PHE-377 and SB-705498) have already entered clinical trials (Wu et al., 2018). However, TRPV1 antagonists can induce abnormal changes in body temperature (hyperthermia and hypothermia (Gavva et al., 2008; Garami et al., 2018, 2020) or decrease heat sensation (Crutchlow et al., 2009). For these reasons, many TRPV1 antagonists have failed to be developed as painkillers.

Nevertheless, TRPV1 inhibitors are still being discovered. Resolvins, a class of lipid mediators, has been identified in this capacity. Resolvins are produced from omega-3 fatty acids and therefore have a great potential for reducing the incidence of side effects associated with pain management. Further, the fact that resolvins can attenuate powerful type cardiovascular disease enhances its potential as a useful analgesic (Salic et al., 2016; Capo et al., 2018). The analgesic role of resolvins has already been proven at nanomolar levels, resolvins are excellent potential therapeutic targets for pain. However, most current studies of resolvins in an analgesic role have been limited to inflammatory pain. Therefore, more studies on the analgesic effect of resolvins for neuropathic pain are needed.

AUTHOR CONTRIBUTIONS

C-KP and YHK conceived and supervised the project. JR, EJG, J-WP, YHK, and C-KP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Contribution of TRPC Channels in Neuronal Excitotoxicity Associated With Neurodegenerative Disease and Ischemic Stroke

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The seven canonical members of transient receptor potential (TRPC) proteins form cation channels that evoke membrane depolarization and intracellular calcium concentration ($[Ca^{2+}]_i$) rise, which are not only important for regulating cell function but their deregulation can also lead to cell damage. Recent studies have implicated complex roles of TRPC channels in neurodegenerative diseases including ischemic stroke. Brain ischemia reduces oxygen and glucose supply to neurons, i.e., Oxygen and Glucose Deprivation (OGD), resulting in $[Ca^{2+}]_i$ elevation, ion dyshomeostasis, and excitotoxicity, which are also common in many forms of neurodegenerative diseases. Although ionotropic glutamate receptors, e.g., *N*-methyl-D-aspartate receptors, are well established to play roles in excitotoxicity, the contribution of metabotropic glutamate receptors and their downstream effectors, i.e., TRPC channels, should not be neglected. Here, we summarize the current findings about contributions of TRPC channels in neurodegenerative diseases, with a focus on OGD-induced neuronal death and rodent models of cerebral ischemia/reperfusion. TRPC channels play both detrimental and protective roles to neurodegeneration depending on the TRPC subtype and specific pathological conditions involved. When illustrated the mechanisms by which TRPC channels are involved in neuronal survival or death seem differ greatly, implicating diverse and complex regulation. We provide our own data showing that TRPC1/C4/C5, especially TRPC4, may be generally detrimental in OGD and cerebral ischemia/reperfusion. We propose that although TRPC channels significantly contribute to ischemic neuronal death, detailed mechanisms and specific roles of TRPC subtypes in brain injury at different stages of ischemia/reperfusion and in different brain regions need to be carefully and systematically investigated.

Keywords: neurological disease, TRPC4 knockout, calcium, neuroprotection, neurodegeneration, neuronal death

INTRODUCTION

Stroke occurs when a part of the brain is deprived of oxygen and glucose. Each year, about 795,000 Americans suffer a new or recurrent stroke, making it the No. 5 cause of death and a leading cause of disability in the United States (Virani et al., 2020). In 70–80% of the cases, the precipitating cause of stroke is a blood clot that blocks the supply of oxygenated blood to a region of the brain, a situation termed ischemic stroke (Woodruff et al., 2011). The damage to neurons during ischemia is caused by a reduction of oxygen and glucose supply, i.e., oxygen and glucose deprivation (OGD) (Ying et al., 1997; Lipton, 1999).

Cell death after cerebral ischemia may result from a number of events, including acidosis, inflammation, generation of arachidonic acid, elevation in intracellular calcium concentrations ($[Ca^{2+}]_i$), loss of cellular ion homeostasis, free radical-mediated toxicity, energy failure, infiltration of leukocytes, cytokine-mediated cytotoxicity and glutamate-induced excitotoxicity (Woodruff et al., 2011). Particularly, excessive extracellular glutamate can elicit multiple neurotoxic effects. Initially, energy depletion-induced depolarization of the neuronal membrane leads to the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (VGCCs), which triggers Ca^{2+}-dependent glutamate release from axonal terminals of excitatory neurons (Bonde et al., 2005). In the past, much of the focus on glutamate-induced excitotoxicity had been on ionotropic glutamate receptors (iGluRs), mainly NMDA (*N*-methyl-D-aspartate) receptors and AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, but more recently, metabotropic glutamate receptors (mGluRs) have also been recognized to play a crucial role in excitotoxicity (Hilton et al., 2006).

The mGluR family consists of three groups, group I-III, and 8 subtypes, mGluR1-8, of which the group I mGluRs, i.e., mGluR1 and mGluR5, are coupled to $G_{q/11}$-phospholipase C (PLC) pathway and the rest are all linked to $G_{i/o}$ proteins (Swanson et al., 2005). On one hand, the activation of $G_{q/11}$-PLC pathway by glutamate through stimulation of mGluR1/5 is to enhance neuronal excitability via a number of mechanisms, such as the suppression of K^+ channels caused by the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid that supports the activity of many K^+ channels (Hille et al., 2015), and the production of inositol 1,4,5-trisphosphate (IP_3), a second messenger that acts at the IP_3 receptors to mobilize Ca^{2+} from the endoplasmic reticulum (ER) Ca^{2+} stores (Berridge, 2009). On the other hand, the activation of $G_{i/o}$-coupled group II and group III mGluRs are generally thought to inhibit neuronal excitation through, among others, activation of G protein-gated inwardly rectifying K^+ (GIRK) channels and inhibition of VGCCs (Logothetis et al., 2015). Therefore, the excessive extracellular glutamate associated with OGD may lead to both excitatory and inhibitory effects through mGluRs depending on the abundance and types of the mGluRs and downstream signaling pathways involved.

A major class of Ca^{2+}-permeable non-selective cation channels activated downstream from mGluRs is the Transient Receptor Potential Canonical (TRPC) channels. The TRPC channel family consists of 7 members, TRPC1-7, in which TRPC2 is a pseudogene in humans (Montell et al., 2002). These channels are typically activated downstream from receptors linked to PLC signaling, with $G_{q/11}$-PLCβ pathway being the most common. Thus, glutamate activation of $G_{q/11}$-coupled mGluR1/5 is likely linked to TRPC channel activation, leading to consequent membrane depolarization and $[Ca^{2+}]_i$ elevation. Ironically, despite the widespread expression of TRPCs in brain neurons (Riccio et al., 2002) and their much-longer lasting activities in response to $G_{q/11}$-PLCβ signaling than iGluRs (Hartmann et al., 2008; Riccio et al., 2009), not much is known about the contributions of TRPC channels in excitotoxicity to neurons except for a few examples. It was shown that in the pilocarpine-induced epilepsy model, the epileptiform burst firing in lateral septal and hippocampal neurons involves the activation of TRPC channels downstream from mGluRs, which is critical for the glutamate excitotoxicity (Phelan et al., 2012, 2013; Zheng and Phelan, 2014). In brain injury induced by focal cerebral ischemia, the roles of TRPC channels can be rather complex. For instance, while TRPC1 and TRPC4 mediate glutamate-induced neuronal death, TRPC6 may exert a protective role against ischemic neuronal death (Du et al., 2010).

Here, we explore the current evidence on contributions of TRPC channels in ischemic neuronal death, taking into account that these channels are activated downstream from mGluRs and likely work in concert with VGCCs and iGluRs to induce neuronal excitation and produce $[Ca^{2+}]_i$ signals, and such activities can have a pivotal impact on glutamate excitotoxicity. Because excitotoxicity occurs commonly in many types of neurodegenerative diseases, including Alzheimer's disease, epilepsy, Huntington's disease, and Parkinson's disease, we also discuss the involvement of TRPC channels in these diseases in attempt to shed some lights on the mechanistic insights of TRPC regulation of excitotoxicity. Finally, we present some of our own data demonstrating the specific role of TRPC4/C5 channels in ischemic cell death and neuroprotective potential of targeting these channels using small molecular probes.

THE BASIS OF EXCITOTOXIC NEURONAL DEATH IN ISCHEMIC STROKE

Cerebral ischemic stroke is caused by interruption of blood supply through the middle cerebral artery (MCA). The MCA has large surface branches to supply blood into its territory that encompasses almost the entire boundary of the cortex and white matter of the hemisphere, including the major lobes (frontal, parietal, temporal, and occipital) and insula cortex (Brust, 2013). Occlusion of the MCA (MCAO) is the most frequently encountered stroke signs and symptoms, which may produce paralysis on the left or right side of the body, vision loss, and speech impairment (Rathore et al., 2002). The neurological deficits in ischemic stroke resulted from the deprivation of glucose and oxygen supply, or OGD, which leads to brain tissue damages. When the MCAO is prolonged, neurons lose glucose-dependent ATP generation, which in turn disrupts electrogenic

pumps, e.g., plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/K^+ ATPase.

The failure of the ATPases subsequently results in $[Ca^{2+}]_i$ increase and membrane depolarization, which causes glutamate release, as well as an elevation of extracellular K^+ concentration. These also alter the ionic composition of the cytoplasm, with increases in Na^+, Ca^{2+}, and Cl^- and a decrease in K^+ levels, leading to an osmolarity change and more commonly hyperosmolarity (Tanaka et al., 1997; Tanaka et al., 1999; Brisson and Andrew, 2012). Consequently, the inflow of water into the neuron in response to the osmolality change causes cell swelling and disruption of the membrane structure, which constitutes one of the multiple causes of neuronal death (Toyoda et al., 2020).

In addition to ionic imbalance caused by the failure of ATPases, excessive extracellular accumulation of glutamate also leads to toxic increases in $[Ca^{2+}]_i$, which activate multiple signaling pathways and ultimately lead to cell death (Durukan and Tatlisumak, 2007; Doyle et al., 2008). For many years, NMDA and AMPA receptors have been considered the pivotal targets of excitotoxicity associated with ischemic stroke. The activation of postsynaptic NMDA and AMPA receptors by synaptic glutamate causes membrane depolarization and Ca^{2+} influx, which definitely accounts for excitotoxicity. However, this is not the only way by which glutamate can elicit excitotoxicity. The abundant presence of mGluRs in both pre- and postsynaptic membranes, as well as extrasynaptic membranes, suggests that these metabotropic receptors are also activated under conditions of excessive glutamate release and accumulation both inside and outside the synapse. At least for mGluR1 and mGluR5, the activation will increase excitability through $[Ca^{2+}]_i$ elevation first by causing ER Ca^{2+} release. This will be followed by store- and/or receptor-operated Ca^{2+} entry, and in the case of coupling to TRPC channels, the activation of these mGluRs will also lead to membrane depolarization, given the non-selective nature of these channels, and the subsequent Ca^{2+} influx through both TRPC channels and VGCCs. Although permeable to both Na^+ and Ca^{2+} as the NMDA and AMPA receptors, the conductance and activation kinetics of TRPC channels are vastly different from that of the iGluRs, typically being slower and longer lasting than the iGluRs as has been demonstrated during synaptic transmission (Hartmann et al., 2008; Riccio et al., 2009; Pressler and Regehr, 2013; Reiner and Levitz, 2018). Thus, in addition to iGluRs, the mGluR-TRPC coupling represents another main mechanism by which glutamate can induce excitotoxicity through $[Ca^{2+}]_i$ elevation and membrane depolarization, along with the consequent ionic imbalance, osmolarity changes and Ca^{2+} signaling (Toyoda et al., 2020), resulting in spatiotemporally distinct signaling effects that work in concert with those generated by iGluRs to induce cell demise.

Importantly, in spite of the promising results in reducing ischemic damage of brain tissues in animal models and the tremendous efforts made, antagonists of NMDA and AMPA receptors have not been effective in terms of neuroprotection against brain injury associated with ischemic stroke in clinical trials. Therefore, it would be beneficial to gain a better understanding of ischemic stroke pathology of the brain by exploring additional pathways, which will help identify new therapeutic candidates and approaches. Based on the current literature and our own experimental findings, we propose that TRPC channels play a critical part in neurological damage associated with ischemic stroke and should be evaluated as viable therapeutic targets for neuroprotection (**Figure 1**).

THE ROLES OF TRPC CHANNELS IN CELL DEATH

The seven mammalian TRPC members are divided into 4 subgroups (TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5). Among them, TRPC1, C3, C4, C5, and C6 are highly expressed in neurons at various brain regions, with distinct distributions and functions; for example, TRPC3 is highly expressed in cerebellar Purkinje neurons and involved in motor coordination, TRPC4 and TRPC5 are highly expressed in cerebral cortex, hippocampus and amygdala and functionally implicated in anxiety and fear learning (Clapham, 2003; Venkatachalam and Montell, 2007; Hartmann et al., 2008; Riccio et al., 2009; Riccio et al., 2014). In general, the TRPC channels in neurons are activated downstream from stimulation of $G_{q/11}$-coupled receptors, including mGluR1/5 (Kim et al., 2003; El-Hassar et al., 2011; Phelan et al., 2012), M1 muscarinic receptors (Yan et al., 2009; Tai et al., 2011), 5-HT2 serotonin receptor (Munsch et al., 2003; Sohn et al., 2011; Gao et al., 2017), H1 histamine receptor (Tabarean, 2012), kisspeptin receptor (Zhang et al., 2008), cholecystokinin (CCK) type 1 and type 2 receptors (Riccio et al., 2009; Wang et al., 2011), and thyrotropin-releasing hormone (TRH) receptors (Zhang et al., 2015). In addition, receptor tyrosine kinases, such as TrkB receptor through stimulation by brain-derived neurotrophic factor (BDNF) (Amaral and Pozzo-Miller, 2007; Li et al., 2010) and leptin receptor by leptin (Qiu et al., 2010), are also linked to TRPC channel activation in neurons through activation of PLCγ's. However, there are some exceptions. For instance, in olfactory bulb granule cells, the TRPC1/C4 heteromeric channels are dependent on NMDA receptors, instead of mGluRs, for activation that causes long lasting depolarization and sustained Ca^{2+} influx (Storch et al., 2012); the activation of TRPC-like channels in thalamic paraventricular nucleus neurons requires not only the $G_{q/11}$-coupled thyrotropin-releasing hormone receptors but also $G_{i/o}$-coupled cannabinoid receptors CB1 and CB2 (Zhang et al., 2015). This latter observation is very interesting in light of the recent findings that receptor-operated activation of TRPC4-containing channels requires coincident stimulation of $G_{i/o}$ protein signaling and PLC activities (Thakur et al., 2016, 2020; Jeon et al., 2020). In addition, some TRPC channels are also reported to be activated by nitric oxide, reactive oxygen species (ROS), thioredoxin, and Ca^{2+} store depletion (Yoshida et al., 2006; Xu et al., 2008; Ogawa et al., 2016). For more comprehensive discussions on activation mechanisms of TRPC channels, readers are referred to our recent review article (Wang et al., 2020).

All TRPC proteins form Ca^{2+}-permeable non-selective cation channels, which are tetramers composed of either identical (homotetramers) or different (heterotetramers) TRPC subunits.

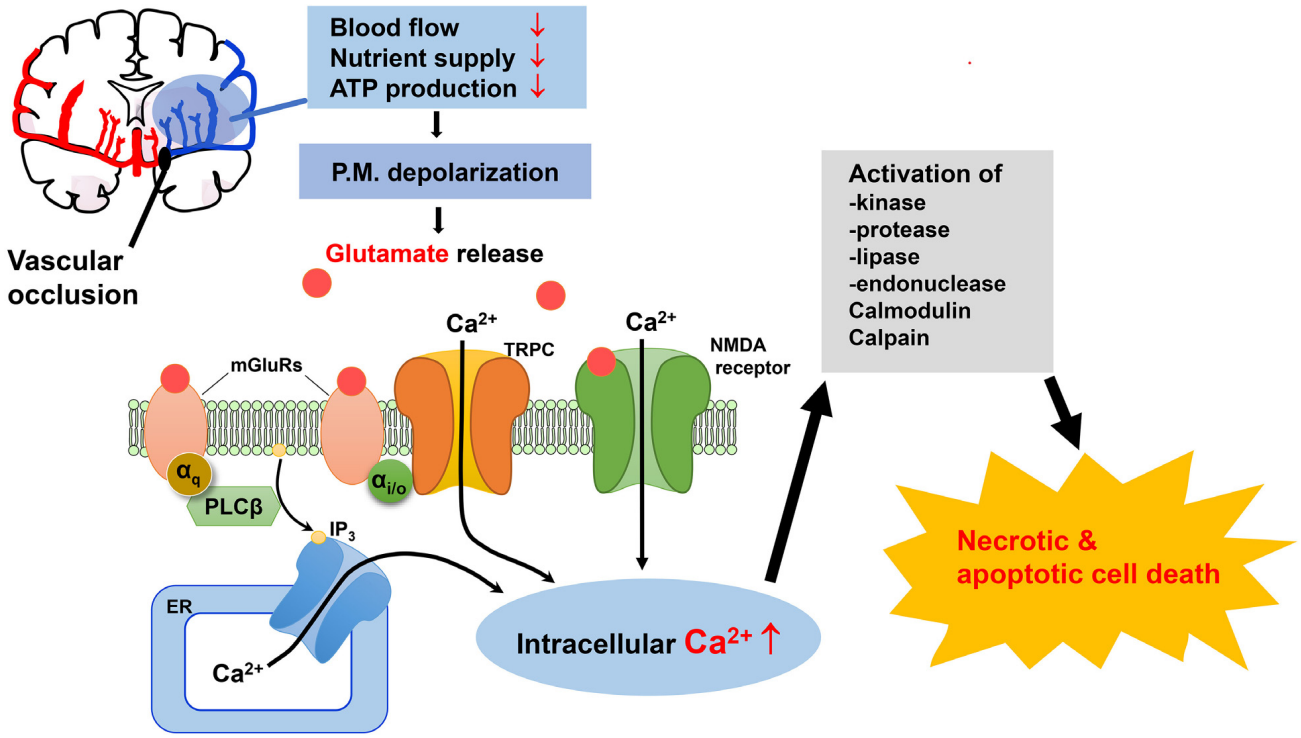

FIGURE 1 | Possible mechanisms for the involvement of TRPC channels in excitotoxic neuronal death in ischemic stroke. Oxygen and glucose deprivation due to the blockade of blood flow to brain areas in ischemic stroke causes excess accumulation of extracellular glutamate. The subsequent activation of NMDA receptors and mGluRs causes elevation of intracellular Ca^{2+} levels through Ca^{2+} influx from extracellular space and Ca^{2+} release from the ER, respectively. TRPC channels are activated downstream from mGluRs, causing more sustained Ca^{2+} elevation by mediating additional Ca^{2+} influx. TRPC4 and TRPC5 channels are also activated downstream from mGluR subtypes that couple to PTX-sensitive $G_{i/o}$ proteins, further expanding the repertoire of receptors that convey glutamate stimulation to Ca^{2+} signaling. Moreover, membrane depolarization resulting from activation of NMDA receptors and TRPC channels leads to excitation which also triggers additional Ca^{2+} influx through voltage-gated Ca^{2+} channels. The rise in intracellular Ca^{2+} due to these mechanisms causes activity changes of a plethora of enzymes, ion channels and transcription factors, as well as mitochondria, which when dysregulated induce cell death via either necrosis or apoptosis. ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; mGluRs, metabotropic glutamate receptors; NMDA, *N*-methyl-D-aspartate; P.M., plasma membrane; PTX, pertussis toxin.

Among the seven TRPC isoforms, TRPC1 has been the most frequently studied in the context of heteromeric channels (Storch et al., 2012), especially in the forms of TRPC1/C4 and TRPC1/C4/C5 heteromers (Phelan et al., 2012; Storch et al., 2012; Broker-Lai et al., 2017). However, TRPC1/C3 heteromers also form, at least in cortical astrocytes where they regulate astrogliosis in response to traumatic brain injury (Belkacemi et al., 2017). As is the case of TRPC1/C4 and TRPC1/C5 channels where TRPC1 reduced the channel conductance (Strubing et al., 2001), TRPC1 also serves to dampen the Ca^{2+} signal and the astrogliosis-promoting effect of TRPC3 (Belkacemi et al., 2017).

The activation of TRPC channels causes Na^+ and Ca^{2+} influxes into the cell, leading to membrane depolarization and $[Ca^{2+}]_i$ elevation. Both effects are important for TRPC channels to carry out their physiological functions. However, under conditions when these effects were not properly controlled, they can also contribute to pathology, reminiscent of iGluRs and other channels that damage cells through disrupting the membrane potential and/or causing Ca^{2+} overload. Due to differences in the biophysical properties, mechanisms of regulation, cell-type specific expression and subcellular distributions, the amplitudes, durations and subcellular locations of the depolarization and Ca^{2+} signals brought about by the activation of TRPC channels are likely very different from that by other channels. Furthermore, besides membrane depolarization and Ca^{2+} signals, TRPC channels also exert their effects on cell survival through other mechanisms, such as direct physical interactions with proteins involved in the cell death pathways (see later). Thus, the TRPC channels can have unique contributions to cell damage in difference diseases and different experimental models.

At least for TRPC4, a self-propagating positive-feedback mechanism has been proposed that allows persistent and prolonged channel activation (Thakur et al., 2020). In lateral septal neurons, this results in typically a depolarization plateau reaching to about −5 mV that lasts for about 1 s even when the stimulus, an agonist of mGluR1/5, is given for only 30 ms (Tian et al., 2014a). The all-or-none feature of the depolarization plateau is also consistent with the positive feedback mechanism. It was shown that this mechanism involves complex interactions among several intracellular messengers and protein partners, e.g., Ca^{2+}, H^+, and PLCδ1 (Thakur et al., 2020), the specific ranges of Ca^{2+} and PIP_2 concentrations as well as the optimal

membrane potentials required for channel activation (Thakur et al., 2016). The functional coupling with PLCδ1 is probably a major reason for the self-propagating activation of the TRPC4 channel, as the PLC isozyme is targeted by its own products, Ca^{2+} and H^+ (Thakur et al., 2020). PLC catalyzes the hydrolysis of PIP_2, producing diacylglycerol (DAG), IP_3 and H^+ (Huang et al., 2010). Subsequently, IP_3 releases Ca^{2+} from the ER store through activation of IP_3 receptors and depletion of the ER Ca^{2+} store is often accompanied with store-operated Ca^{2+} entry from the extracellular space (Putney, 2017). When coupled to TRPC4, Ca^{2+} influx through the TRPC4 channel also provides an additional source for greater and more prolonged $[Ca^{2+}]_i$ increase, and the Ca^{2+} then feeds back to reinforce the activities of both PLCδ1 and TRPC4. In excitable cells, VGCCs also strongly impact native TRPC4 activation by bringing additional Ca^{2+}, and perhaps also a part of the voltage sensitivity even though the TRPC channels are intrinsically voltage sensitive on their own (Gordienko and Zholos, 2004; Tian et al., 2014a). In addition, PIP_2, which is continuously replenished upon hydrolysis through the actions of phosphatidylinositol 4 kinases and phosphatidylinositol-4-phosphate 5-kinases (Myeong et al., 2018), serves as both the membrane anchor and substrate of PLCδ1, as well as the source of DAG, IP_3 and H^+, which are all implicated in TRPC4 activation one way or the other (Storch et al., 2017; Thakur et al., 2020). However, at high concentrations, PIP_2 also exerts a tonic block on TRPC4 activation (Otsuguro et al., 2008; Thakur et al., 2016), and similarly, high cytosolic Ca^{2+} concentrations also suppress the channel activity (Thakur et al., 2016, 2020). Moreover, the optimal membrane potentials for TRPC4 activation, especially native TRPC4-containing channels, have been observed at −40 to −60 mV, and are influenced by $[Ca^{2+}]_i$ (Gordienko and Zholos, 2004). Therefore, an intricate balance is created through complex interactions among Ca^{2+}, PIP_2, DAG, H^+, and membrane voltage to support the coupled activities of PLCδ1 and TRPC4, leading to sustained channel activity that lasts much longer than the time of exposure to the triggering stimuli. Notably, for TRPC4, the triggering stimuli include coincident activations of $G_{i/o}$ proteins and PLC signaling through either $G_{q/11}$-PLCβ or receptor tyrosine kinase-PLCγ pathways (Jeon et al., 2020). For other TRPC channels, $G_{i/o}$ proteins may not be involved and the depolarization amplitude and duration may not be as strong and as long, respectively, as that of TRPC4. However, the dual (both stimulatory and inhibitory) regulation by PIP_2 and Ca^{2+}, and the sensitivity to DAG are common among the TRPCs (Wang et al., 2020), although whether or not they exhibit a specific dependence on a specific PLC isozyme, as in the case of PLCδ1 for TRPC4 (Thakur et al., 2016), remains to be elucidated. It is possible that other TRPC channels also exhibit self-propagating activation like TRPC4, but with different kinetics, amplitudes, and durations.

At the cellular level, the most direct evidence of TRPC channel contribution to cell death comes from cytotoxic effect of (−)-englerin A, a guaiane sesquiterpenoid found in the bark of *Phyllanthus engleri*, on renal carcinoma cells (Akbulut et al., 2015; Carson et al., 2015) and triple-negative breast cancer (TNBC) cell lines (Grant et al., 2019) that express high levels of TRPC4 and/or TRPC1. It was found that englerin A exerts its cytotoxic effect through activation of TRPC4 and TRPC1/C4 channels endogenously expressed in the cancer cells. Intuitively, the cell killing action of englerin A was attributed to TRPC4-mediated Ca^{2+} influx and then intracellular Ca^{2+} overload, as Ca^{2+} overload by TRPC channels has indeed been reported to regulate apoptosis or other forms of programmed cell death in some cell types (Kondratskyi et al., 2015; Maher et al., 2018; Elzamzamy et al., 2020). However, a later study suggested that the cytotoxicity effect might largely result from the excessive Na^+ influx (Ludlow et al., 2017). It would appear that both the Na^+ and Ca^{2+} influxes mediated by TRPC channels are potentially harmful to the cell, if not properly controlled.

THE ROLE OF TRPCs IN NEURODEGENERATIVE DISEASES ASIDE FROM STROKE

Pathologically, excess glutamate accumulation is relevant to brain injury in many forms of neurodegenerative diseases. Therefore, before discussing the possible contributions of TRPC channels to brain injury associated with ischemic stroke, it is worthwhile to consider the involvement of TRPC channels in neuronal cell death in other neurodegenerative diseases, as they likely share some common mechanisms of excitotoxicity with ischemic stroke in damaging brain neurons. TRPC channels have been reported to play roles in a number of neurological disorders. Depending on the type of disease and the specific TRPC subtype, the activation of TRPC channels is not always detrimental to the neuron. Some TRPCs may actually be neuroprotective in certain neurodegenerative diseases.

A likely consequence of pathological accumulation of excess glutamate is coincident stimulation of both $G_{q/11}$- and $G_{i/o}$-coupled mGluRs. This provides conditions for strong and prolonged self-propagating activation of TRPC4 and TRPC5 channels, leading to membrane potential disruption and Ca^{2+} overload. Indeed, TRPC1/C4 and TRPC5 channels have been implicated in seizure-induced neuronal death in mouse lateral septum and hippocampus, respectively, in the pilocarpine-induced model of epilepsy (Phelan et al., 2012). Inhibiting TRPC5 also protected neurons in pyriform cortex, amygdala, and hippocampus from death in kainate-treated rats with prolonged seizures (Park et al., 2019) and in hippocampal CA3 neurons in a tramatic brain injury model (Park et al., 2020). In these models, TRPC5 was thought to be activated by oxidation which first triggered a rise in cytosolic Zn^{2+} levels and then opening of TRPC5 channels to mediate Ca^{2+} influx, leading to neuronal cell death (Park et al., 2019). In the Huntington's disease model, activation of TRPC5 through glutathionylation has also been shown to cause the loss of striatal neurons (Hong et al., 2015), and this effect can be mitigated through destabilizing the presence of TRPC5 on plasma membrane via depalmitoylation (Hong et al., 2020).

On the other hand, TRPC4-containing channels are not always associated with neuronal cell injury. TRPC1/C4 heteromeric channels have been reported to exert a protective role in neuronal cell death induced by subarachnoid hemorrhage

(Wang et al., 2016). This was suggested to occur through Ca^{2+} activation of calcineurin which suppresses NMDA receptor activity through dephosphorylation. Likewise, TRPC1 has been found to be neuroprotective in animal models of Parkinson's disease and Alzheimer's disease. In dopaminergic neurons of substantia nigra, TRPC1 inhibits L-type VGCCs by facilitating the interaction of STIM1 with Cav1.3 in response to ER Ca^{2+} store depletion and thereby dampening the neurotoxin-induced VGCC function that causes neuronal death (Sun et al., 2017). In addition, through store-operated Ca^{2+} entry, TRPC1 protects the dopaminergic neurons from neurotoxin-induced ER stress and the decrease of AKT/mTOR signaling (Selvaraj et al., 2012). Conversely, dopaminergic neurons respond to neurotoxin with decreased store-operated Ca^{2+} entry via attenuation of TRPC1 transcription, which is regulated by NF-κB (Sukumaran et al., 2018). Furthermore, the inhibition of TRPC1-mediated Ca^{2+} entry by neurotoxin is facilitated by sigma 1 receptor, an ER membrane protein acting as a chaperone to regulate Ca^{2+} release and other functions, which ultimately leads to the loss of dopaminergic neurons (Sun et al., 2020). In the mouse model of Alzheimer's disease, the deletion of *trpc1* gene was found to aggravate amyloid-β (Aβ)-induced learning and memory deficits and TRPC1 was shown to interact with Aβ precursor protein (APP) at the transmembrane region, resulting in reduced Aβ levels in hippocampal neurons and attenuation of apoptosis (Li et al., 2018). By contrast, the upregulated TRPC1 expression seen in SK-N-SH human neuroblastoma cells expressing a polyglutamine Huntingtin mutant was found to support a store-operated non-selective cation current and this activity contributed to glutamate-induced apoptosis of primary cultured striatal medium spiny neurons prepared from YAC128 mice that mimic neurodegeneration of Huntington's disease (Wu et al., 2011). Indeed, eliminating TRPC1 in YAC128 mice improved motor performance and rescued neuronal spines from progressive loss (Wu et al., 2018). Therefore, TRPC1 may be either detrimental or beneficial to brain neurons depending on the models and neuronal cell types.

TRPC3 has been speculated to play a part in ischemic injury of the cerebellum owning to it higher expression in this than in other brain regions (Cederholm et al., 2019). However, this regional specific effect of TRPC3 was found to change in aging monkeys and transgenic mice overexpressing human α-synuclein, where TRPC3 was found to become enriched in the mitochondria of striatal neurons and contribute to the disruption of mitochondrial membrane potential and cell apoptosis commonly seen in Parkinson's disease (Chen M. et al., 2017). Thus far, TRPC3 represents the only TRPC isoform that has been localized to mitochondria, in addition to its common plasma membrane localization, and involved in Ca^{2+} uptake into the organelle (Feng et al., 2013). Thus, the link of TRPC3 expression and function in mitochondria to α-synuclein upregulation implicates a role of TRPC3 in the pathogenesis of Parkinson's disease (Chen M. et al., 2017). In a rodent model of pilocarpine-induced status epilepticus, while the expression of TRPC3 was found to be increased, that of TRPC6 was decreased in CA1 and CA3 pyramidal neurons and dentate granule cells. It was reported that either inhibiting TRPC3 or enhancing TRPC6 function and/or expression protected neurons from seizure-induced injury (Kim et al., 2013). These findings support the detrimental effect of TRPC3 hyperactivation on brain neurons, but implicate that TRPC6 may exert an opposite effect.

Indeed, although closely related to TRPC3, TRPC6 has mainly been shown to be protective against neurodegeneration. For example, a system wide reduction of TRPC6 expression was found in patients with Alzheimer's disease and mild cognitive impairment, including blood cells, which negatively correlated with the cognitive performance (Lu et al., 2018; Chen et al., 2019). Moreover, neurons differentiated from induced pluripotent stem cells (iPSCs) derived from peripheral blood of sporadic Alzheimer's disease patients also exhibit decreased TRPC6 expression, as well as elevated Aβ and phosphorylated tau levels, hallmarks of Alzheimer's disease (Tao et al., 2020). Mechanistically, TRPC6 has been proposed to physically interact with APP via its second transmembrane segment, through which it inhibits the cleavage of APP by γ-secretase, leading to reduced Aβ production (Wang et al., 2015). That increasing TRPC6 expression or using a membrane penetrating peptide representing TRPC6's second transmembrane segment effectively lowered the levels of Aβ and phosphorylated tau in iPSC neurons derived from sporadic Alzheimer's disease patients supports a role of TRPC6 in suppressing the disease pathogenesis (Tao et al., 2020).

In a separate study, TRPC6 was found to form a complex with Orai2 that is regulated by STIM2 to conduct store-operated Ca^{2+} influx in dendritic mushroom spines of hippocampal neurons (Zhang et al., 2016). When this is impaired, the mushroom spines become unstable and disrupted, leading to memory loss. The beneficial effect of TRPC6 to Alzheimer's disease was demonstrated by showing that stimulating TRPC6 or the store-operated Ca^{2+} entry improved hippocampal long-term potentiation of the APP-presenilin 1 mutant mice, an experimental model of Alzheimer's disease (Zhang et al., 2016; Popugaeva et al., 2019). However, in a different model, where the expression of a familial Alzheimer's disease (FAD) presenilin 1 mutation bearing the deletion of the 9th exon (PSEN1ΔE9) in hippocampal neurons caused mushroom spine loss through enhancing, instead of suppressing, TRPC6-mediated store-operated Ca^{2+} entry, inhibiting such activity then becomes beneficial (Chernyuk et al., 2019). Therefore, depending on the nature of dysregulation, TRPC6 can be either beneficial or detrimental to neurons, highlighting the importance of maintaining the proper Ca^{2+} homeostasis. Previously, an inhibitory effect of presenilin 2 and its Alzheimer's-disease-linked variants on receptor-operated TRPC6 function has been demonstrated in heterologous expression systems, suggesting functional interaction between presenilins and TRPC6 (Lessard et al., 2005). In status epilepticus, TRPC6 serves to protect granule neurons of dentate gyrus from degeneration through activation of ERK1/2 and the subsequent phosphorylation of dynamin-related proteins 1 (DRP1) at Ser-616 (Ko and Kang, 2017) and an increase in the expression of a mitochondrial protease, Lon protease-1 (Kim et al., 2019). In the absence of TRPC6, the hypo-phosphorylation of DRP1 and reduction in Lon protease-1 then lead to mitochondrial elongation and dysfunction, which

increase the vulnerability of granule cells to seizure-induced death (Kim and Kang, 2015).

Taken together, the above findings suggest that TRPC channels exert differential effects on neurodegeneration in isoform and disease specific fashions. Although $[Ca^{2+}]_i$ dysregulation and membrane depolarization may be the common effects of TRPC channel activation, other mechanisms, including protein–protein interactions that involve specific regions of particular TRPC subtypes with certain disease-related protein partners and unique signaling pathways pertaining to the regulation of specific TRPC channels, are also involved. These diverse mechanisms underlie the differential outcomes resulting from TRPC channel function, ranging from neuroprotection to neurodegeneration. Therefore, it cannot be generalized that the extended activation of TRPC channels always leads to cell death, despite the high risk of Ca^{2+} overload.

THE ROLE OF TRPCs IN ISCHEMIC BRAIN INJURY

Brain injury after focal ischemic insult has been linked to the reduction of oxygen and nutrient (glucose) supply to the affected brain region (Lipton, 1999; Lo et al., 2003). Both *in vivo* MCAO model and the *in vitro* OGD model are commonly used to evaluate the involvement and mechanisms of TRPC channels in neuronal cell death associated with ischemic stroke. Like with the other neurodegenerative diseases discussed above, these studies revealed complex roles of different TRPC channels in ischemic brain injury.

For TRPC1, both the MCAO model and OGD assay suggested that TRPC1 plays a protective role against neuronal injuries caused by cerebral ischemia/reperfusion through suppression of ROS generation (Xu et al., 2018). TRPC1 expression was shown to be downregulated not only in brain tissues of mice subject to 90 min MCAO followed by 24 h reperfusion, but also in the cultured murine hippocampal cell line, HT22, that was exposed to an OGD culture for 4 h and then placed in the reoxygenated normal medium for 6–24 h. It was demonstrated that the amplitude of store-operated Ca^{2+} entry in the hippocampal cells was positively corrected with the expression TRPC1, but negatively correlated with the NADPH oxidase activity, and unrelated to the expression of STIM1 and Orai1 as well as mitochondrial ROS generation. However, no evidence was provided to suggest a direct inhibitory effect of Ca^{2+} on NADPH oxidase activity. Rather, TRPC1 inhibited NADPH oxidase-mediated ROS production through physically interacting with a catalytic component of the oxidase, Nox4, which facilitated Nox4 degradation along with cytoplasmic retention of the cytosolic subunits of the NADPH oxidase complex, p47phox and p67phox (Xu et al., 2018). This protective role of TRPC1 is opposite from the results of an earlier work, which showed that SKF96365 prevented cortical neuron death induced by 1.5 h OGD and 24 h reoxygenation (Wang et al., 2016). Since SKF96365 is a non-specific drug that inhibits all TRPC channels, and all forms of store- and receptor-operated Ca^{2+} entry, it did not inform which specific channel type was responsible for the protective effect.

On the other hand, the TRPC3/6/7 triple knockout mice were reported to be resistant to brain injury induced by MCAO followed by reperfusion (Chen X. et al., 2017). Here, the detrimental role of TRPC3/6/7 channels was attributed at least in part to astrocytes, in which these channels contributed to the enhanced NF-κB phosphorylation, reduced AKT phosphorylation and increased cell apoptosis following OGD and reoxygenation treatment (Chen X. et al., 2017). How this function of TRPC3/6/7, which promotes the death of astrocytes, is related or coordinated with astrogliosis, an abnormal proliferation of brain astrocytes observed after stroke and shown to be positively regulated by TRPC3 (Shirakawa et al., 2010; Munakata et al., 2013; Belkacemi et al., 2017), is an interesting question that needs to be addressed in future studies.

Although TRPC6 expression in mouse cortical neurons has been reported to be increased following brain ischemia/reperfusion *in vivo* and OGD/reoxygenation *in vitro*, and this increase contributed to neuronal injury in an NMDA receptor-dependent manner (Chen J. et al., 2017), nearly all other studies argue for a protective role of TRPC6 in neurological damage associated with ischemic stroke. The neuroprotective role of TRPC6 was suggested to occur through activation of cAMP response element-binding protein (CREB) signaling, which is disrupted following cerebral ischemia due to degradation of TRPC6 proteins in neurons by proteolytic cleavage mediated by calpain, non-lysosomal cysteine proteases activated as a result of $[Ca^{2+}]_i$ rise from NMDA receptor activation by elevated extracellular glutamate (Du et al., 2010). It was found that attenuating the calpain-mediated TRPC6 degradation could underlie the neuroprotective effects of several natural compounds, such as resveratrol, neuroprotectin D1, and (–)-epigallocatechin-3-gallate (the main ingredient of green tea), and calycosin (Lin et al., 2013a; Yao et al., 2013, 2014; Guo et al., 2017). That these protective effects were obliviated by inhibiting mitogen-activated protein kinase kinase (MEK) and/or calmodulin kinases suggests that these kinases mediate CREB activation downstream from TRPC6 (Lin et al., 2013b; Yao et al., 2013). On the other hand, increasing TRPC6 expression or its function through overexpression or treatment with hyperforin also inhibited NMDA receptor function and suppressed calpain activity (Li et al., 2012; Lin et al., 2013b), indicative of reciprocal regulations between TRPC6 and NMDA receptors/calpain. Once suggested as a TRPC6 agonist (Leuner et al., 2007), hyperforin was found to increase TRPC6 expression in mouse hippocampus following ischemia/reperfusion (Lin et al., 2013b). Hyperforin was also reported not to change TRPC6 channel activity, but acts independently of TRPC6 as a protonophore, which may explain its anti-depressive effects (Sell et al., 2014). Furthermore, the calpain cleavage of TRPC6 has also been reported to be activated by interleukin 17 (IL-17) (Zhang et al., 2014), a T-cell derived pro-inflammatory cytokine shown to contribute to ischemic brain injury (Gelderblom et al., 2012), suggesting an immune-neural interaction that exacerbates ischemic neurological damage through downregulating TRPC6.

In contrast to the rich information on TRPC6, little is known about the roles of TRPC4 and TRPC5 in brain injury associated with ischemic stroke. In one study, it was reported that TRPC4

protein expression was increased in rat striatal and hippocampal neurons at 12 h to 3 days after MCAO (Gao et al., 2004). Other studies speculating a detrimental function of TRPC5 in neurological deficits following stroke had based on the activation of TRPC5 by oxidation, without experimentation in the stroke model (Ishii et al., 2011; Park et al., 2019, 2020). In a very recent study, TRPC5 was found to directly interact with phospholipid scramblase 1 (PLSCR1) on the plasma membrane to facilitate externalization of phosphatidylserine (PS) and apoptosis of cortical neurons in response to cerebral ischemia/reperfusion (Guo et al., 2020). The same protein complex likely also includes TRPC1 and TRPC4. While the examination of cerebral slices of animals subject to ischemia/reperfusion revealed reduced PS externalization and apoptosis in TRPC5 knockout mice, whether these translate into protection against cerebral infarction and/or behavioral neurological impairments remains to be elucidated (Guo et al., 2020). On the other hand, overexpression of TRPC5 in spinal cord via adeno-associated viruses attenuated spinal cord ischemia/reperfusion injury in rats (Shen et al., 2020). This could result from the angiogenic function of TRPC5 which dampens the injury induced inflammation. Indeed, TRPC5 has been reported to promote endothelial cell sprouting, angiogenesis, and blood perfusion in ischemic tissues through activation of nuclear factor of activated T cell (NFAT) isoform c3 and angiopoietin-1 (Zhu et al., 2019). Therefore, the effect of TRPC4/C5 on ischemic brain injury may be very complex, including both positive and negative regulations that either exacerbate or prevent neuronal cell death.

TRPC4 IN ISCHEMIC BRAIN INJURY – OUR OWN STUDY

The above analysis indicates that despite the common mechanisms that link G protein and PLC pathways to TRPC channel activation and the likelihood that these channels participate in excitotoxicity triggered by excessive extracellular accumulation of glutamate through mGluRs, it is not easy to predict whether a specific TRPC channel type is detrimental or beneficial to brain neurons under conditions of cerebral ischemia/reperfusion. Depending on the TRPC isoforms, cell types involved, and mechanisms of actions, the TRPC channel may be neuroprotective or destructive.

To define the overall contributions of TRPC channels in brain damage associated with cerebral ischemia, we first compared neurological scores and brain infarction volumes of wild type and quadruple TRPC1, C4, C5, C6 knockout (QuadKO) mice subject to 40 min of MCAO using the standard intraluminal suture (thread) method, followed by 24 h reperfusion. In this method, a monofilament nylon surgical suture with a heat-rounded tip is inserted from an opening at external carotid artery and advanced through internal carotid artery until it reaches the middle cerebral artery to block the blood flow there. After the desired time period of occlusion, the filament was withdrawn to allow resupply of the blood to the blocked brain area (Alkayed et al., 1998; Sun et al., 2019). Since TRPC2 is mainly expressed in the vomeronasal system (Liman et al., 1999), TRPC3 mainly present in the cerebellum (Hartmann et al., 2008), and TRPC7 mostly found in peripheral tissues (Okada et al., 1999), we consider TRPC1, C4, C5, and C6 as the main cerebral TRPC subtypes expressed in areas typically affected in the MCAO model. As described above, both TRPC1 and C6 have been suggested to play protective roles against ischemic brain injury, but the function of TRPC4 and C5 remains unclear. Therefore, the QuadKO mice provide a good model to learn if TRPC channels generally serve a protective or detrimental role in cerebral ischemia. We found that on average, TRPC-QuadKO mice had better neurological scores (**Figure 2A**) and smaller brain infarct areas (shown by staining with 2,3,5-triphenyltetrazolium chloride, TTC) than the wild type mice (**Figures 2B,C**), suggesting that overall, TRPC channels contribute to causing damage of the brain in response to cerebral ischemia/reperfusion. Given that TRPC1 and TRPC6 may play some neuroprotective function during ischemia/reperfusion, it is plausible that the detrimental actions are mainly mediated by TRPC4 and/or TRPC5, and these two TRPC isoforms may dominate the cortical neuronal responses to ischemic insults. This interpretation is consistent with the finding that TRPC4/C5 expression is more abundant than other TRPC isoforms in several major areas of rat brain, including the prefrontal cortex (Fowler et al., 2007). Clearly, the distinct contributions of different TRPC isoforms in brain injury at different stages of cerebral ischemia/reperfusion warrant more detailed investigation.

Given that among the major brain TRPC isoforms, only TRPC4 has not been evaluated for its role in ischemic stroke, we then set out to compare brain damages caused by cerebral ischemia/reperfusion in wild type and TRPC4 knockout mice. Here, we performed transient focal middle cerebral artery/common carotid artery occlusion (MCA/CCAO) on the animals for 30, 90, and 120 min, followed by 24 h reperfusion. The MCA/CCAO is surgically performed using craniotomy to directly occlude distal MCA with a thin stainless wire (Aronowski et al., 1994; Zhao et al., 2015). This type of method was reported to produce reversible infarction and confined focal cerebral ischemia at distal MCA territory (Braeuninger and Kleinschnitz, 2009; Tavafoghi et al., 2016). We found that the infarct areas were markedly smaller in brains of *Trpc4*$^{-/-}$ mice than wild type animals, especially with shorter time of ischemia, i.e., 30 and 90 min (**Figures 2D,E**). Specifically, with the 30 min ischemia followed by 24 h reperfusion, whereas the infarctions were readily visible in wild type brains, they were hardly detectable in *Trpc4*$^{-/-}$ specimens. However, with a longer time (120 min) ischemia, the protective effect of TRPC4 knockout diminished (**Figures 2D,E**). These data indicate that TRPC4-containing channels contribute positively to ischemic brain damage, particularly during the early time period of the ischemia.

Next and to attribute underlying mechanisms more directly to TRPC4 function in neurons, we used cortical neuron cultures prepared from newborn wild type, *Trpc1*$^{-/-}$, *Trpc4*$^{-/-}$ and TRPC QuadKO mice to examine the effect of OGD-reoxygenation on cultured neurons *in vitro* (Ying et al., 1997). As illustrated in **Figure 3A**, cells were cultured for 16–18 days *in vitro* (DIV). On the day of the experiment, the culture medium was replaced with either a deoxygenated bicarbonate buffer without glucose (OGD) or an oxygenated bicarbonate

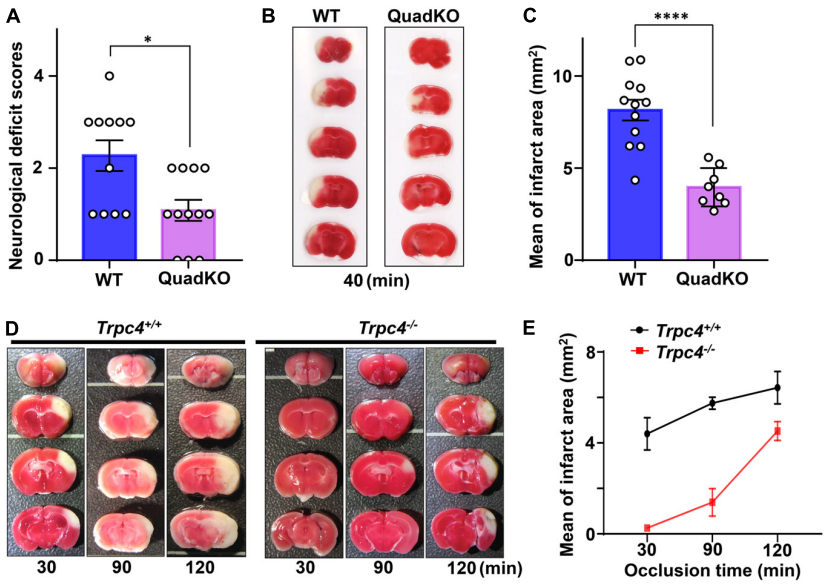

FIGURE 2 | Transient receptor potential canonical (TRPC) deficiency attenuates cerebral ischemic damage. **(A–C)** TRPC1, C4, C5, C6 quadruple knockout (QuadKO) and wild type control mice were subjected to reversible MCAO for 40 min using intraluminal suture method, followed by 22–24 h reperfusion. After assessing neurological deficits as described (Li et al., 2011) **(A)**, the animals were sacrificed and brain sections stained with TTC for quantification of infarct areas **(B,C)**. **(A)** Neurological scores of animals subjected to ischemia/reperfusion. Data represent means ± SEM for $n = 11$ (wild type) and 12 (QuadKO) mice. $^{*}P < 0.05$ by Mann–Whitney test. **(B)** Representative photographs of TTC-stained brain sections demarcating infarction. **(C)** Infarct areas at rostrocaudal plains were determined from morphometric analyses of TTC-stained brain sections. Data represent means ± SEM for $n = 12$ (wild type) and 8 (QuadKO) mice. $^{****}P < 0.0001$ by unpaired *t*-test. **(D,E)** *Trpc4*$^{-/-}$ and wild type control mice were subjected to reversible 30, 90, and 120 min MCA/CCAO, followed by 24 h reperfusion. **(D)** Representative photographs of TTC-stained brain sections. **(E)** Infarct areas at rostrocaudal plains were determined from morphometric analyses of TTC-stained brain sections. Data are presented as means ± range for $n = 2$ mice for each time point.

buffer with glucose (Control), and the cultures were maintained in a 37°C hypoxic incubator for 2 h with the oxygen level kept at 1% atmosphere. After the treatment, the neurons were either immediately evaluated for cell death by staining with propidium iodide (PI) or returned to the normal culture medium and maintained in the regular incubator at the normal oxygen level for 24 h before the PI staining. All cells were also stained with DAPI (4′,6-diamidino-2-phenylindole) to label the nuclei (**Figure 3B**). A comparison of the ratios of PI-positive/DAPI-positive labels, which represent percental cell death, revealed that wild type neurons were more vulnerable than the TRPC knockout neurons to the 2 h exposure of the bicarbonate buffer no matter if the buffer was oxygenated or not (**Figure 3C**). The reason for this is unclear, but could be related to the differences in the developmental status between the wild type and TRPC knockout cortical neurons cultured *in vitro*. Indeed, TRPCs play roles in several different aspects of neuronal development (Greka et al., 2003; Jeon et al., 2013; Tian et al., 2014b) and some of these could influence the sensitivity of the neurons to nutrient deprivation, given that the bicarbonate buffer does not contain serum and amino acids. This protective effect of TRPC depletion on neuronal survival in the serum and amino acid-deprived but glucose-containing buffer requires further investigation. Despite this, while the 2-h OGD treatment (without reoxygenation) did not immediately increase cell death further in wild type cortical neurons, it did so in all TRPC knockout neurons (**Figure 3C**), suggesting that OGD may interfere with the protective effect of TRPC knockout on cultured cortical neurons subject to serum and amino acid deprivation.

With the 24 h reoxygenation and nutrient replenishment (herein referred to as refeeding), cell death nearly doubled in wild type neurons that had been treated with OGD but not those exposed to the control buffer. The response to refeeding, however, was quite different between the three different TRPC knockout lines. The control buffer-treat QuadKO neurons displayed a marked increase in cell death after 24 h refeeding, but OGD-treated ones virtually did not change. However, while the *Trpc1*$^{-/-}$ neurons did not show any increase in death after refeeding regardless of the oxygen and glucose status during the starvation, the *Trpc4*$^{-/-}$ neurons exhibited a marked refeeding-dependent increase in cell death only when OGD treatment was applied (**Figure 3C**). These results, although difficult to interpret without more extensive investigations, underscore the complex and diverse roles different TRPC channels play in neuronal cell responses to nutrient shortage, OGD, and the subsequent replenishment of all nutrients, including oxygen.

To avoid the complications with the cultured neurons and the developmental effects of the TRPC knockout, we also performed OGD experiments using brain slices following a previous protocol (Taylor et al., 1999; Tasca et al., 2015). As illustrated in **Figure 4A**, the cerebral slices from wild type mice were recovered in artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2 and 5% CO_2 at 35°C for 1–2 h after cutting. Then they were transferred to a modified aCSF omitting glucose

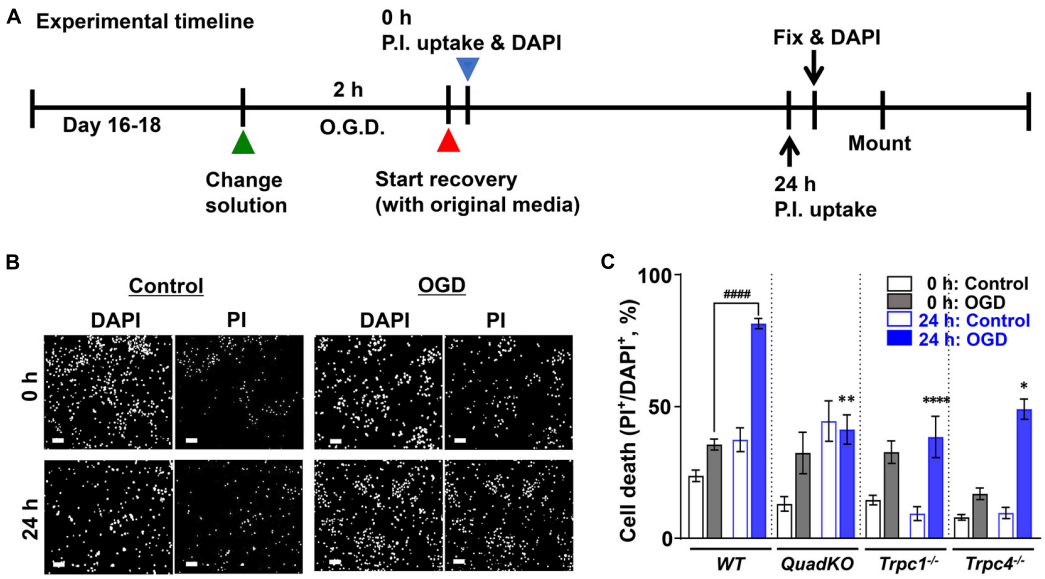

FIGURE 3 | Oxygen and glucose deprivation (OGD)-induced cell death of cultured cortical neurons from wild type, *Trpc1*$^{-/-}$, *Trpc4*$^{-/-}$, and *Trpc1/c4/c5/c6*$^{-/-}$ mice. **(A)** Schematic of the experimental timeline. Primary cultures of cortical neurons (16–18 DIV) from WT, *Trpc1*$^{-/-}$, *Trpc4*$^{-/-}$, and *Trpc1/c4/c5/c6*$^{-/-}$ (QuadKO) mice were treated without or with OGD for 2 h and then allowed to grow in normal medium for 0 or 24 h. **(B)** Representative images of DAPI staining (Bocksch et al., 2010) and PI uptake of cultured cortical neurons from wild type (WT) mice grown under control conditions or subjected to OGD for 2 h. Cells were stained either immediately after the treatment (0 h) or returned to normal culture conditions for 24 h before staining (24 h). Scale bars, 50 μm. **(C)** Summary data of PI-positive cells, normalized to the total cell number determined by DAPI staining. Data represent means ± SEM of $n = 20$ to 30 fields of views for each condition pooled from 4 coverslips of neurons from two separately prepared primary cultures. $^{\#\#\#\#}P < 0.0001$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. WT 24 h: OGD by one-way ANOVA.

and bubbled with N_2 (OGD) and maintained for 30 min at the same temperature. Control slices were treated with fresh oxygenated aCSF in parallel. After that, all slices were returned to fresh oxygenated aCSF and incubated for another 3 h. PI and Hoechst were added at 40 and 10 min, respectively, before the end of the incubation to label dead cells and nuclei of all cells. This was followed by fixation with 4% paraformaldehyde (12 h at 4°C) and mounting. In order to examine to what extent inhibiting TRPC4/C5 channels can protect against OGD-induced neuronal death and the possible therapeutic windows of targeting these channels, we also applied TRPC4/C5 antagonists, ML204 (Miller et al., 2011) and compounds 9 and 13 (cpd 9, cpd 13, analogs of M084) (Zhu et al., 2015), either at the beginning or the end of the OGD treatment. As shown in **Figures 4B,C**, after 30 min OGD and 3 h reoxygenation, the number and intensity of PI labeling in the cortex area were dramatically increased as compared to the control. Including the TRPC4/C5 antagonists either during or post OGD significantly decreased the damage. Since the TRPC4/C5 antagonists inhibit both TRPC4 or TRPC5 homomeric channels and TRPC1/C4 and TRPC1/C5 heteromeric channels, these data support the general idea that targeting TRPC1/C4/C5 channels can be beneficial to stroke therapy including post ischemic protection. Clearly, additional studies are needed to illustrate the details on how these TRPC channels contribute to neurological damage in response to ischemia and reperfusion and the appropriate intervention strategies that bring the most benefit with minimal detrimental effects.

DISCUSSION

Recent studies have demonstrated that TRPC channels play complex and even opposing functions in neuronal cell survival and death. Depending on the different TRPC subtypes, brain locations and cell types, as well as the specific pathological conditions, TRPC channels can be either detrimental or beneficial to neurons. However, the general theme is that for nearly every neurodegenerative disease, TRPC channels play crucial roles, and these include ischemic stroke and other conditions that cause brain injury. Because of the diverse mechanisms that have been revealed so far, it is also clear that no generalized mechanism should be used to explain or predict how a specific TRPC channel contributes to brain injury. Even for TRPC6, although most studies have indicated a pro-survival role, the molecular and cellular mechanisms elucidated from various studies have been very different, ranging from a direct interaction of the channel with a specific disease-causing protein (Wang et al., 2015), regulation of mitochondrial fission (Ko and Kang, 2017), to CREB-dependent transcription (Du et al., 2010) and suppression of NMDA receptor and calpain function (Li et al., 2012; Lin et al., 2013b). Therefore, future studies should focus not only on unique mechanism(s) that underlies the regulation of a specific TRPC channel in a particular form of neuronal injury, but also make attempts to reconcile the mechanism with existing knowledge and identify a common theme(s), if possible, that would enrich the mechanistic understanding of how the TRPC channel contributes to disease pathogenesis.

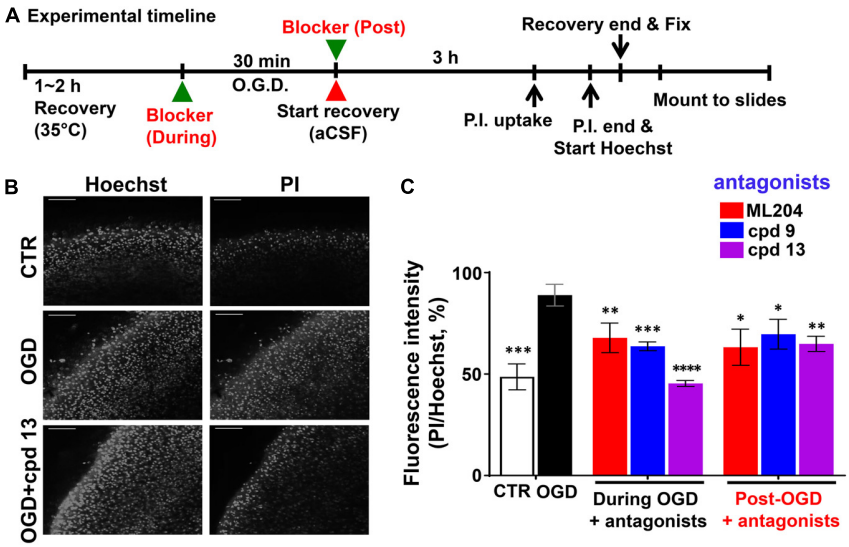

FIGURE 4 | Oxygen and glucose deprivation -induced neuronal death in the cerebral cortex of mouse brain slices. TRPC4/C5 blockers rescued OGD-induced neuronal death in the cerebral cortex of brain slices. **(A)** Schematic of the experimental timeline. **(B)** Representative images of Hoechst staining (left panels) and PI uptake (right panels) of acute brain slices (P15) from wild type (WT) mice without (CTR) or with OGD for 30 min in the absence or presence of TRPC4/C5 blocker, compound 13 (cpd 13). After the treatment, the slices were recovered for 3 h and stained with PI and Hoechst at 40 and 10 min, respectively, before the end of the 3 hr incubation. Scale bars, 100 μm. **(C)** Summary data of PI/Hoechst fluorescence ratios (random unit, but the same settings used for all conditions). TRPC4/C5 blockers, ML204 (at 50 μM), cpd 9, and cpd 13 (at 30 μM) prevented the OGD-induced cell death to varying degrees no matter if they were added during or after (post) the OGD treatment. Data represent means ± SEM of $n = 8$ to 15 fields of view of independently treated slices. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. OGD without antagonist by Student *t*-test.

Particularly for brain damage associated with ischemic stroke, the roles of TRPC channels at different stages of the ischemia/reperfusion and in different brain areas are far from clear. The acute deprivation of oxygen and glucose, as well as other nutrient supplies, certainly exerts a tremendous stress on neurons, causing many changes in signal transduction and intracellular and extracellular messengers that are linked to TRPC channels through either functional or physical coupling or both. These can be factors that directly activate the channel, such as DAG or ROS, or transmitters that work through G protein-coupled receptors, like glutamate, as well as neurotrophic factors that stimulate tyrosine kinases, e.g., BDNF. The combination of some of these factors may even bring synergistic effect, making some otherwise insignificant molecules into important contributors to TRPC channel activation that either induces neuronal death or protects them from the damages caused by other signals. Importantly, changes that occur in the ischemic stage do not just revert back to their original states after reperfusion or oxygenation. The restoration of nutrient supply and blood circulation also causes inflammation and oxidation, leading to stress responses that may evoke TRPC activation as well. Thus, a TRPC subtype may be involved in either the ischemic or reperfusion stage, or both stages, of the ischemia/reperfusion injury but with different outcomes. Furthermore, while irreversible damages can occur immediately at the ischemic core during OGD through necrosis, neurons in the neighboring regions (ischemic penumbra) more often display delayed cell death or apoptosis (Rami and Kogel, 2008). It is not known how different TRPC isoforms are involved in the different forms of cell death pathways, and therefore, it remains to be elucidated whether and how each of them plays a part in different stages and different brain regions (core *vs*. penumbra) in neurological damages associated with ischemia/reperfusion. These, together with the diverse mechanisms that have been reported for TRPC channel activation under pathological conditions, argue for the need of more careful and systematic examinations of TRPC channels in ischemic brain damage.

In practice, a stroke therapy is more useful in post-stroke than during or pre-stroke application since in most cases, the need for treatment is only realized after the stroke has occurred. Therefore, a better understanding of mechanisms that contribute to neuronal death during reperfusion and/or in ischemic penumbra instead of the core area, or any intervention that would facilitate the recovery of damaged neurons, if possible, from the death pathway will be desirable for therapeutic development. However, despite all the studies that have been discussed above, nothing is available on whether any TRPC channel would be a good target of post-stroke neuroprotection. Our *in vitro* experiment (**Figure 4**) suggests that it might be possible for the TRPC4/C5 inhibitors to have such a function, even though it might not be a complete protection. It may be common that targeting a single pathway or molecule post-stroke or post-OGD will not completely protect all neurons from ischemic injury, since even if the targeting is effective and represents the most critical one, irreversible damages that have occurred during ischemia will unlikely be rescued. Recently, the TRPC channel field has experienced an explosion of newly identified small molecular probes that can act as either selective agonists or antagonists of specific TRPC

channel subtypes (Wang et al., 2020). This gives an opportunity to test whether selectively enhancing or suppressing the function of a specific TRPC subtype at different time points post MCAO will exacerbate or inhibit brain damage and neurological deficits. Of course, these experiments will need to be followed by cell type specific manipulation of TRPC channel function or expression and other approaches in order to gain mechanistic insights on how the channel contributes to the delayed neuronal death.

Mechanistically, even though Ca^{2+} signal is an obvious downstream event of TRPC channel activation, there can be many different kinds of consequences which may or may not be related to the Ca^{2+} signal. The downstream targets of Ca^{2+} also remain largely undefined, although both CaMKII and calcineurin have been implicated. Ca^{2+}-dependent kinases could also include those that phosphorylate NMDA receptor NR2B subunits, e.g., DAPK1, which is known to play a role in stroke (Nair et al., 2013). TRPC channels can mediate DAPK1 activation through calcineurin or calmodulin (Shamloo et al., 2005; Nair et al., 2013). Also, as has been illustrated for TRPC6, calpain is another possible candidate of Ca^{2+} regulation and it is known to play a role in NMDA receptor-mediated cell death (Lankiewicz et al., 2000; Simpkins et al., 2003). Therefore, specific inhibitors of the above Ca^{2+}-regulated enzymes, calpain, CaMKII, calcineurin, and DAPK1 may be used to dissect their roles.

In summary, although TRPC channels appear to be ideally suited for integrating mGluR signaling to contribute to excitotoxicity to neurons under conditions of ischemic stroke, critical data demonstrating their involvement in neuronal damage or protection are only beginning to emerge. The pathological significance and roles of TRPC channels in ischemia/reperfusion remain to be fully elucidated. More detailed studies on regulatory mechanisms of TRPC channels will also shed lights on their functional significance in stroke pathology and future drug development.

METHODS

Animals

All animal procedures were carried out in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston. TRPC1 KO (*Trpc1*$^{-/-}$) and quadruple TRPC1, C4, C5, C6 KO (QuadKO) mice (129/SvEv and C57BL/6J mixed background) were created and maintained as described (Dietrich et al., 2007; Formoso et al., 2020); TRPC4 KO (*Trpc4*$^{-/-}$) in C57BL/6 background were generated and maintained as previously described (Freichel et al., 2001).

Mouse Ischemic Models

Focal ischemia (intraluminal suture) model of MCAO was carried out for 40 min followed by reperfusion as previously described (Sun et al., 2019). Animals were male QuadKO mice (8–9 weeks, ∼25 g) and age-matched wild type controls (C57BL/6J). In the MCA\CCAO model, unilateral occlusion of the left middle cerebral artery (MCA) and the left common carotid artery (CCA) was made as previously described (Aronowski et al., 1994; Zhao et al., 2015) using male wild type C57BL/6J and TRPC4 KO mice (8 weeks old). The occlusion times were 30, 90, and 120 min followed by reperfusion. With this method, the infarction is limited to the cortical tissue.

Oxygen and Glucose Deprivation (OGD) Models

Primary cultures of cortical neurons were established using postnatal day 0 mouse pups (M/F) according to the description (Facci and Skaper, 2012). Cortical tissues from wild type (C57BL/6J), TRPC1 KO, TRPC4 KO, and QuadKO pups were triturated in a papain dissociation solution (Worthington) supplemented with DNase (200 units/ml) and incubated for 1 h in 37°C CO_2 incubator. After stopping the reaction by adding ovomucoid (10 mg/ml), DNase (200 units/ml), and 10% fetal bovine serum in Earle's balanced salt solution, cells were plated at 2×10^5 per well in 500 μl of neurobasal medium supplemented with B27 (Invitrogen) and Glutamax (invitrogen) on poly-D-lysine (Sigma)-coated 12-mm round coverslips in 24-well culture plates. Another 500 μl of medium with arabinofuranosyl cytidine (1 μM final) was then added, and 20–30% of the medium was exchanged every other day until the cells were used for experiments (usually 16–18 days). For OGD, the medium was replaced with the deoxygenated glucose-free bicarbonate solution and the plate transferred to an anaerobic incubator containing 5% CO_2 and 94% N_2 (1% O_2) atmosphere for 2 h. After OGD, the cells were rinsed with oxygenated glucose (20 mM)-containing bicarbonate solution, fed with the original neurobasal medium, and cultured for 0 or 24 h at 37°C, 5% CO_2 (Ying et al., 1997).

Acute brain slices were prepared from wild type and TRPC4 KO mice (M, P15) as previously described (Tian et al., 2014a). Coronal slices (350 μm) were incubated in normal artificial cerebrospinal fluid (aCSF) consisting of (in mM): 125 NaCl, 26 $NaHCO_3$, 1 $MgSO_4$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 10 glucose, bubbled with 95% O_2/5% CO_2 at 35°C for at least 90 min before experiments. To induce OGD, slices were transferred to 15 ml conical tubes containing aCSF without glucose, which were bubbled with N_2/CO_2 at 35°C for 30 min. After the OGD treatment, the slices were returned to the glucose containing aCSF bubbled with 95% O_2/5% CO_2 at 35°C for 3 h.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare Committee of University of Texas Health Science Center at Houston.

AUTHOR CONTRIBUTIONS

JJ prepared illustrations and wrote the manuscript. MZ wrote the manuscript. All authors proofread and approved the manuscript.

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MINI REVIEW
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The Role of TRP Channels and PMCA in Brain Disorders: Intracellular Calcium and pH Homeostasis

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Brain disorders include neurodegenerative diseases (NDs) with different conditions that primarily affect the neurons and glia in the brain. However, the risk factors and pathophysiological mechanisms of NDs have not been fully elucidated. Homeostasis of intracellular Ca^{2+} concentration and intracellular pH (pH_i) is crucial for cell function. The regulatory processes of these ionic mechanisms may be absent or excessive in pathological conditions, leading to a loss of cell death in distinct regions of ND patients. Herein, we review the potential involvement of transient receptor potential (TRP) channels in NDs, where disrupted Ca^{2+} homeostasis leads to cell death. The capability of TRP channels to restore or excite the cell through Ca^{2+} regulation depending on the level of plasma membrane Ca^{2+} ATPase (PMCA) activity is discussed in detail. As PMCA simultaneously affects intracellular Ca^{2+} regulation as well as pH_i, TRP channels and PMCA thus play vital roles in modulating ionic homeostasis in various cell types or specific regions of the brain where the TRP channels and PMCA are expressed. For this reason, the dysfunction of TRP channels and/or PMCA under pathological conditions disrupts neuronal homeostasis due to abnormal Ca^{2+} and pH levels in the brain, resulting in various NDs. This review addresses the function of TRP channels and PMCA in controlling intracellular Ca^{2+} and pH, which may provide novel targets for treating NDs.

Keywords: TRP channels, brain pathology, neurodegenerative diseases, calcium, pH, homeostasis, neuron

INTRODUCTION

Calcium (Ca^{2+}) is a second messenger involved in numerous signal transduction pathways, including cell proliferation, cell growth, neuronal excitability, metabolism, apoptosis, and differentiation (Berridge et al., 2000; Gleichmann and Mattson, 2011; Maklad et al., 2019). Intracellular Ca^{2+} has a complex role in brain signaling and regulates brain physiology to maintain neuronal integrity (Marambaud et al., 2009; Bezprozvanny, 2010; Kawamoto et al., 2012). Ca^{2+} influx across the plasma membrane is important for fundamental brain functions which are mainly mediated by glutamate receptor channels, voltage-gated Ca^{2+} channels, sodium-calcium exchanger, and transient receptor potential (TRP) channels (Bezprozvanny, 2010; Cross et al., 2010; Gees et al., 2010; Cuomo et al., 2015; Kumar et al., 2016). Thus, Ca^{2+} signaling affects a variety of neuronal functions in diverse physiological roles, and Ca^{2+} must be tightly regulated to avoid uncontrolled responses that can lead to pathological conditions (Kumar et al., 2016). However, sustained increase in Ca^{2+} influx induces endoplasmic reticulum stress, mitochondrial dysfunction, and various proteases, resulting in neuronal cell death

(Bezprozvanny, 2010; Kawamoto et al., 2012). Indeed, impaired cell function caused by reactive nitrogen (oxygen) species and abnormal pH homeostasis also underpins the pathophysiology of neurodegenerative diseases (NDs) (Piacentini et al., 2008; Bezprozvanny, 2010; Gleichmann and Mattson, 2011; Zundorf and Reiser, 2011; Harguindey et al., 2017, 2019; Popugaeva et al., 2017). In particular, the maintenance of Ca^{2+} and pH levels is involved in a variety of NDs, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and age-related disorders (Harguindey et al., 2007; Kumar et al., 2009; Smaili et al., 2009; Ruffin et al., 2014; Hong et al., 2020; Thapak et al., 2020). Extensive literature indicates that an excessive increase in cytosolic Ca^{2+} and H^+ constitutes both direct and indirect ND-induced processes (Marambaud et al., 2009; Smaili et al., 2009; Bezprozvanny, 2010; Ruffin et al., 2014; Zhao et al., 2016; Harguindey et al., 2017).

TRP channels constitute a large family of membrane Ca^{2+} channels involved in a wide range of processes including thermoregulation, osmosis, pH, stretch, and chemical signaling (Kaneko and Szallasi, 2014). Functionally, activation of TRP channels influences Ca^{2+} signaling by allowing Ca^{2+} to enter the cell (cell depolarization), which may activate voltage-gated Ca^{2+} channels (Nilius and Owsianik, 2011; Vennekens et al., 2012). TRP channels in neuronal cells regulate voltage-gated Ca^{2+}, K^+, and Na^+ channels, whereas TRP channel regulation in glial cells results in reduced Ca^{2+} entry via ORAI by membrane depolarization, or increased Ca^{2+} influx through the hyperpolarization of the membrane (Gees et al., 2010). In the central nervous system, TRP channels are widely expressed throughout the brain and play an essential role in regulating Ca^{2+} homeostasis associated with various cellular functions, including synaptic plasticity, synaptogenesis, and synaptic transmission in a specific region of the brain (Venkatachalam and Montell, 2007; Kaneko and Szallasi, 2014; Jardin et al., 2017; Chi et al., 2018; Hong et al., 2020). In addition, TRP subtype channels are expressed simultaneously or separately in neurons and glia, fulfilling critical roles in cell homeostasis, development, neurogenesis, and synaptic plasticity (Vennekens et al., 2012). Several members of the TRP subtype are highly expressed in neurons and glia (Moran et al., 2004; Butenko et al., 2012; Ho et al., 2014; Ronco et al., 2014; Verkhratsky et al., 2014; Liu et al., 2017; Rakers et al., 2017) (**Table 1**). Thus, diverse TRP channels expressed in the brain are involved in the progression of NDs such as Parkinson's and Alzheimer's. In particular, increased intracellular Ca^{2+} via TRP channels contributes to various pathophysiological events (Venkatachalam and Montell, 2007; Kaneko and Szallasi, 2014; Moran, 2018; Hong et al., 2020) as well as brain disorders such as AD, PD, stroke, epilepsy, and migraine (**Table 1**)(Morelli et al., 2013; Kaneko and Szallasi, 2014; Kumar et al., 2016; Moran, 2018; Hong et al., 2020; Liu et al., 2020).

The normal regulation of intracellular Ca^{2+} levels involves mechanisms that control the specific uptake and extrusion mechanisms across the cell membrane (Kawamoto et al., 2012; Strehler and Thayer, 2018). Ca^{2+} influx is mediated by several voltage- and ligand-gated channels as well as transporters. Conversely, Ca^{2+} extrusion is dependent on Ca^{2+} pumps and Na^+/Ca^{2+} exchangers (Strehler and Thayer, 2018). Among these, plasma membrane Ca^{2+} ATPases (PMCAs) actively extrude Ca^{2+} ions out of cells (Boczek et al., 2019). Thus, these pumps are important gatekeepers for maintaining intracellular Ca^{2+} homeostasis in cells (Stafford et al., 2017; Boczek et al., 2019). However, PMCA dysfunction causes altered Ca^{2+} homeostasis and leads to a persistent increase in cytosolic Ca^{2+}, which can be neurotoxic and can accelerate the development of NDs and cognitive impairments as the person ages (Strehler and Thayer, 2018; Boczek et al., 2019). In particular, it is possible that the regulation of Ca^{2+} concentration might be more sensitive in which the cells are expressed both TRP and PMCA in the particular brain region (**Figure 1**). Thereby, abnormal expression of either TRP or PMCA subtype may be more likely to cause ND than other parts of the brain (**Figure 2**) (Minke, 2006; Stafford et al., 2017). In addition, PMCA activity is associated with intracellular acidification (Hwang et al., 2011) which is associated with neurological conditions observed among AD patients and other ND patients (Kato et al., 1998; Hamakawa et al., 2004; Mandal et al., 2012; Ruffin et al., 2014; Tyrtyshnaia et al., 2016).

It is crucial to investigate whether increased Ca^{2+} and (or) acidification are risk factors that affects ND-induced processes (Chesler, 2003; Hwang et al., 2011; Ruffin et al., 2014; Cuomo et al., 2015; Stafford et al., 2017; Boczek et al., 2019). Here, we review the involvement of TRP channels and PMCA in the pathophysiology of NDs.

BRAIN DISORDERS

Neurodegenerative Diseases

NDs such as AD, PD, HD, and ALS are age-related conditions characterized by uncontrolled neuronal death in the brain (Hong et al., 2020; Slanzi et al., 2020; Thapak et al., 2020). To date, several studies have reported that NDs are associated with protein aggregation, oxidative stress, inflammation, and abnormal Ca^{2+} homeostasis (Sprenkle et al., 2017). The impairment of Ca^{2+} homeostasis is known to result in increased susceptibility to NDs (Kumar et al., 2009; Smaili et al., 2009; Bezprozvanny, 2010; Gleichmann and Mattson, 2011; Kawamoto et al., 2012; Bagur and Hajnoczky, 2017). In particular, this impairment is associated with changes in Ca^{2+} buffering capacity, deregulation of Ca^{2+} channel activity, and alteration in other calcium regulatory proteins that occur in some types of neurons and glial cells in certain brain regions (Zundorf and Reiser, 2011; Nikoletopoulou and Tavernarakis, 2012). There is also increased Ca^{2+} influx mediated by abnormal TRP channel activation (Sawamura et al., 2017). Similarly, Ca^{2+} extrusion through PMCA has been shown to decrease in aged neurons (Jiang et al., 2012). For this reason, these NDs are associated with Ca^{2+} channels in neurons and glial cells (astrocytes, microglia, and oligodendrocytes), which are important for neuronal survival, myelin formation, neuronal support, and regulation of local neuron activity (neurons-glial signaling) (Zhang and Liao, 2015; Cornillot et al., 2019; Enders et al., 2020).

TABLE 1 | A summary of the transient receptor potential (TRP) subtypes found in distribution of central nervous system (CNS) cell types.

TRP channels		Expression in brain	Expression in glia	Disorders	References
TRPC subfamily	TRPC1	- Cerebellum, hippocampus, forebrain - Dopaminergic neuron (Human/mouse)	Astrocyte, microglia,	NDs, ADs, PD, HD,	Riccio et al., 2002; Bollimuntha et al., 2005, 2006; Selvaraj et al., 2009, 2012; Hong et al., 2015
	TRPC3	- Cerebellum, hippocampus, forebrain - Dopaminergic neuron (Human)	Astrocyte,	NDs, ADs, PDs	Rosker et al., 2004; Wu et al., 2004; Yamamoto et al., 2007; Mizoguchi et al., 2014
	TRPC4	Cerebellum, hippocampus, forebrain	Astrocyte,	Epilepsy	Wang et al., 2007; Wu et al., 2008; Von Spiczak et al., 2010; Tai et al., 2011
	TRPC5	- Cerebellum, forebrain - Hippocampus (mouse)	Astrocyte,	NDs, PDs, Epilepsy	Shin et al., 2010; Tai et al., 2011; Kaczmarek et al., 2012
	TRPC6	Cerebellum, hippocampus, forebrain, striatum	Astrocyte, microglia	NDs, ADs	Lessard et al., 2005; Wang et al., 2015; Liu et al., 2017; Lu et al., 2017
TRPM subfamily	TRPM2	- Hippocampus, forebrain - Cerebellum (human), cortex (rat)	Astrocyte, microglia	NDs, ADs, PDs	Fonfria et al., 2005; Kaneko et al., 2006; Hermosura et al., 2008; Ostapchenko et al., 2015
	TRPM7	- Cerebellum, forebrain, - Hippocampus (human) - cortex (mouse)	Astrocyte, microglia	NDs, ADs, PDs, Epilepsy	Aarts and Tymianski, 2005; Hermosura et al., 2005; Chen X. et al., 2010; Coombes et al., 2011; Oakes et al., 2019
TRPV subfamily	TRPV1	- Basal ganglia, hindbrain Cerebellum - Hippocampus (rat/mouse),	Astrocyte, microglia	NDs, AD, HD, epilepsy	Lastres-Becker et al., 2003; Kim et al., 2005; Gibson et al., 2008; Li et al., 2008; Lee et al., 2011; Balleza-Tapia et al., 2018
	TRPV4	Cerebellum, hippocampus,	Astrocyte, microglia	NDs, AD,	Auer-Grumbach et al., 2010; Chen D. H. et al., 2010; Landoure et al., 2010; Klein et al., 2011; Wang et al., 2019
TRPA subfamily	TRPA1	Cerebellum, hippocampus,	Astrocyte, oligodendrocyte	AD	Shigetomi et al., 2011; Lee et al., 2016; Saghy et al., 2016; Bolcskei et al., 2018

PMCA, plasma membrane Ca^{2+} ATPase; AD, Alzheimer's disease; PD, Parkinson's disease; ND, neurodegenerative disease.

Pathophysiological Role of TRP Channels

TRP channels are non-selective, Ca^{2+}-permeable channels that regulate diverse cellular functions in neurons (Nilius, 2007; Venkatachalam and Montell, 2007; Sawamura et al., 2017). Based on functional characterization of TRP channels by a wide range of stimuli (Zheng, 2013), aberrant activity of TRP channels likely initiates and/or propagates ND processes, especially cell death, via increased intracellular Ca^{2+} in various brain regions (Moran, 2018; Hong et al., 2020; Huang et al., 2020). Here, we focus on the function of TRP channels associated with Ca^{2+} signaling in neurons and glial cells (**Figure 1A**) (Nilius, 2007; Bollimuntha et al., 2011; Zheng, 2013; Zhang and Liao, 2015; Jardin et al., 2017; Sawamura et al., 2017; Hasan and Zhang, 2018; Samanta et al., 2018; Cornillot et al., 2019; Enders et al., 2020; Wang et al., 2020). Based on sequence homology, the TRP family currently comprises 28 mammalian channels and is subdivided into six subfamilies: TRP canonical (TRPC), TRP vanilloid (TRPV), TRP ankyrin (TRPA), TRP melastatin (TRPM), TRP polycystin (TRPP), and TRP mucolipin (TRPML) (Nilius, 2007; Selvaraj et al., 2010; Nishida et al., 2015; Sawamura et al., 2017). Most TRP channels are non-selective channels with consistent Ca^{2+} permeability (Samanta et al., 2018) and each TRP subtype responds to various temperatures, ligands, as well as specific agonists and activators (**Figure 1B**) (Luo et al., 2020). TRP channels are tetramers formed by monomers that share a common structure comprising six transmembrane domains and containing cation-selective pores (Hellmich and Gaudet, 2014). Numerous studies have reported that these TRP channels are related to neuronal cell death that is associated with abnormal Ca^{2+} homeostasis (Gees et al., 2010; Sawamura et al., 2017).

TRPC (Classic or Canonical)

TRPC was the first TRP group identified in mammals (Selvaraj et al., 2010). The TRPC subfamily contains members: TRPC1-7

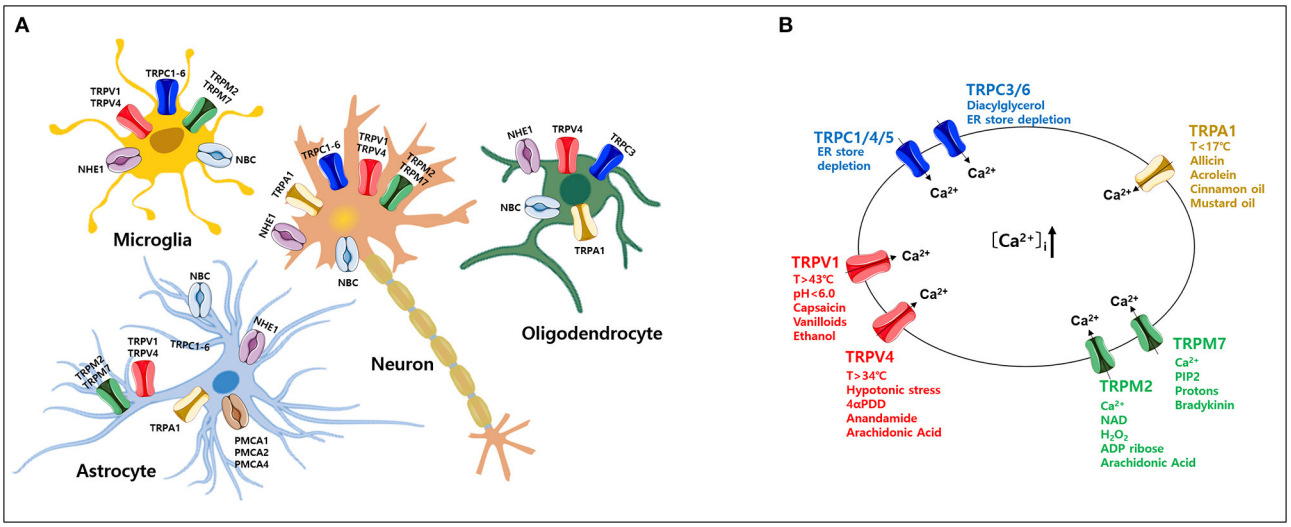

FIGURE 1 | Expression of various transient receptor potential (TRP) subtypes and calcium (Ca^{2+}) influx by their agonists in the mammalian central nervous system (CNS). **(A)** Expression profile of various TRP channels, NHE1, and NBC, in mammalian CNS cell types. **(B)** Ca^{2+} influx through activation of TRP subtypes by various agonists or activators in the mammalian CNS. TRP, transient receptor potential; PMCA, plasma membrane Ca^{2+} ATPase; NBC, Na^+/HCO_3^- cotransporters; NHE, Na^+/H^+ exchangers.

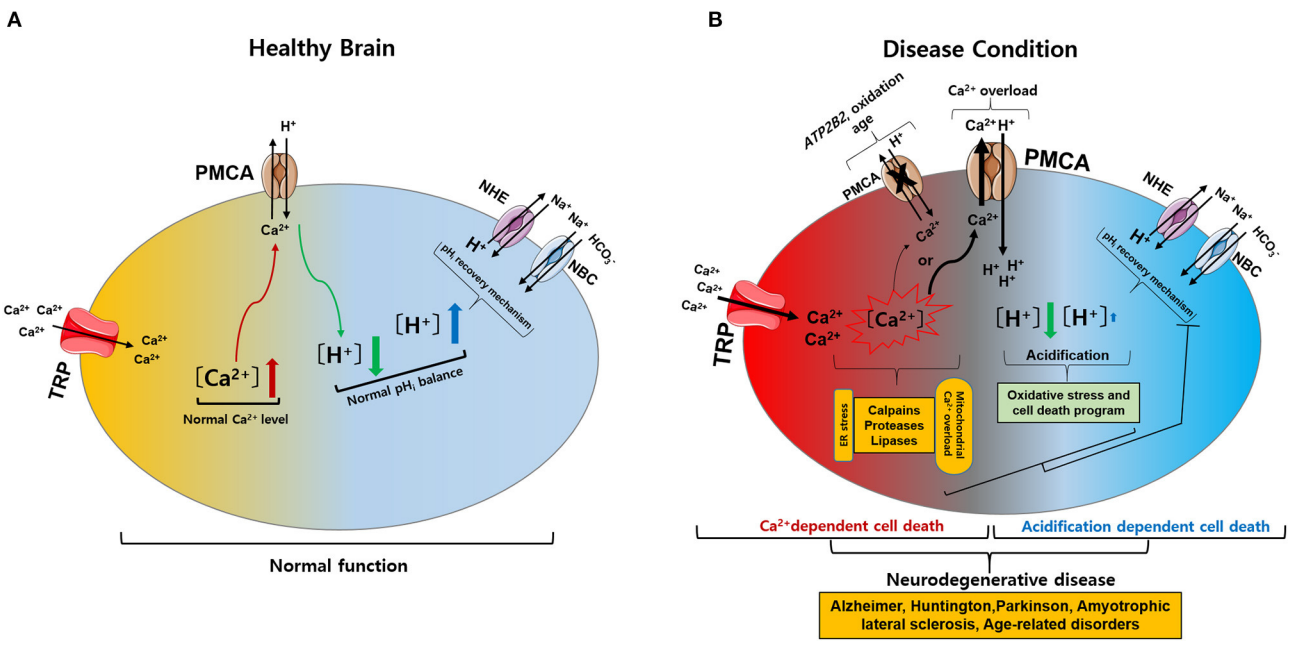

FIGURE 2 | Intracellular calcium (Ca^{2+}) and pH (pH_i) signaling by activation of TRP and PMCA in healthy and diseased condition of the brain. **(A)** Normal physiological function of intracellular Ca^{2+} and pH_i homeostasis. The activation of TRP channels leads to Ca^{2+} influx into the cytosol. Increased Ca^{2+} levels are regulated by PMCA. The activation of PMCA can cause acidification. Acidification conditions are mediated by pH_i recovery functions regulated by NBC and NHE. **(B)** Neurodegenerative diseases caused by pathophysiological functions of intracellular Ca^{2+} and pH_i homeostasis. (1) The activation of TRP channels leads to excess Ca^{2+} influx and overload Ca^{2+} is maintained due to *ATP2B2*, oxidation, and age-related downregulation of PMCA: Ca^{2+}-dependent cell death. (2) PMCA overexpression due to cytoplasmic Ca^{2+} overload cause persistent acidification from inhibition of the pH_i recovery mechanism by oxidative stress or cell death program: acidification dependent cell death. Ultimately, abnormal intracellular Ca^{2+} and pH_i levels impair neuronal function, resulting in neurodegenerative diseases. TRP, transient receptor potential; PMCA, plasma membrane Ca^{2+} ATPase; NBC, Na^+/HCO_3^- cotransporters; NHE, Na^+/H^+ exchangers.

(Wang et al., 2020). With the exception of TRPC2, all TRPC channels are widely expressed in the brain from the embryonic period to adulthood (Douglas et al., 2003). TRPC channels can form functional channels by heteromeric interactions, functioning as non-selective Ca^{2+} entry channels with distinct activation modes (Villereal, 2006). Thus, TRPC channels play an important role in regulating basic neuronal processes. TRPC1 is highly expressed and involved in the early development and proliferation of neurons (Yamamoto et al., 2005; Hentschke et al., 2006) as well as synaptic transmission (Broker-Lai et al., 2017; Wang et al., 2020). TRPC1 and TRPC4 have been reported to regulate neuronal cell death in response to seizures in the hippocampus and septum (Broker-Lai et al., 2017). The TRPC1/4/5 channel has been expressed in the somatosensory cortex, hippocampus, and motor cortex of adult rats (Riccio et al., 2002; Moran et al., 2004; Fowler et al., 2007). In particular, the dense expression of TRPC3 regulates hippocampal neuronal excitability and memory function (Neuner et al., 2015). The abnormal increase in sustained cytosolic Ca^{2+} by TRPC5 activation causes neuronal damage through the calpain-caspase-dependent pathway and the CaM kinase as seen in HD (Hong et al., 2015). Spinocerebellar ataxia type 14 (SCA14) is an autosomal dominant ND caused by a mutation in protein kinase Cγ (Wong et al., 2018). This mutation of SCA14 has been demonstrated to cause phosphorylation failure in TRPC3 channels, resulting in persistent Ca^{2+} entry that may contribute to neurodegeneration (Adachi et al., 2008). On the other hand, TRPC3 or TRPC6 promotes neurotrophin action on brain-derived neurotrophic factor (BDNF) by improving neuronal survival through Ca^{2+} influx (Huang et al., 2011). All TRPC channels are expressed in astrocytes and TRPC1 and TRPC3 play a critical role in astrocyte store-operated Ca^{2+} entry, which is induced by endoplasmic reticulum depletion (Verkhratsky et al., 2014). TRPC1 and TRPC6 are also expressed in rat microglia (Zhang and Liao, 2015). Thus, some TRPC channels exhibit different functions in normal physiological or pathological events, depending on Ca^{2+} signaling in the brain (Huang et al., 2011; Li et al., 2012; Neuner et al., 2015).

TRPM (Melastatin)

Of all TRP channels, the TRPM subfamily has the largest and most diverse expression levels and has been strongly implicated in NDs (Samanta et al., 2018). The TRPM channel consists of eight members (TRPM1-8) and shares common structural characteristics with other TRP channels (Huang et al., 2020). However, they have a variety of C-terminal sections with active enzyme domains and a unique N-terminal without ankyrin repeats involved in channel assembly and trafficking (Huang et al., 2020). A distinctive feature of TRPM channels is the regulation of Ca^{2+} and magnesium (Mg^{2+}) homeostasis, and TRPM (2–7) are mainly expressed in the CNS. In addition, TRPM2 is activated by a wide range of factors including NAD^{+}-related metabolites, adenosine diphosphate-ribose, oxidative stress, and depletion of glutathione (GSH) (Sita et al., 2018). Increased levels of reactive oxygen species (ROS) due to GSH depletion causes TRPM2-dependent Ca^{2+} influx to induce neuronal cell death, suggesting that several neurological disorders, including AD, PD, and bipolar disorder (Akyuva and Naziroglu, 2020). In addition, an increase in intracellular Ca^{2+} and Aβ induced by TRPM2 activity induces neuronal cell death in the rat striatum (Belrose and Jackson, 2018). Mg^{2+} is the second most abundant cation and essential cofactor in various enzymatic reactions (Ryazanova et al., 2010). TRPM2 is expressed by both microglia and astrocytes, which regulate gliosis and immune cell function (Wang et al., 2016; Huang et al., 2017). TRPM7 is permeable to Mg^{2+} and maintains Mg^{2+} homeostasis (Ryazanova et al., 2010). In mouse cortical neurons, inhibition of TRPM7 expression protects against neuronal cell damage (Asrar and Aarts, 2013; Huang et al., 2020). TRPM7 is also found in astrocytes and microglia to control migration, proliferation, and invasion (Siddiqui et al., 2014; Zeng et al., 2015).

TRPV (Vanilloid)

TRPV channels form homo- or heterotetrameric complexes and are non-selective cation channels (Startek et al., 2019). The TRPV subfamily consists of six members (TRPV1-6) that are located mostly on the plasma membrane (Zhai et al., 2020). Recent studies on pathological TRPV1 expression in the brain have been performed (Mickle et al., 2015). TRPV1 activation induces caspase-3 dependent programmed cell death through Ca^{2+}-mediated signaling, resulting in cell death of cortical neurons (Ho et al., 2012; Song et al., 2013) and also triggers cell death through L-type Ca^{2+} channels and Ca^{2+} influx in rat cortical neurons (Shirakawa et al., 2008). The activation of cannabinoid 1 (CB1) receptors stimulates TRPV1 activity, leading to increased intracellular Ca^{2+} and cell death of mesencephalic dopaminergic neurons (Kim et al., 2005, 2008). TRPV1 activation induces apoptotic cell death in rat cortical neurons, leading to chronic epilepsy distinguished by abnormal brain activity (Fu et al., 2009). TRPV1 activation in microglia plays a positive role in promoting microglial phagocytosis in damaged cells while disrupting mitochondria and increasing ROS production (Kim et al., 2006; Hassan et al., 2014). TRPV1 has been shown to affect the migration of astrocytes (Ho et al., 2014). Abnormal function of TRPV4 leads to neuronal dysfunction and axonal degeneration due to increased Ca^{2+} via Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Woolums et al., 2020). TRPV4 plays a role in regulating the osmotic pressure in the brain and is highly expressed throughout glial cells associated with ND (Liedtke and Friedman, 2003; Rakers et al., 2017). Thus, these channels play an important role in Ca^{2+} homeostasis and are therapeutic targets for various disorders.

TRPA (Ankyrin)

TRPA1 was first identified as an ankyrin-like transmembrane protein and the solitary member of the mammalian TRPA subfamily (Yang and Li, 2016). TRPA1 is a non-selective cation channel formed by homo- or heterotetramer subunits with a cytosolic N-terminal domain (16 ankyrin repeat sequence) and C-terminal Ca^{2+}-binding domains (Nilius et al., 2011; Fernandes et al., 2012). The TRPA1 channel responds to a variety of ligands, such as temperature, osmotic changes, and endogenous compounds (Nishida et al., 2015). To date, the reported role of TRPA1 in neurons is the mediation of pain,

cold, inflammation, and itch sensation (Fernandes et al., 2012). Recent reports indicate that TRPA1 hyperactivation causes Aβ oligomer-mediated rapid Ca^{2+} signaling (Bosson et al., 2017; Hong et al., 2020). Additionally, ablation of TRPA1 in APP/PS1 transgenic mice attenuated the progression of AD, improved learning and memory conditions, and reduced Aβ plaques and cytokines (Lee et al., 2016). Similarly, TRPA1 channels promote Ca^{2+} hyperactivity of astrocytes and then contribute to synaptic dysfunction due to the oligomeric forms of Aβ peptide (Lee et al., 2016; Bosson et al., 2017; Logashina et al., 2019; Alavi et al., 2020). In addition, TRPA1 mediates Ca^{2+} signaling in astrocytes, resulting in dysregulation of synaptic activity in AD (Bosson et al., 2017).

Other Channels

TRPML and TRPP have limited similarity to other TRP family members (Samanta et al., 2018; Huang et al., 2020). TRPML channels (TRPML1-3) are Ca^{2+} permeable cation channels that each contain six transmembrane segments with helices (S1–S6) and a pore site comprised of S5, S6, and two pore helices (PH1 and PH2) (Schmiege et al., 2018; Tedeschi et al., 2019). TRPML channels are mostly located in intracellular compartments instead of the plasma membrane (Clement et al., 2020). TRPP channels share high protein sequence similarity with TRPML channels and are located in the primary cilia consisting of TRPP1 (also known as PKD1) and TRPP2 (PKD2) (Samanta et al., 2018). To date, evidence indicates that various TRP channels are expressed in the CNS and play important roles in the development of several NDs (Sawamura et al., 2017; Samanta et al., 2018). In particular, TRP channels and Ca^{2+} homeostasis (Bezprozvanny, 2010) are likely to underpin Ca^{2+}-dependent neuronal death in NDs (Sawamura et al., 2017; Hong et al., 2020).

PATHOPHYSIOLOGICAL ROLE OF PLASMA MEMBRANE CALCIUM ATPases

Of the various proteins involved in Ca^{2+} signaling, PMCA is the most sensitive Ca^{2+} detector that regulates Ca^{2+} homeostasis (Boczek et al., 2019). PMCA exists in four known isoforms (Boczek et al., 2019). In both mice and humans, PMCAs 1–4 exhibit anatomically distinct expression patterns, such that isoforms 1 and 4 are ubiquitously expressed in all tissue types, whereas PMCA2 and PMCA3 are tissue-specific and exclusive in neurons of the brain (Kip et al., 2006). In addition, PMCA1, 2, and 4 were detected in rat cortical astrocytes (Fresu et al., 1999) (**Table 2**). The general structure of PMCA consists of 10 transmembrane domains (TM) with the N- and C-terminal ends on the cytosolic side (Stafford et al., 2017). The physiological functions of PMCA include the regulation and maintenance of optimal Ca^{2+} homeostasis (Bagur and Hajnoczky, 2017). PMCA is an ATP-driven Ca^{2+} pump that maintains low resting intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) to prevent cytotoxic Ca^{2+}overload-mediated cell death through activation of ion channels such as TRP (Zundorf and Reiser, 2011). In addition, PMCA is involved in Ca^{2+}-induced intracellular acidification by countertransport of H^+ ions (Vale-Gonzalez et al., 2006; Majdi et al., 2016). Thus, PMCA plays a vital role in controlling cell survival and cell death (Stafford et al., 2017). PMCA expression changes significantly during brain development

TABLE 2 | A summary of the transient receptor potential (TRP) subtypes found in distribution of central nervous system (CNS) cell types.

PMCA subfamily	Expression in brain	Expression in glia	Disorders	References
PMCA1	- Ubiquitous in brain (human and rat). - Cerebellum, cerebral cortex, brain stem (Human)	Rat cortical astrocytes	AD, PD	Stauffer et al., 1995; Fresu et al., 1999; Brini et al., 2013
PMCA2	- Cerebellar purkinje neurons (human/mouse) - cerebellum, cerebral cortex, brain stem (Human)	Rat cortical astrocytes	AD, PD, cerebellar ataxias, sensory neuron diseases	Stauffer et al., 1995; Fresu et al., 1999; Kurnellas et al., 2007; Empson et al., 2010; Hajieva et al., 2018; Strehler and Thayer, 2018
PMCA3	- Cerebellum, cerebral cortex (Human) - Cerebellum and hippocampus (Rat)	Limited	Cerebellar ataxias, sensory neuron diseases	Stauffer et al., 1995; Zanni et al., 2012; Strehler and Thayer, 2018
PMCA4	- Ubiquitous in brain (human/rat) - Cerebellum, cerebral cortex, brain stem (Human)	Rat cortical astrocytes	AD, PD	Stauffer et al., 1995; Fresu et al., 1999; Brini et al., 2013; Zaidi et al., 2018

PMCA, plasma membrane Ca^{2+} ATPase; AD, Alzheimer's disease; PD, Parkinson's disease.

(Boczek et al., 2019). One of the characteristics of brain aging is a Ca^{2+} homeostasis disorder, which can result in detrimental consequences on neuronal function (Boczek et al., 2019). Overall, PMCAs have been attributed a housekeeping role in maintaining intracellular Ca^{2+} levels through precise regulation of Ca^{2+} homeostasis (Strehler et al., 2007). However, the altered composition of PMCA is associated with a less efficient Ca^{2+} extrusion system, increasing the risk of neurodegenerative processes (Strehler and Thayer, 2018). *ATP2B2* is a deafness-associated gene that encodes PMCA2 (Smits et al., 2019). A recent study reported a link between PMCA2 and autism spectrum disorder (ASD) (Yang et al., 2013). ASD is a group of neurodevelopmental disorders that results in deficits in social interaction (Chaste and Leboyer, 2012; Fatemi et al., 2012). Intracellular Ca^{2+} levels are crucial for regulating neuronal survival, differentiation, and migration (Bezprozvanny, 2010). Perturbations in these processes underlie the pathogenesis of autism spectrum disorders (Gilbert and Man, 2017). *ATP2B3* mutations are associated with X-linked cerebellar ataxia and Ca^{2+} extrusion disorders in patients with cerebellar ataxia and developmental delay (Zanni et al., 2012; Mazzitelli and Adamo, 2014; Cali et al., 2015). Several neurotoxic agents, such as oxidation and age, downregulate PMCA function and increase susceptibility to NDs (Zaidi, 2010). In particular, the internalization of PMCA2 initiated by protease function in rat hippocampal pyramidal cells after glutamate exposure or kainate-induced seizures, in which loss of PMCA function occurs, may contribute to Ca^{2+} dysregulation and lead to neuronal cell death (Pottorf et al., 2006; Stafford et al., 2017). A decrease in PMCA activity and increased Ca^{2+} may cause cell death depending on the degree of cytosolic accumulation of tau and Aβ in AD (Boczek et al., 2019). In addition, PMCA expression is decreased in the cortex of postmortem brains of patients with AD (Berrocal et al., 2019; Boczek et al., 2019).

pH REGULATION BY PMCA IN NEURODEGENERATIVE DISEASES

As mentioned above, PMCAs have a Ca^{2+} extrusion function on the membrane and another important function, namely H^+ uptake (Stafford et al., 2017). Since PMCA is responsible for control of Ca^{2+} extrusion and H^+ uptake rates, it provides an important link between Ca^{2+} signaling and intracellular pH (pH_i) in neurons (Hwang et al., 2011). Mechanisms that maintain strict pH homeostasis in the brain control neuronal excitability, synaptic transmission, neurotransmitter uptake, nociception, and inflammation (Chesler, 2003; Dhaka et al., 2009; Casey et al., 2010; Hwang et al., 2011). Changes in pH caused via pH-sensitive or pH-regulated ion channels are detrimental to brain function and can cause multiple degenerative diseases (Ruffin et al., 2014). Neuronal excitability is particularly sensitive to changes in intracellular and extracellular pH mediated by various ion channels (Parker and Boron, 2013). The activation of TRPV1 has been reported to induce a rise in Ca^{2+} and cause intracellular acidification via the activation of PMCA in the rat trigeminal ganglion (Hwang et al., 2011). Under normal conditions, acidification conditions are promptly returned to and maintained at normal pH levels through a physiological pH_i recovery mechanism involving the regulation of Na^+/H^+ exchangers (NHE) and Na^+-HCO_3^- cotransporter (NBCs) in the brain (Chesler, 2003; Sinning and Hubner, 2013; Ruffin et al., 2014; Bose et al., 2015). NHE1 is abundantly expressed in all neuronal cells and astrocytes, regulating cell volume homeostasis and pH_i (Song et al., 2020). NBC1 is also widely expressed in astrocytes throughout the brain (Annunziato et al., 2013) (**Figure 1A**). However, functional inhibition of pH_i recovery mechanism in pathological conditions leads to excessive intracellular acidification (Majdi et al., 2016). Therefore, although the exact underlying mechanism that causes intracellular acidification in brain neurons is unknown. However, it appears that persistent intracellular acidification condition promotes irreversible neuronal damage and induces amyloid aggregation in the brains of patients with AD (Xiong et al., 2008; Ruffin et al., 2014).

CONCLUSION

Intracellular Ca^{2+} and pH regulation play vital roles in both physiological and pathological conditions. Abnormal changes in Ca^{2+} or pH typically cause cell death. TRP channels are involved in Ca^{2+} influx, which affects neuronal and glial functions under normal physiological conditions. However, altered expression of TRP channels can lead to excess Ca^{2+} influx, and intracellular Ca^{2+} overload is maintained due to *ATP2B2*, oxidation, and aging-related downregulation of PMCA, leading to Ca^{2+}-dependent cell death. Alternatively, overexpression of PMCA due to cytoplasmic Ca^{2+} overload causes continuous acidification from inhibition of the pH_i recovery mechanisms by oxidative stress or programmed cell death, resulting in acidification-dependent cell death (**Figure 2**) (Harguindey et al., 2017, 2019). To date, TRP channels have been investigated for their role in NDs. However, targeting TRP channels and PMCA, including Ca^{2+} and pH regulation, as a treatment for NDs requires a deeper understanding of their function in both health and disease. This review describes potential therapeutic targets for NDs by discussing TRP channels and PMCA responsible for the disruption of intracellular Ca^{2+} and pH homeostasis that underpin ND development.

AUTHOR CONTRIBUTIONS

C-KP and YK conceived and supervised the project. S-MH, JL, C-KP, and YK wrote the paper. All authors contributed to the article and approved the submitted version.

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TRPC6 Attenuates Cortical Astrocytic Apoptosis and Inflammation in Cerebral Ischemic/Reperfusion Injury

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Transient receptor potential canonical 6 (TRPC6) channel is an important non-selective cation channel with a variety of physiological roles in the central nervous system. Evidence has shown that TRPC6 is involved in the process of experimental stroke; however, the underlying mechanisms remain unclear. In the present study, the role of astrocytic TRPC6 was investigated in an oxygen–glucose deprivation cell model and middle cerebral artery occlusion (MCAO) mouse model of stroke. HYP9 (a selective TRPC6 agonist) and SKF96365 (SKF; a TRPC antagonist) were used to clarify the exact functions of TRPC6 in astrocytes after ischemic stroke. TRPC6 was significantly downregulated during ischemia/reperfusion (IR) injury in cultured astrocytes and in cortices of MCAO mice. Application of HYP9 *in vivo* alleviated the brain infarct lesion, astrocytes population, apoptosis, and interleukin-6 (IL-6) and IL-1β release in mouse cortices after ischemia. HYP9 dose-dependently inhibited the downregulation of TRPC6 and reduced astrocytic apoptosis, cytotoxicity and inflammatory responses in IR insult, whereas SKF aggravated the damage *in vitro*. In addition, modulation of TRPC6 channel diminished IR-induced Ca^{2+} entry in astrocytes. Furthermore, decreased Ca^{2+} entry due to TRPC6 contributed to reducing nuclear factor kappa light chain enhancer of activated B cells (NF-κB) nuclear translocation and phosphorylation. Overexpression of astrocytic TRPC6 also attenuated apoptosis, cytotoxicity, inflammatory responses, and NF-κB phosphorylation in modeled ischemia in astrocytes. The results of the present study indicate that the TRPC6 channel can act as a potential target to reduce both inflammatory responses and apoptosis in astrocytes during IR injury, subsequently attenuating ischemic brain damage. In addition, we provide a novel view of stroke therapy by targeting the astrocytic TRPC6 channel.

Keywords: apoptosis, astrocytes, Ca^{2+}, inflammation, ischemic stroke, NF-κB, TRPC6

INTRODUCTION

The transient receptor potential (TRP) channels are cation-permeable membrane proteins with common structural features of six transmembrane segments. Based on the homology of amino acid sequences, the TRP superfamily can be subdivided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPN (*Drosophila* NOMPC), TRPP (polycystin), and TRPML (mucolipin) (Venkatachalam and Montell, 2007). Among the channels, the TRPC channel, which is most closely related to *Drosophila* TRPs, is widely distributed in different tissues

and governs the fate and functions of various cell types (Curcic et al., 2019). TRPCs are broadly expressed in brain, lung, heart, kidney, liver, spleen, and other organs in mammalian animals like human, mouse, rat, and rabbit (Montell, 2001; Venkatachalam and Montell, 2007). In terms of similarity to amino acid sequences and function, TRPC members can be further classified into four subgroups: TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7 (Wang et al., 2020a).

The TRPC6 channel is emerging as an important target for the control of Ca^{2+} currents in a wide range of disorders, including immune-mediated diseases, pulmonary arterial hypertension, atherosclerosis, and central nervous system (CNS)-related diseases, such as autism spectrum disorders, glioma, depression, traumatic brain injuries, seizure, Alzheimer's disease, and ischemic stroke (Hamid and Newman, 2009; Ding et al., 2010; Du et al., 2010; Griesi-Oliveira et al., 2015; Kim and Kang, 2015; Zhang et al., 2015, 2016; Pochwat et al., 2018; Ramirez et al., 2018; Chen et al., 2019). TRPC6 is abundantly expressed in various anatomical regions of the CNS, such as the cerebellum, hippocampus, middle frontal gyrus, and cortex (Riccio et al., 2002; Du et al., 2010). As a regulator of Ca^{2+} influx, TRPC6 is involved in neuronal survival, synapse formation, neuronal nerve-growth-cone guidance, and sensory transduction (Li et al., 2005; Jia et al., 2007; Zhou et al., 2008; Quick et al., 2012). Dysfunction of the TRPC6 channel may trigger a series of downstream events and neurobiological disorders.

Ischemic stroke is a life-threatening condition caused by a vascular embolism due to cardiac events, artery-to-artery embolism, or *in-situ* small artery disease (Hankey, 2017). Several underlying mechanisms, including excitotoxicity, ionic imbalance, oxidative and nitrative stress, inflammation, and apoptosis, are involved in the pathophysiological process of cerebral ischemia (Khoshnam et al., 2017). Ca^{2+} overload has a critical role and initiates the ischemic cascade during brain ischemia/reperfusion (IR) injury. Increasing evidence indicates an important role of TRPC6 in cerebral IR injury (Liu et al., 2020). TRPC6 is identified on cortical neurons and astrocytes, and is downregulated in neurons after brain ischemic injury (Du et al., 2010; Guo et al., 2017; Qu et al., 2017; Shirakawa et al., 2017). Notably, maintaining the TRPC6 protein level in neurons improves neuronal survival and behavioral performance, thus alleviating ischemic brain damage (Du et al., 2010; Guo et al., 2017). However, the roles of the TRPC6 channel in mouse cortical astrocytes following IR injury have not been evaluated.

In the present study, the specific effects of astrocytic TRPC6 on ischemic stroke were investigated. TRPC6 protein expression in primary mouse astrocytes was downregulated following IR injury. Inhibition of TRPC6 downregulation via HYP9 or TRPC6 overexpression protected astrocytes and the brain against IR insults. These results indicate that the TRPC6 channel contributes to neuroprotection in cerebral ischemia by promoting astrocyte survival. Furthermore, this study provides therapeutic evidence for the treatment of ischemic stroke by targeting the TRPC6 channel.

MATERIALS AND METHODS

Animals

C57BL/6J male mice 8–10 weeks of age were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments were approved by the Animal Care Committee of the First Affiliated Hospital at Zhejiang University. Mice were housed in polypropylene cages and maintained at $25 \pm 1^{\circ}C$ under 12 h light/12 h dark cycles with free access to rodent chow and water. Mice were randomly allocated to each group before any treatment. Proper anesthetic procedures were used to ensure that the mice did not suffer unnecessarily during or after the experimental procedure. A total of 82 mice were used in this study (including 10 mice that died).

In vivo Model of Focal Cerebral Ischemia

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). Animals were anesthetized with 1% sodium pentobarbital (75–100 mg/kg, intraperitoneal injection); body temperature was maintained at $37 \pm 0.5^{\circ}C$ during the operation using a heating pad. Transient MCAO was generally performed as previously reported (Lin et al., 2013a). Briefly, the left common carotid artery was exposed to separate the internal carotid artery (ICA) and the external carotid artery (ECA). Next, a 6-0 monofilament nylon suture (RWD Life Science Co., LTD, Shenzhen, China) with a rounded tip was inserted through the exposed left ICA and advanced into the middle cerebral artery. After 1 h of occlusion, the filament was gently withdrawn to allow reperfusion. At 24 h after reperfusion, animals were sacrificed and the brain tissues were obtained for future assays.

Intracerebroventricular Injection

Mouse intracerebroventricular injection was performed using a stereotaxic instrument (RWD Life Science Co., Ltd., Shenzhen, China) with a micro-syringe pump under anesthesia. Drugs or vehicle (5 μL in total) were slowly injected (1 μL/min) into the left ventricle at a depth of 2.5 mm below the brain surface, 1.0 mm lateral and 0.5 mm posterior to the bregma. After injection, animals were allowed to recover from anesthesia under a heating pad.

Cell Culture

As previously reported(Shen et al., 2016), primary astrocytes from cerebral cortices of 0–1-day-old post-natal C57BL/6 mice were isolated under sterile conditions. Astrocytes were grown in culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin]. Dissociated cortical cells were seeded onto poly-D-lysine (PDL, 10 μg/mL; # P7405; Sigma-Aldrich St. Louis, MO, USA)-coated T-75 flasks (Costar; Corning Inc., Corning, NY, USA) at a density of three cortices per flask and incubated at 37°C with 5% CO_2 in a humidified incubator. The medium was changed every other day. After 8–10 days, confluent cultures were shaken (250 rpm at 37°C) for 12 h to reduce microglial contamination. Then, purified astrocytes were cultured in medium supplemented with 20 μM cytosine-1-β-D-arabinofuranosid (Sigma-Aldrich) for the next 2–3 days. The remaining attached cells were digested with 0.25% trypsin

(#25200056; Gibco, Grand Island, NY, USA) and then reseeded at a density of 0.03–0.05 × 10^6/cm^2 in PDL-coated six-well plates at 37°C in an incubator with 5% CO_2. After 5–7 days when the astrocytes reached 90–95% confluence, cells were prepared for subsequent experiments. More than 95% of cells in culture were astrocytes (results not shown).

Oxygen-Glucose Deprivation and Re-oxygenation (OGD/R)

OGD/R experiments were established to mimic the *in vitro* condition of IR injury. The cultures were incubated for 1–4 h in DMEM medium without glucose in a humidified incubator chamber at 37°C with 95% N_2 and 5% CO_2. Subsequently, the astrocytes were returned to the original culture conditions and then incubated under normoxic conditions for the next 24 h.

Drugs and Experimental Groups

The TRPC6-specific agonist HYP9 (Leuner et al., 2010) (#H9791; Sigma-Aldrich), dissolved in dimethyl sulfoxide (DMSO; the final maximum DMSO concentration was <0.05%), was used to determine the role of TRCP6 in astrocytes following IR injury. To verify the optimum concentration, 0, 1, 5, 10, 15, 20, or 30 μM HYP9 was preincubated *in vitro*. SKF96365 (SKF; #ab120280; Abcam, Cambridge, MA, USA) was originally recognized as a major inhibitor of TRPC channels (Singh et al., 2010). In the present study, SKF (dissolved in deionized water) at 0, 0.05, 0.5, 5, 10, 20, 30, or 40 μM concentration was used to treat primary mouse astrocytes. The *in vitro* groups consisted of eight subgroups: (1) control group (CON + Naive); (2) control combined with vehicle (DMSO) group (CON + Vehicle); (3) control combined with HYP9 (15 μM) group (CON + HYP9); (4) control combined with SKF (30 μM) group (CON + SKF); (5) OGD/R group (OGD + Naive); (6) OGD/R combined with vehicle (DMSO) group (OGD + Vehicle); (7) OGD/R combined with HYP9 (15 μM) group (OGD + HYP9); (8) OGD/R combined with SKF (30 μM) group (OGD + SKF).

Animals were randomly assigned into eight groups before any procedure as follows: (1) sham operation group (sham + Naive) ($n = 9$); (2) sham operation combined with vehicle (DMSO) group (sham + Vehicle) ($n = 3$); (3) sham operation combined with HYP9 (5 μg) group (sham + HYP9) ($n = 3$); (4) sham operation combined with SKF (20 μg) group (sham + SKF) ($n = 3$); (5) MCAO operation group (MCAO + Naive) ($n = 18$); (6) MCAO operation combined with DMSO group (MCAO + Vehicle) ($n = 12$); (7) MCAO operation combined with HYP9 (5 μg) group (MCAO + HYP9) ($n = 12$); (8) MCAO operation combined with SKF (20 μg) group (MCAO + SKF) ($n = 12$). **Figure 1** shows the experiment paradigm.

Lentivirus Infection

Lentivirus vectors were chemically synthesized by Obio Technology (Shanghai) Corp., Ltd. To overexpress astrocytic TRPC6, we infected primary astrocytes with lentiviruses carrying FLAG-tagged full-length mCherry-WT-TRPC6 (WT-TRPC6) or mCherry-WT-TRPC6-null (Vehicle1). Besides, to knock down TRPC6 in primary astrocytes, lentiviruses carrying mCherry-shRNA-TRPC6 (sh-TRPC6) or mCherry-shRNA-TRPC6-null (Vehicle2) were used to infect primary cortical astrocytes. Targeted sequences for TRPC6 and Vehicle2 siRNAs are: GCTTGCCAACATTGAGAAA and TTCTCCGAACGTGTCACGT, respectively. Astrocytes at 70% confluence were infected with lentiviruses according to the vendor's protocol. After 72–96 h lentiviral infection, astrocytes were used for subsequent experiments. The efficiency of infections was detected by western bolts.

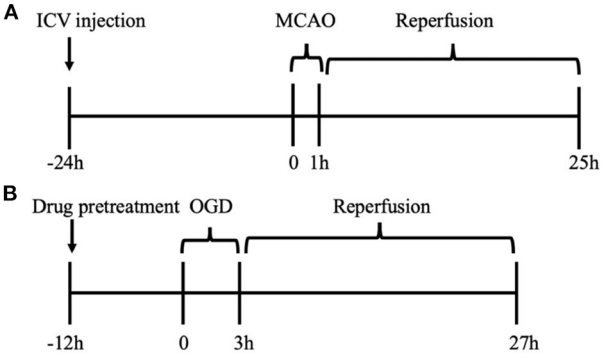

FIGURE 1 | Experiment paradigm. **(A)** *In vivo* cohort for MCAO model of ischemic stroke in mice. The drugs were administrated into left ventricles 24 h before MCAO. **(B)** *In vitro* cohort for OGD/R experiment in primary mouse astrocytes. After 12 h pre-incubation of drugs, OGD/R was developed in cultured astrocytes.

TTC Staining

Animals were sacrificed at 24 h after reperfusion. The infarct volume was tested based on 2,3,5-triphenyltetrazolium chloride (TTC) staining. The brains were sectioned into 2-mm coronal slices using a brain matrix. The brain slices were incubated in 2% TTC solution staining for 20 min at 37°C and then fixed in 4% paraformaldehyde (PFA). The ischemic volume was quantified using ImageJ software (version 2.0). The infarct percentage was shown as the ratio of the ischemic area to the entire slice area.

Analysis of Cell Viability and Lactate Dehydrogenase (LDH) Release

An LDH release quantification kit (#11644793001; Sigma-Aldrich) was used to evaluate cell viability. The cell-free supernatant was obtained by centrifugation at ∼250 × g for 10 min. Subsequently, the supernatant was incubated in working solutions in the dark for 1 h at room temperature (∼22°C). The absorbance of the samples was measured at 490 nm using a microplate reader (SpectraMax i3x; Molecular Devices, Sunnyvale, CA, USA). To determine the cytotoxicity percentage, all groups were compared with the control groups.

Flow Cytometry

The flow cytometry measurement of astrocyte apoptosis and Ca^{2+} concentration was performed using a BD flow cytometer (BD Biosciences, San Jose, CA, USA). Astrocytes were digested

with trypsin. The harvested cells were washed with phosphate-buffered saline (PBS) twice before incubating in buffer containing 5 μL of fluorescein isothiocyanate-annexin V and 5 μL of propidium iodide or 0.5 μg/ml DAPI for 15 min at room temperature in the dark. Subsequently, the cells were detected and analyzed.

The Ca^{2+} concentration was also measured using the BD flow cytometer. Cortical astrocytes were loaded with 2 μM Fluo-4 AM (#F14217; Invitrogen, Carlsbad, CA, USA) in culture medium for 1 h at 37°C in an incubator with 5% CO_2. Next, the medium was changed to Hank's balanced salt solution (#14025092; Gibco) and the astrocytes were incubated for another 30 min at 37°C. Then, the astrocytes were collected and analyzed using flow cytometry.

Western Blot

Western blot analysis was performed as previously described with slight modifications (Li et al., 2020). Samples (20–40 μg) were separated on 12% gels and transferred onto 0.45-μm polyvinylidene difluoride membranes (#IPVH00010; Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk for 1 h and then incubated with the following primary antibodies at 4°C overnight: TRPC6 (1;1,000; #21403; SAB Biotherapeutics, Sioux Falls, SD, USA), caspase-3 (1:1,000; #ab214430; Abcam, Cambridge, MA, USA), NF-κB (1:1,000; #6956S; Cell Signaling Technology), phospho-NF-κB (1:1,000; #3033S, Cell Signaling Technology), IL-1β (1:2,000; #ab9722; Abcam), Flag (1:1000; # F1804, Sigma-Aldrich), β-actin (1:2,000; #60008-1-Ig; Proteintech, Rosemont, IL, USA). Then, the membranes were incubated for 1 h with secondary antibodies (1:8,000; #SA00001-1 and #SA00001-2; Proteintech). Protein-specific signals were visualized using the Bio-Rad ChemiDoc™ MP imaging system (Hercules, CA, USA).

Immunofluorescence

Brain tissues were cut into 20 μm slices using a cryostat (#CM1950; Leica, Wetzlar, Germany). Tissues were blocked in 5% goat serum containing 0.3% Triton X-100 for 1 h at 37°C and then incubated with the following primary antibodies overnight at 4°C: GFAP (1:400; #80788S and #3670S; Cell Signaling Technology), TRPC6 (1:100; #ab62461; Abcam). Next, the slices were washed with PBS and incubated with secondary fluorescent antibodies (1:100; #SA00013-1, SA00013-2, SA00013-3, or SA00013-4; Proteintech) for 1 h at room temperature. In addition, 4′,6-iamidino-2-phenylindole, dihydrochloride (DAPI; 1 μg/mL; #4083; Cell Signaling Technology) staining was used to visualize the nuclei. Images were acquired using a confocal laser-scanning microscope (Nikon A1 Ti, 600× magnification; Leica TCS SP8, 400× magnification).

Astrocytes plated on glass coverslips 12 mm in diameter were collected and fixed in 4% PFA for 15 min at room temperature. Subsequently, the fixed cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature followed by blocking in 10% goat serum at 37°C for 30 min. Before incubation with secondary fluorescent antibodies, the astrocytes were labeled with the following primary antibodies: GFAP (1:400; #80788S and #3670S; Cell Signaling Technology), NF-κB (1:100; #6956S; Cell Signaling Technology). Next, DAPI (1 μg/mL) was used to stain nuclei. Specific fluorescent signals were tested using a confocal microscope (Leica TCS SP8, 630× or 1,890× magnification).

Enzyme-Linked Immunosorbent Assay

The release of interleukin-6 (IL-6) and IL-1β from cortices and cultured astrocytes was quantified using enzyme-linked immunosorbent assay (IL-6, #70-EK206HS; IL-1β, #70-EK201BHS-96; MULTI SCIENCES, Hangzhou, China). The experiments were performed according to the manufacturer's instructions. The final absorbance was detected at 450 nm and the background correction was set at 570 nm on a microplate reader (SpectraMax i3x; Molecular Devices).

Statistical Analyses

Data are presented as the means ± standard error of the mean (SEM) of at least three independent analyses. SPSS ver. 25.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA) were used for statistical analyses. One-way analysis of variance with Tukey's test for *post-hoc* analysis were performed. P-value <0.05 was considered statistically significant.

RESULTS

TRPC6 in Mouse Cortical Astrocytes Is Downregulated After Ischemia

In several studies, TRPC6 in neurons reportedly declines after cerebral ischemia, and maintaining the expression of neuronal TRPC6 protein contributes to neuronal survival and reduced cerebral ischemic insult (Du et al., 2010; Lin et al., 2013a; Guo et al., 2017). However, the role of the astrocytic TRPC6 channel after stroke remains unclear.

Astrocytes have provided important insights into ischemic stroke (Liu and Chopp, 2016). To confirm the vital functions of the astrocytic TRPC6 channel in cerebral ischemia, we established an *in vitro* model of stroke in primary mouse astrocytes using an OGD/R experiment. Astrocytes were exposed to 1–4 h of OGD followed by 24 h reperfusion. The apoptosis rate was elevated and cell viability was decreased in astrocytes following hypoxia injury (**Figures 2A–C**). The TRPC6 protein levels in astrocytes after OGD/R were significantly downregulated (**Figures 2D,F**). Conversely, the expression of the apoptosis-specific biomarker, cleaved caspase-3, was gradually increased after stroke compared with the control groups (**Figures 2D,E**). As shown in **Figures 2A–D**, 3 h OGD and 24 h reperfusion triggered appropriate hypoxic damage in astrocytes; thus, the subsequent *in vitro* OGD/R experiments were performed under this condition.

The *in vivo* stroke model is mimicked by MCAO in mice. Immunofluorescence signals showed that astrocytic TRPC6 was downregulated in the ischemic penumbra (**Figures 2G,H**). In parallel with the *in vitro* results, the TRPC6 protein level in peri-infarct areas was decreased compared with that in contralateral cortices (**Figures 2I,K**). In addition, after ischemic stroke, the cleaved caspase-3 expression in the ipsilateral hemisphere was upregulated compared with the contralateral hemisphere (**Figures 2I,J**). Furthermore, immunofluorescence

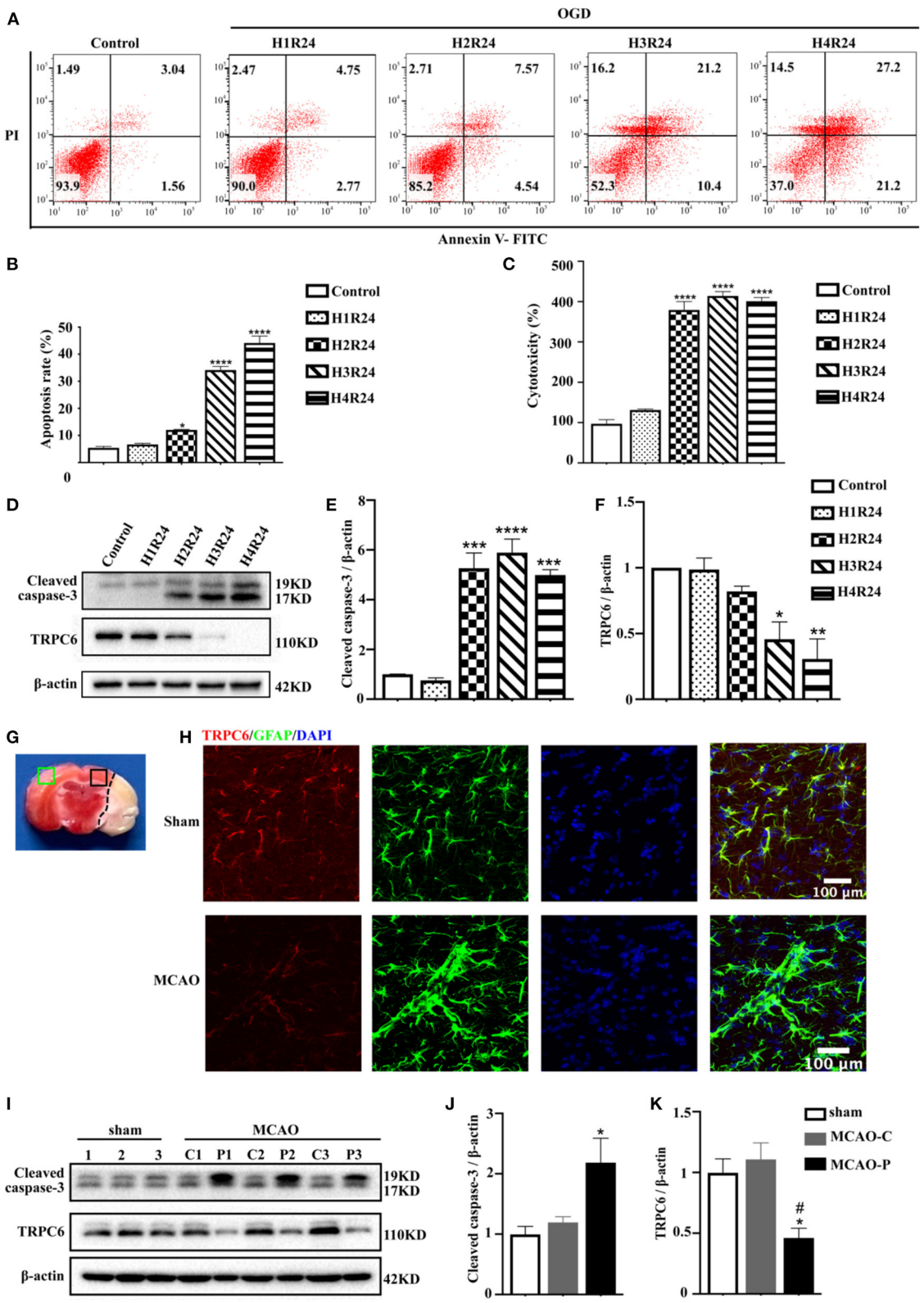

FIGURE 2 | TRPC6 in the cortical astrocytes is downregulated in ischemia. **(A)** Cultured astrocytes were exposed or not to OGD/R, and their apoptosis and death was detected by FITC/PI flow cytometry analysis. The percentage of apoptosis in scatter plots was presented as the proportion of apoptotic cells vs. total cell

(Continued)

FIGURE 2 | population (H1R24: 1 h OGD followed by 24 h reperfusion; H2R24: 2 h OGD followed by 24 h reperfusion; H3R24: 3 h OGD followed by 24 h reperfusion; H4R24: 4 h OGD followed by 24 h reperfusion). **(B)** Bar chart showed the statistical results of apoptosis rate measured by flow cytometry in each group in three independent experiments. Data were shown as mean ± SEM ($n = 3$). **(C)** LDH assay was used to measure the cytotoxicity of astrocytes in control and OGD groups. Data were shown as mean ± SEM ($n = 4$). **(D)** The protein level of TRPC6 and cleaved caspase-3 in astrocytes were tested by western blot analysis. β-actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean ± SEM ($n = 3$). **(G)** Brain slices with TTC staining. Green rectangle: contralateral cortex; Black rectangle: peri-infarct cortex. **(H)** Representative images of the cortex after sham or MCAO operation (peri-infarct cortex) double-stained with the TRPC6 (Red) and GFAP (Green) antibodies (Scar bar: 100 μm; 600× magnification). **(I)** Mice were exposed to sham operation or MCAO procedure ($n = 3$). The expressions of TRPC6 and cleaved caspase-3 in sham group (left hemisphere) and in contralateral (C) or peri-infarct (P) cortex were detected by western blotting. β-actin was used as a loading control. The number "1," "2," "3" represents cortices in each group were extracted form three different mice. **(J,K)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(I)**. Data were shown as mean ± SEM ($n = 3$). MCAO-C, contralateral cortices in MCAO mice; MCAO-P, peri-infarct cortices in MCAO mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (all comparisons to control group or sham group). $^{\#}p < 0.05$ (comparisons to MCAO-C group).

signals showed that astrocytic TRPC6 was downregulated in the ischemic penumbra (**Figure 2H**).

These results indicate that IR injury-induced changes in TRPC6 channel protein levels may have important functions in brain ischemia.

Reducing IR-Mediated TRPC6 Downregulation With HYP9 Alleviates Ischemic Insults in the Mouse Cortex

Consequently, to determine the role of TRPC6 in an *in vivo* model of ischemic stroke, intraventricular administration of HYP9 or SKF was performed in mice. MCAO resulted in increased infarct size and pro-inflammatory cytokine release compared with the sham groups (**Figure 3**). Pretreatment with HYP9 alleviated cortical injury, reduced infarct volume (**Figures 3A,B**), astrocytic population (**Figure 3C**), cleaved caspase-3 expression (**Figures 3D,E**), and inhibited pro-inflammatory cytokine (IL-6 and IL-1β) generation after MCAO (**Figures 3G,H**). However, the effects of SKF on an animal model of stroke regarding infarction volume, astrocytic population, cleaved caspase-3 protein level, and cytokine generation were not significantly different. In conclusion, the results show that application of the TRPC6 agonist HYP9 can alleviate IR-induced cerebral injury in an animal model of stroke.

Dose-Dependent HYP9 Inhibition of TRPC6 Downregulation in Astrocytes Promotes Astrocyte Survival During IR Injury

HYP9 is a specific TRPC6 channel activator (Leuner et al., 2010). Treatment with HYP9 (0, 1, 5, 10, 15, 20, or 30 μM) was used to verify whether TRPC6 channel degradation is responsible for IR-induced astrocyte insults. Astrocytes were pre-incubated with different concentrations of HYP9 for 12 h in a CO_2 chamber and then exposed to normal or OGD/R conditions. Application of 15 μM HYP9 contributed to a significant decrease in OGD/R-induced astrocyte apoptosis and cytotoxicity (**Figures 4A–C**). Western blot results showed that HYP9 (15 μM) suppressed the downregulation of TRPC6 and decreased cleaved caspase-3 protein levels in primary mouse astrocytes in the OGD group (**Figures 4D–F**). These results indicate that maintaining the protein level of the TRPC6 channel in astrocytes with HYP9 diminishes OGD/R-induced astrocyte apoptosis and improves cell vitality.

TRPC Antagonist SKF Aggravates Astrocyte Damage Dose-Dependently in the OGD/R Experiment

Next, whether blockage of the TRPC6 channel by SKF results in increased astrocyte damage after OGD/R was investigated. Cultured astrocytes were pretreated with 0, 0.05, 0.5, 5, 10, 20, 30, or 40 μM SKF for 12 h before the OGD/R experiment. As shown in **Figures 5A,B**, the apoptosis of astrocytes was markedly increased in the OGD group supplemented with 30 or 40 μM SKF compared with the OGD group without SKF pretreatment. In addition, the effects of SKF on astrocyte cytotoxicity in the control and OGD groups were investigated using the LDH assay. Notably, IR injury-mediated astrocyte cytotoxicity was elevated after pre-incubation with 20, 30, or 40 μM SKF in OGD groups (**Figure 5C**). In addition to the increased cleaved caspase-3 expression, the OGD/R-induced degradation of TRPC6 protein was not rescued after treatment with SKF (**Figures 5D–F**). Taken together, pretreatment of cultured astrocytes with 30 or 40 μM SKF aggravates apoptosis and cytotoxicity in OGD groups.

TRPC6 Protects Astrocytes Against IR Injury in Modeled Ischemia

To further clarify the protective role of astrocytic TRPC6 during IR injury, we then infected primary cortical astrocytes with lentivirus vectors to overexpress (WT-TRPC6) or knock down (sh-TRPC6) TRPC6. The efficiency of lentiviral infections was shown in **Figure 6D**. OGD/R notably decreased the protein levels of TRPC6 compared with that in control groups in astrocytes (**Figure 6D**). Overexpression of TRPC6 strikingly reduced OGD/R-induced astrocyte apoptosis (**Figures 6A,B**), cytotoxicity (**Figure 6C**), and cleaved caspase-3 protein levels (**Figures 6D,E**). However, knocking down TRPC6 via sh-TRPC6 did not exacerbate IR injury-mediated apoptosis, cytotoxicity or cleaved caspase-3 protein levels in astrocytes (**Figure 6**). In general, TRPC6 attenuates astrocytes IR injury in modeled ischemia *in vitro*.

Maintaining the Protein Level of the TRPC6 Channel in Astrocytes Alleviates Astrocytic Inflammatory Responses

Astrocytic inflammatory response also has a critical effect on regulating the progress and prognosis of ischemic stroke (Cekanaviciute and Buckwalter, 2016; Deng et al., 2018). In

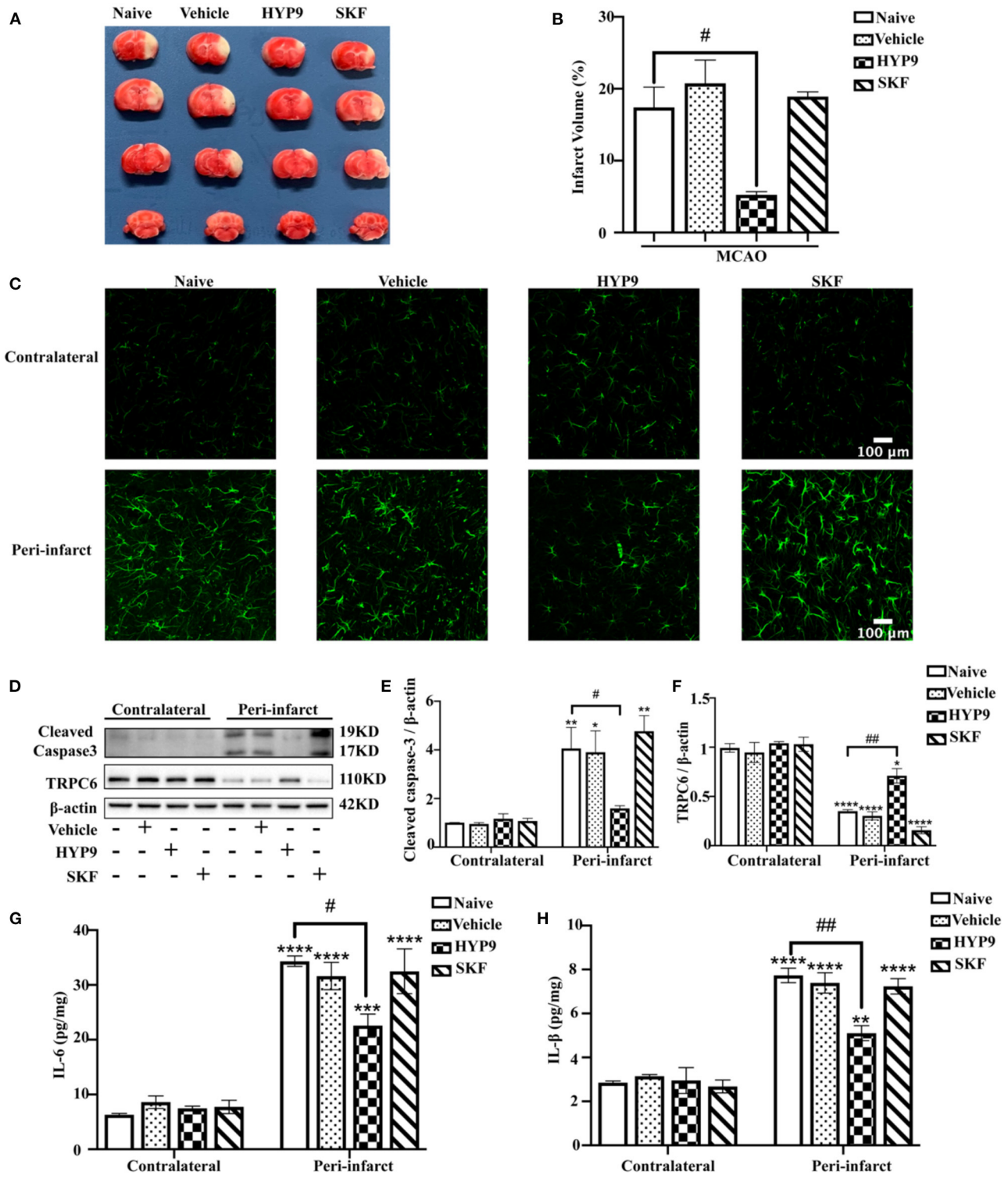

FIGURE 3 | Reducing IR-mediated TRPC6 downregulation with HYP9 alleviates ischemic damage in mice. Vehicle (DMSO), 5 μg HYP9, or 20 μg SKF were injected into left ventricles of mice 24 h before sham or MCAO operation. **(A)** TTC-stained cerebral slices indicated the infarct size in mice. **(B)** Quantification of infarct volumes after ischemia. Data were shown as mean ± SEM ($n = 3$). $^{\#}p < 0.05$ vs. MCAO + Naive group. **(C)** Representative images of astrocytes population with GFAP (green) staining in contralateral or peri-infarct cortices in MCAO mice (Scar bar: 100 μm; 400× magnification). **(D)** Western blot detecting TRPC6 and cleaved caspase-3 in mouse cortices. β-actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were

(Continued)

FIGURE 3 | shown as mean ± SEM ($n = 3$). **(G,H)** The release of IL-6 and IL-1β was determined by ELISA assay. Data were shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. MCAO + Naïve group in contralateral cortices; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MCAO + Naive group in peri-infarct area.

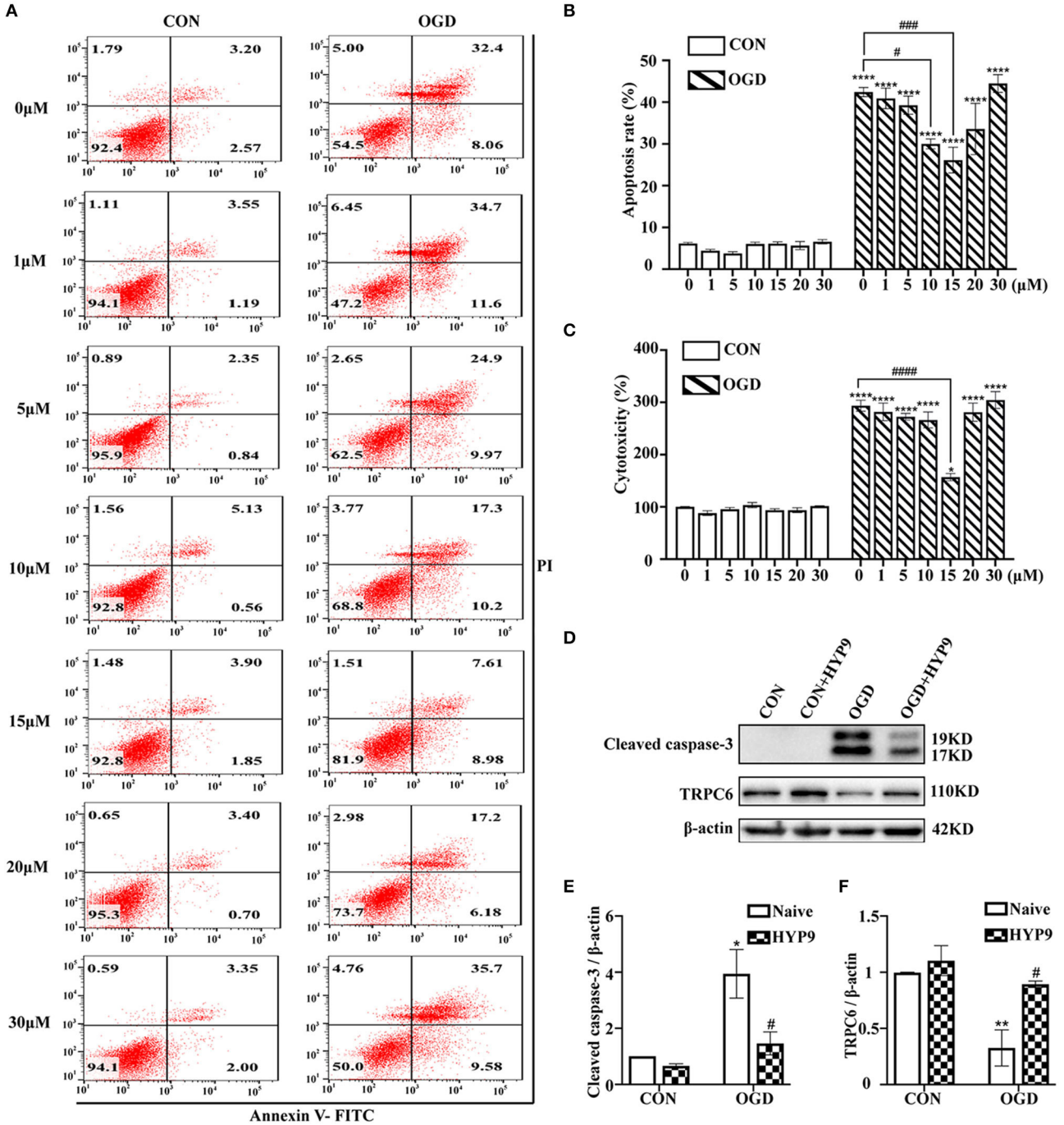

FIGURE 4 | Inhibition of TRPC6 downregulation by HYP9 protects astrocytes from ischemic damage. Astrocytes were pre-incubated with 0, 1, 5, 10, 15, 20, and 30 μM HYP9 for 12 h, and then subjected to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative scatter plots of astrocytic apoptosis measured by Annexin-V FITC/PI flow cytometry in each group. **(B)** Cell apoptosis rate was analyzed by Annexin-V FITC/PI flow cytometry, and the data were shown as mean ± SEM ($n = 4$). **(C)** Quantification of OGD/R-mediated cell cytotoxicity by LDH assay. Data were shown as mean ± SEM ($n = 4$). **(D)** Immunoblots for TRPC6 and cleaved caspase-3 of the extracts from CON or OGD-treated cortical astrocytes acquiring 15 μM HYP9 or not. β-actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. CON (HYP9 = 0 μM) group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. OGD (HYP9 = 0 μM) group.

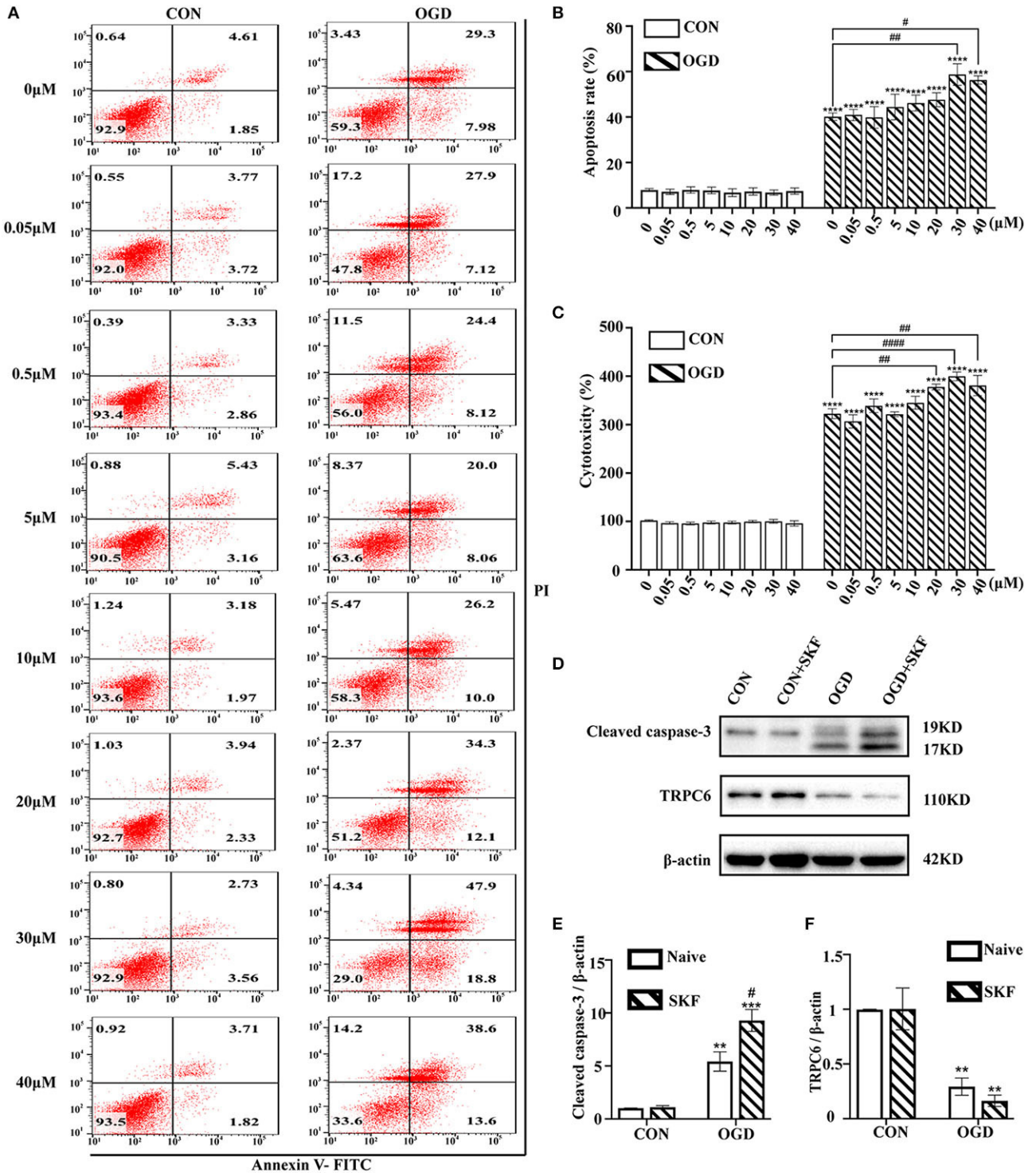

FIGURE 5 | TRPC antagonist SKF aggravates OGD/R induced astrocytes insults. Astrocytes were pre-incubated with 0, 0.05, 0.5, 5, 10, 20, 30, and 40 μM SKF for 12 h, and then subjected to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative scatter plots of astrocytic apoptosis measured by Annexin-V FITC/PI flow cytometry in each group. **(B)** Cell apoptosis rate was analyzed by Annexin-V FITC/PI flow cytometry, and the data were shown as mean ± SEM ($n = 4$). **(C)** Quantification of OGD/R-mediated cell cytotoxicity by LDH assay. Data were shown as mean ± SEM ($n = 4$). **(D)** Immunoblots for TRPC6 and cleaved caspase-3 of the extracts from CON or OGD-treated cortical astrocytes acquiring 30 μM SKF or not. β-actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean ± SEM ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. CON (SKF = 0 μM) group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ vs. OGD (SKF = 0 μM) group.

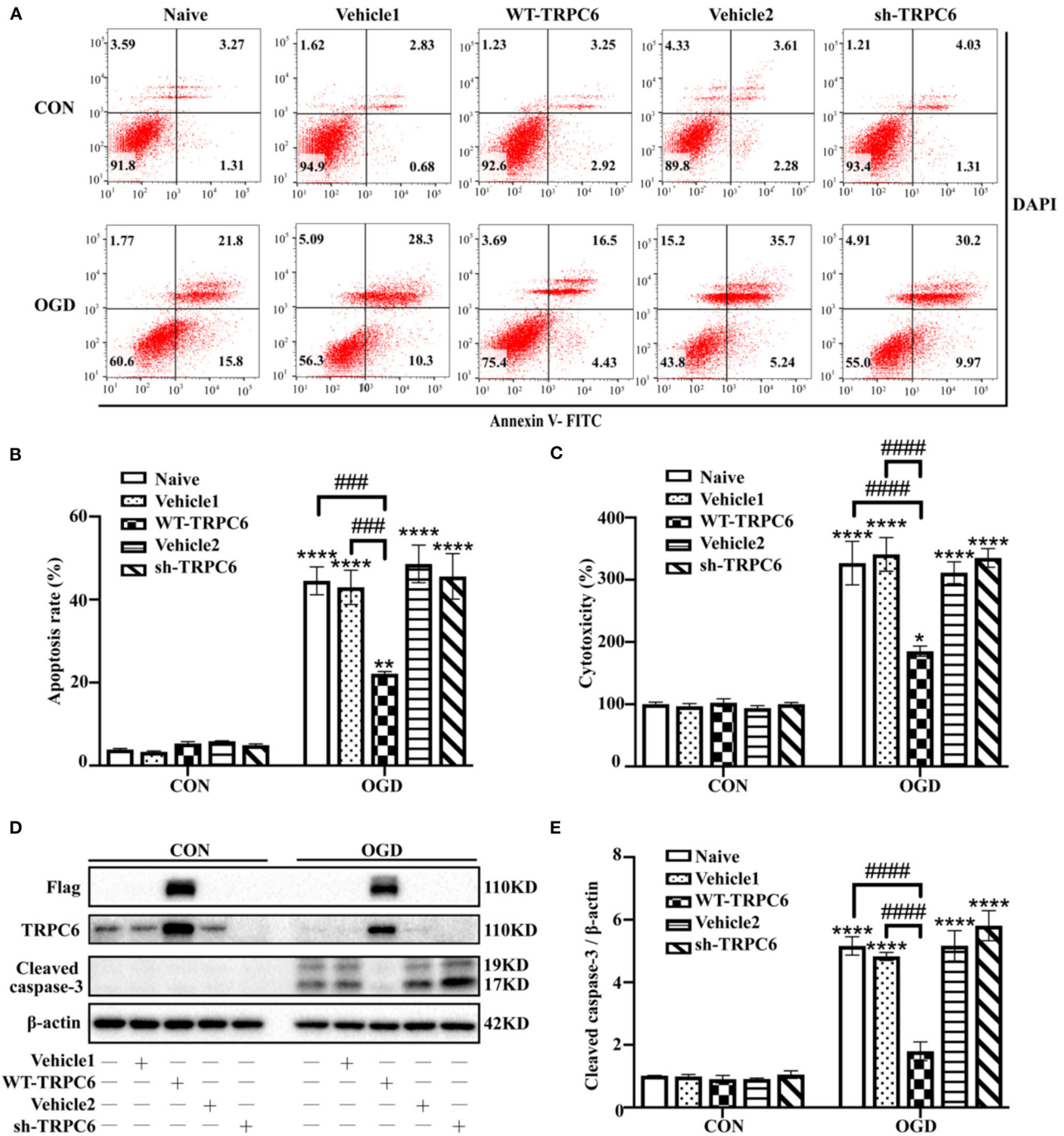

FIGURE 6 | TRPC6 protects astrocytes against IR injury in modeled ischemia. Astrocytes were infected with lentiviruses carrying mCherry-WT-TRPC6-null (Vehicle1), FLAG-tagged full-length mCherry-WT-TRPC6 (WT-TRPC6), mCherry-shRNA-TRPC6-null (Vehicle2), or mCherry-shRNA-TRPC6 (sh-TRPC6), and then exposed to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative scatter plots of astrocytic apoptosis measured by Annexin-V FITC/DAPI flow cytometry in each group. **(B)** Cell apoptosis rate was analyzed by Annexin-V FITC/DAPI flow cytometry, and the data were shown as mean ± SEM ($n = 4$). **(C)** Quantification of OGD/R-mediated cell cytotoxicity by LDH assay. Data were shown as mean ± SEM ($n = 4$). **(D)** Immunoblots for Flag, TRPC6, and cleaved caspase-3 of the extracts from control or OGD-treated cortical astrocytes acquiring Vehicle1, WT-TRPC6, Vehicle2, or sh-TRPC6 vectors. β-actin was used as a loading control. **(E)** Quantification of cleaved caspase-3 protein levels shown in **(D)**. Data were shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. CON + naive group; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. OGD + naive or OGD + Vehicle1 group.

addition to cell apoptosis and vitality, the expression of the pro-inflammatory cytokines IL-6 and IL-1β were measured to confirm the effects of HYP9 and SKF on ischemic astrocytic inflammation. The IL-6 and IL-1β protein levels were significantly increased in the OGD groups compared with the control groups. Notably, HYP9 and WT-TRPC6 reduced the elevated level of IL-6 and IL-1β in astrocytes in the OGD groups (**Figures 7A–F**).

The data show that inhibition of TRPC6 downregulation with HYP9 or WT-TRPC6 reduces inflammatory responses in astrocytes after OGD/R.

Inhibiting TRPC6 Downregulation Suppresses Intracellular Ca^{2+} Overload in Primary Astrocytes

The above reported results indicate the TRPC6 channel is closely associated with apoptosis, vitality, and inflammatory response in cultured cortical astrocytes exposed to IR damage. As a Ca^{2+} channel, TRPC6 modulates the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Wang et al., 2020a). To understand the potential mechanism of TRPC6 channel against cerebral ischemic insults in astrocytes, the effects of HYP9 and SKF on $[Ca^{2+}]_i$ in astrocytes was measured. Consistent with previous research (Li et al., 2015; Rakers and Petzold, 2017), OGD/R dramatically increased astrocytic $[Ca^{2+}]_i$ compared with control conditions (**Figures 8A,B**). Notably, application of HYP9 statistically reduced the elevated $[Ca^{2+}]_i$ in the OGD group (**Figures 8A,B**). In addition, SKF raised $[Ca^{2+}]_i$ in ischemic astrocytes (**Figures 8A,B**). These results indicate that astrocytic TRPC6 channel triggers a variety of protective cellular responses by inhibiting brain ischemia-induced Ca^{2+} overload.

TRPC6 Diminishes NF-κB Nuclear Translocation and Phosphorylation

NF-κB phosphorylation and nuclear translocation are considered vital pathways that mediate the inflammatory cascade, including cytokine generation (Kopitar-Jerala, 2015; Shih et al., 2015). Therefore, the influence of HYP9 and SKF on NF-κB nuclear translocation and phosphorylation in primary mouse astrocytes exposed to ischemic damage was investigated. Immunofluorescent signals showed the nuclear distribution of NF-κB was increased after OGD/R (**Figure 9**). Notably, the TRPC6-specific activator HYP9 attenuated IR injury-induced NF-κB nuclear translocation (**Figure 9**). Furthermore, HYP9 and WT-TRPC6 inhibited the phosphorylation of NF-κB in astrocytes subjected to OGD/R (**Figures 10A–G**). Collectively, the findings indicate that NF-κB phosphorylation and nuclear translocation may be pivotal downstream pathways that contribute to the protective effects of TRPC6 in astrocytes after stroke.

DISCUSSION

In cerebral ischemia, several clinical trials have failed due to narrow therapeutic window, adverse effects, and individual differences (Petrovic-Djergovic et al., 2016). A breakthrough in stroke therapeutic strategies is considered urgent. In the present study, significant downregulation of TRPC6 protein was observed in both cultured astrocytes and cerebral cortices after IR injury. *In vivo* results confirmed that the TRPC6 channel-specific activator HYP9 attenuated the infarct volume, astrocytes population, apoptosis, and the generation of pro-inflammatory cytokines (IL-6 and IL-1β) caused by MCAO. However, significant effects of SKF on infarct lesion or cortical IL-6 and IL-1β release in MCAO mice were not observed. In addition, HYP9 was shown to attenuate apoptosis rate, improve cell vitality, and reduce the generation of IL-6 and IL-1β in cultured astrocytes subjected to OGD/R. Furthermore, the TRPC channel antagonist SKF aggravated astrocytic apoptosis and cytotoxicity in OGD groups. In addition, inhibition of the TRPC6 channel downregulation with HYP9 suppressed the increase in $[Ca^{2+}]_i$ in ischemic astrocytes. The IR-mediated increased NF-κB phosphorylation and nuclear translocation were inhibited by HYP9 pretreatment in primary mouse astrocytes. In parallel, overexpression of TRPC6 also decreased IR injury-induced astrocytic apoptosis, cytotoxicity, inflammatory responses and NF-κB phosphorylation in modeled ischemia *in vitro*.

Previously, neuron-specific-related mechanisms are primarily considered in the pathogenesis of brain ischemia. As knowledge increased, astrocytes and other CNS cells are shown to be closely involved in ischemic stroke (Pekny et al., 2016; Werner et al., 2020). Astrocytes interact with neurons and participate in structural support, neuronal metabolism, synaptogenesis, synaptic transmission, axonal remodeling, and neurogenesis (Halassa and Haydon, 2010; Liu and Chopp, 2016). IR injury-activated astrocytes initiate the CNS inflammatory response, which in turn exacerbates brain insults during ischemic stroke (Cekanaviciute and Buckwalter, 2016). Thus, maintaining the normal function of astrocytes likely promotes post-ischemic neurological recovery. Therapeutic targeting of astrocytes is expected to be a future area of research regarding treatment and prognosis of brain ischemia (Neuhaus et al., 2017).

Evidence shows that TRPC6 is a crucial regulator of inflammatory cascades, especially in pulmonary inflammation (Chen et al., 2020; Ortiz-Muñoz et al., 2020). TRPC6 contributes to platelet activation, leukocyte transendothelial migration, lung vascular barrier disruption, and airway inflammation (Tauseef et al., 2012; Weber et al., 2015; Chen et al., 2020; Ortiz-Muñoz et al., 2020). In addition, activation of the TRPC6 channel promotes apoptosis in neonatal glomerular mesangial cells and renal tubular epithelial cells under different injury conditions (Soni and Adebiyi, 2016; Hou et al., 2018). TRPC6 inhibits N-methyl-D-aspartate (NMDA)-mediated Ca^{2+} elevation and reduces neuronal ischemic excitotoxity (Li et al., 2012). Furthermore, inhibition of TRPC6 degradation in neurons through cAMP-response element binding protein (CREB) pathway alleviates ischemic cerebral insults (Du et al., 2010). (-)-Epigallocatechin-3-gallate, resveratrol, and neuroprotectin D1 contribute to neuroprotective effect on brain IR damage through TRPC6/CREB pathways (Lin et al., 2013b; Yao et al., 2013, 2014). In particular, Hyperforin, a selective TRPC6 agonist, alleviates cerebral IR injury by suppressing the degradation of TRPC6 and increasing phosphorylated CREB in Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) signaling pathway (Lin et al., 2013a). Collectively, these

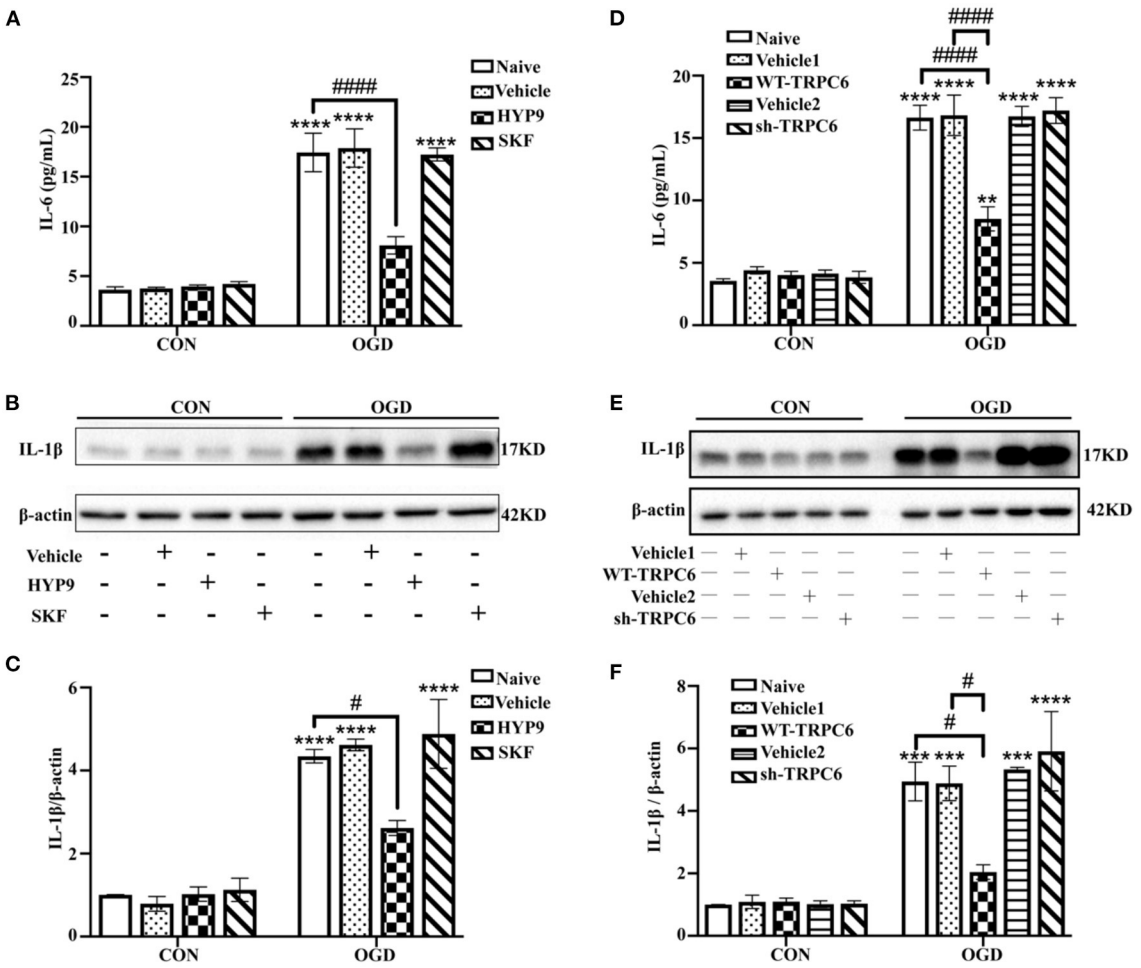

FIGURE 7 | Maintaining the protein level of TRPC6 attenuates astrocytic inflammatory responses. Astrocytes were pre-incubated with DMSO (as vehicle, the final DMSO concentration was 0.025%), 15 μM HYP9 or 30 μM SKF for 12 h, and then exposed to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** The protein level of IL-6 was determined by ELISA assay. Data were shown as mean ± SEM ($n = 4$). **(B)** The expression of pro-inflammatory cytokine IL-1β in cortical astrocytes was measured using western blotting. β-actin was used as a loading control. **(C)** Quantification of IL-1β protein levels shown in **(B)**. Data were shown as mean ± SEM ($n = 3$). Astrocytes were infected with lentiviruses carrying Vehicle1, WT-TRPC6, Vehicle2, or sh-TRPC6, and then exposed to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(D)** The protein level of IL-6 was determined by ELISA assay. Data were shown as mean ± SEM ($n = 4$). **(E)** The expression of pro-inflammatory cytokine IL-1β in cortical astrocytes was measured using western blotting. β-actin was used as a loading control. **(F)** Quantification of IL-1β protein levels shown in **(E)**. Data were shown as mean ± SEM ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. CON + Naive group; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs. OGD + Naive or OGD + Vehicle1 group.

evidences suggest the vital role of TRPC6/CREB pathway in ischemic stroke. Studies have shown that TRPC6 channel is expressed in microglia and involved in microglial activation and neuroinflammatory response in different diseases including hypertension, epilepsy, Alzheimer's disease, infectious diseases and neuropathic pain (Toth et al., 2013; Lee et al., 2014; Liu et al., 2017; Shinjyo et al., 2020; Wang et al., 2020b). However, the relevance of the TRPC6 channel in IR injury-induced astrocytic apoptosis and inflammation remains unclear. In addition, other downstream mechanisms beyond TRCP6/CREB pathways are worth exploring.

Hyperforin, a main compound of St. John's wort, can activate the TRPC6 channel and induce changes in $[Ca^{2+}]_i$ currents (Leuner et al., 2007); however, hyperforin is chemically unstable when subjected to oxygen and light. HYP9 is a stable synthetic phloroglucinol derivative of hyperforin that maintains the biological function of hyperforin in activating the TRPC6 channel (Leuner et al., 2010). Consequently, HYP9 was used in the present study to explore its function in the TRPC6 channel and the associated downstream cellular mechanisms in ischemic stroke. Inflammatory responses and apoptosis in ischemic astrocytes were inhibited by maintaining the protein level of the TRPC6 channel *via* HYP9. Overexpression of TRPC6 also reduced IR injury-induced astrocytic apoptosis, cytotoxicity, and inflammatory responses in experimental stroke *in vitro*. Meanwhile, SKF is a TRPC channel blocker (Song et al., 2014; Jing et al., 2016). SKF also affects other channels belonging to the TRPC channel subfamily. Evidence shows that TRPC1, TRPC3,

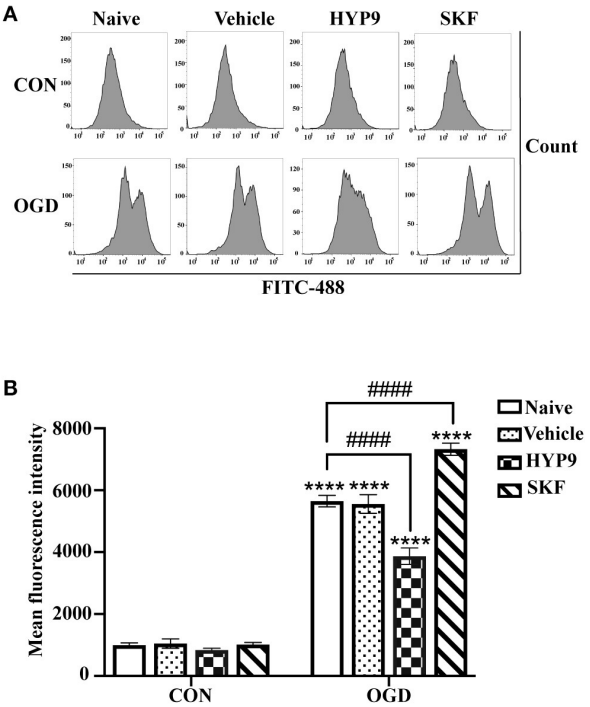

FIGURE 8 | TRPC6-specific agonists HYP9 suppresses intracellular Ca^{2+} overload in primary astrocytes. Astrocytes were pre-incubated with Vehicle (DMSO, the final concentration was 0.025%), 15 μM HYP9 or 30 μM SKF for 12 h, and then exposed to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative charts of average fluorescence intensity. The level of intracellular Ca^{2+} in astrocytes was measured by Ca^{2+} fluorescence probe Fluo-4 AM through FITC-488 flow cytometry. **(B)** Quantification of Ca^{2+} concentration in astrocytes by flow cytometry. Data were shown as mean ± SEM ($n = 4$). $^{****}p < 0.0001$ vs. CON + Naive group; $^{\#\#\#\#}p < 0.0001$ vs. OGD + Naive group.

and TRPC7 are involved in cerebral ischemic stroke (Chen et al., 2017; Xu et al., 2018). In the present study, SKF aggravated astrocyte injury after ischemia *in vitro*. However, significant changes in infarct volume, cleaved caspase-3 expression, and cytokine release were not observed in the MCAO model after SKF application. The inconsistent results between cell and animal models of stroke may be due to the complex effects of SKF on cortical non-astrocytic cells. Noticeably, knocking down TRPC6 via sh-TRPC6 shown no significant effects on apoptosis, cytotoxicity, and inflammatory responses in primary astrocytes after OGD/R. Since TRPC6 is strikingly downregulated in astrocytes exposed to IR insults, knocking down of TRPC6 did not significantly aggravate astrocytic damage in OGD group. The dissimilar effects of SKF and sh-TRPC6 on ischemic astrocytes might be related to the side-effect of SKF on other TRPCs (TRPC3/7). Overall, further *in vivo* studies using astrocytic TRPC6-, siRNA-, or shRNA-targeted transgenic mice are needed to fully clarify the role of the astrocytic TRPC6 channel in stroke.

Activation of the cerebral inflammatory cascade has important roles in acute brain IR insults. The inflammatory response induced by IR injury directly affect the progress of cerebral ischemia (Petrovic-Djergovic et al., 2016; Ramiro et al., 2018). Notably, astrocytes regulate the neuroinflammatory response by affecting the generation and release of diverse cytokines in ischemic stroke (Sofroniew, 2014). Astrocytes generate several pro-inflammatory cytokines and chemokines, including IL-6, IL-1β, tumor necrosis factor-α (TNF-α), C-C motif chemokine ligand (CCL)-2, CCL-5, CCL-7, C-X-C motif chemokine ligand (CXCL)-10, and vasoactive endothelial growth factor (John et al., 2005; Hamby et al., 2012; Sofroniew, 2015). Astrocyte-derived IL-6 and IL-1β are essential for secondary inflammatory damage in brain ischemia (John et al., 2005; Diaz-Cañestro et al., 2019). The pro-inflammatory nuclear transcription factor NF-κB belongs to the detrimental astrocytic signaling pathways (Colombo and Farina, 2016). NF-κB is involved in astrocyte inflammation and apoptosis (Su et al., 2019; Bai et al., 2020). Compelling evidence has shown that inhibition of NF-κB nuclear translocation or phosphorylation alleviates IR-mediated cytokine production and brain inflammation (Colombo and Farina, 2016; Liu et al., 2019; Xie et al., 2019; Bai et al., 2020). NF-κB activation is tightly modulated by various typical upstream signaling molecules, which include toll-like receptors, TNF receptors, IL-1 receptors, CAMKII, and stromal interaction molecule or Orai-mediated Ca^{2+} signals (Verstrepen et al., 2008; Berry et al., 2018; Pires et al., 2018; Ye et al., 2019).

A crucial relationship exists between Ca^{2+} and NF-κB signaling pathways (Wei et al., 2019). As a vital second messenger, Ca^{2+} governs a variety of functions causing cellular pathological and physiological changes. Modulation of the Ca^{2+} current has attracted interest as a promising medical strategy for ischemic stroke (Kalogeris et al., 2016; Secondo et al., 2018). The TRPC6 channel, a Ca^{2+} permeable cation channel, has been associated with Ca^{2+} entry and brain ischemic pathogenesis (Liu et al., 2020). In the present study, TRPC6 was dramatically downregulated during IR injury. The TRPC6-specific agonist HYP9 rescued TRPC6 degradation in primary cultured astrocytes and reduced $[Ca^{2+}]_i$. Consistently, the TRPC channel antagonist SKF contributed to increased $[Ca^{2+}]_i$. How a Ca^{2+} entry activator reduces and a Ca^{2+} channel blocker increases the Ca^{2+} influx in primary astrocytes is notable. TRPC6 has been shown to block the NMDA receptor (NMDAR)-triggered $[Ca^{2+}]_i$ increase and neurotoxicity in neurons after brain IR damage (Li et al., 2012). Overexpression of TRPC6 inhibits $[Ca^{2+}]_i$ overload, prevents neuronal death, lessen infarct size, and improves behavior performance after ischemia. Similarly, another research team suggests that the TRPC6 channel selectively suppresses the NMDAR-mediated current in hippocampal neurons, which is increased *via* TRPC6 knockdown or interference with SKF (Shen et al., 2013). In another study, SKF increases intracellular Ca^{2+} by promoting the reverse mode of the Na^+/Ca^{2+} exchanger (Song et al., 2014). Thus, we hypothesized that the underlying mechanism of TRPC6-induced $[Ca^{2+}]_i$ decrease in astrocytes after ischemic insults may be associated with the complex interaction between the TRPC6 channel and other ion exchangers.

Although the present study showed a protective role of the TRPC6 channel in astrocytes after ischemia, various questions remain unanswered. The effects of the astrocytic TRPC6

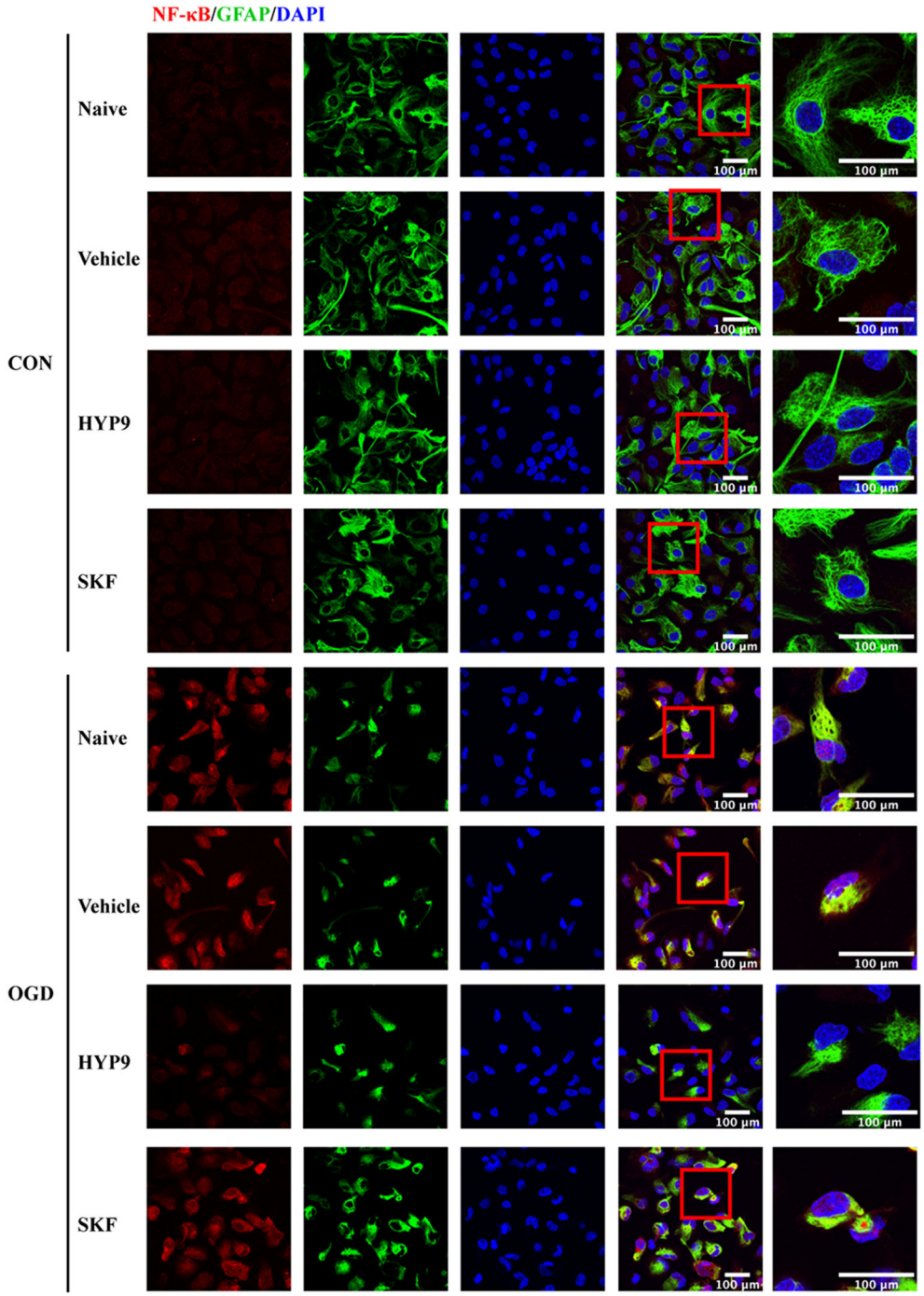

FIGURE 9 | TRPC6 diminishes NF-κB nuclear translocation in astrocytes after ishcemia. Astrocytes were pre-incubated with Vehicle (DMSO, the final concentration is 0.025%), 15 μM HYP9 or 30 μM SKF for 12 h, and then expose to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). Representative images of cortical astrocytes after OGD/R double-stained with the NF-κB (red) and GFAP (green) antibodies (Scar bar: 100 μm; 630× or 1,890× magnification).

channel on ischemic neurons will be addressed in our future work. Furthermore, the underlying mechanisms of TRPC6-triggered astrocytic Ca^{2+} changes in ischemic stroke warrant further investigations.

CONCLUSION

The results of the present study indicate that HYP9 reduces the infarct size, apoptosis, and pro-inflammatory cytokine release in

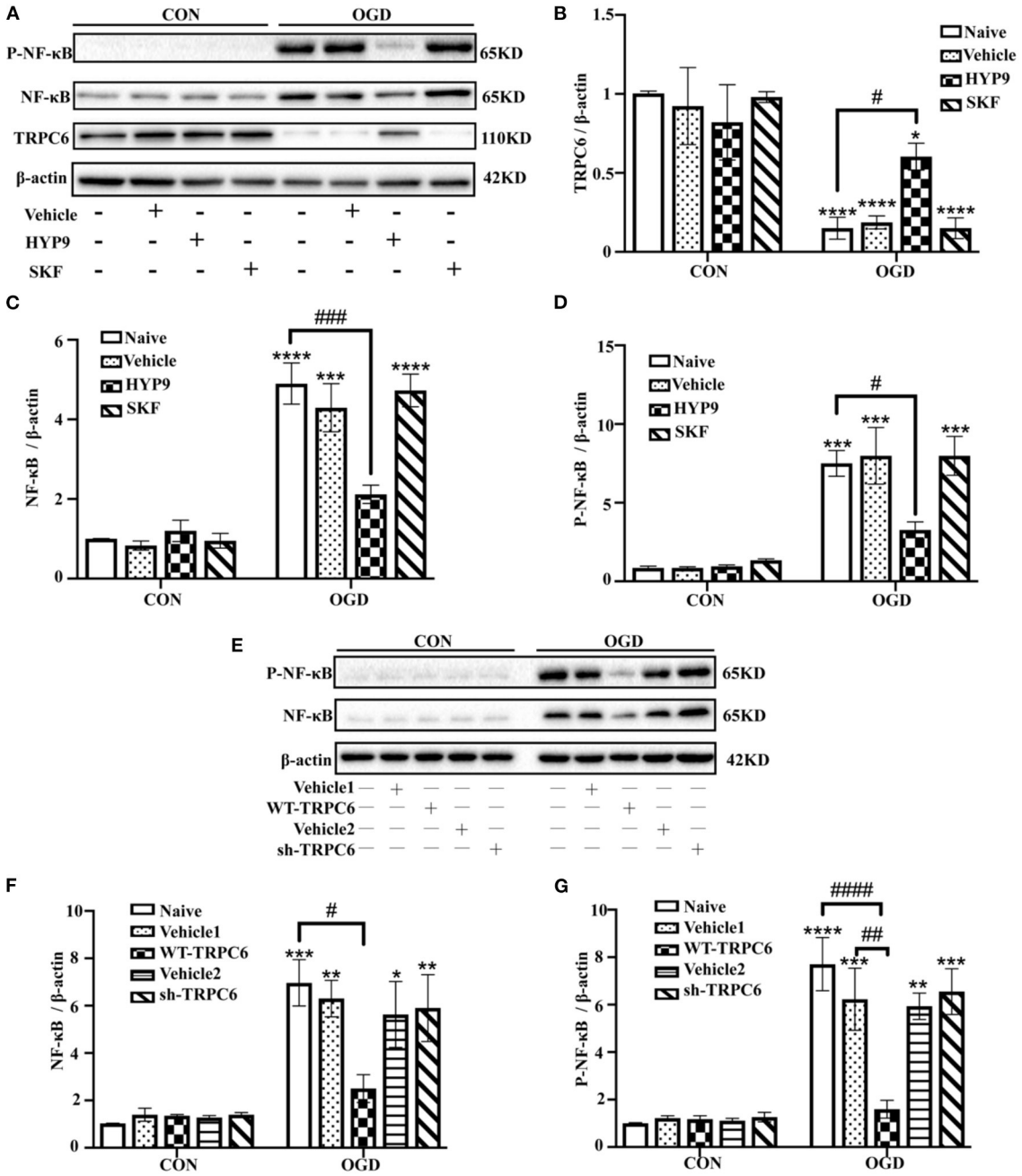

FIGURE 10 | TRPC6 diminishes OGD/R-induced NF-κB phosphorylation in astrocytes. Astrocytes were pre-incubated with Vehicle (DMSO, the final concentration is 0.025%), 15 μM HYP9 or 30 μM SKF for 12 h, and then expose to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Immunoblots for TRPC6, NF-κB, and phosphorylated-NF-κB (P-NF-κB) of the extracts from cortical astrocytes. β-actin was used as a loading control. **(B–D)** Quantification of TRPC6, NF-κB, and P-NF-κB protein levels shown in **(A)**. Data were shown as mean ± SEM ($n = 3$). Astrocytes were infected with lentiviruses carrying Vehicle1, WT-TRPC6, Vehicle2, or sh-TRPC6, and then exposed to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(E)** Immunoblots for NF-κB and P-NF-κB of the extracts from cortical astrocytes. β-actin was used as a loading control. **(F,G)** Quantification of NF-κB, and P-NF-κB protein levels shown in **(E)**. Data were shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. CON + Naive group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. OGD + Naive or OGD + Vehicle1 group.

a mouse model of ischemic stroke. Inhibition of TRPC6 channel downregulation *via* HYP9 or TRPC6 overexpression alleviates apoptosis and inflammatory response in astrocytes exposed to ischemic damage. Furthermore, the protective role of TRPC6 in stroke is associated with Ca^{2+}/NF-κB-dependent pathways. The results of this study indicate that the astrocytic TRPC6 channel and TRPC6 agonist HYP9 might be a novel therapeutic approach to prevent ischemic stroke induced by brain injury.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the First Affiliated Hospital at Zhejiang University.

AUTHOR CONTRIBUTIONS

LL wrote the manuscript. LL, MC, and KL finished the experiment. SZ and XXio designed the general idea. All authors edited the drafts of the manuscript.

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REVIEW
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Role of Oxidative Stress and Ca^{2+} Signaling in Psychiatric Disorders

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Psychiatric disorders are caused by complex and diverse factors, and numerous mechanisms have been proposed for the pathogenesis of these disorders. Accumulating evidence suggests that oxidative stress is one of the general factors involved in the pathogenesis/pathophysiology of major psychiatric disorders, including bipolar disorder, depression, anxiety disorder, and schizophrenia. Indeed, some clinical trials have shown improvement of the symptoms of these disorders by antioxidant supplementation. However, the molecular basis for the relationship between oxidative stress and the pathogenesis of psychiatric disorders remains largely unknown. In general, Ca^{2+} channels play central roles in neuronal functions, including neuronal excitability, neurotransmitter release, synaptic plasticity, and gene regulation, and genes that encode Ca^{2+} channels have been found to be associated with psychiatric disorders. Notably, a class of Ca^{2+}-permeable transient receptor potential (TRP) cation channels is activated by changes in cellular redox status, whereby these TRP channels can link oxidative stress to Ca^{2+} signals. Given the unique characteristic of redox-sensitive TRP channels, these channels could be a target for delineating the pathogenesis or pathophysiology of psychiatric disorders. In this review, we summarize the outcomes of clinical trials for antioxidant treatment in patients with psychiatric disorders and the current insights into the physiological/pathological significance of redox-sensitive TRP channels in the light of neural functions, including behavioral phenotypes, and discuss the potential role of TRP channels in the pathogenesis of psychiatric disorders. Investigation of redox-sensitive TRP channels may lead to the development of novel therapeutic strategies for the treatment of psychiatric disorders.

Keywords: oxidative stress, Ca^{2+} signaling, TRP channels, behavior, psychiatric disorders

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INTRODUCTION

Psychiatric disorders, which are chronic, recurrent, and devastating disorders, are one of the main causes of disability worldwide, with the current understanding of psychiatric disorders remaining limited due to the complex and diverse nature of these disorders. Based on a 2017 survey, the number of patients with psychiatric disorders is extremely high worldwide (284 million cases of anxiety disorders; 264 million cases of depression; 46 million cases of bipolar disorder; 20 million cases of schizophrenia) (Ritchie and Roser, 2018); therefore, therapeutic strategies for the treatment of psychiatric disorders are urgently required.

Accumulating evidence suggests that oxidative stress is one of the general factors involved in the pathogenesis/pathophysiology of psychiatric disorders (Ng et al., 2008), with antioxidant levels seeming to correlate with the degree of severity of psychiatric disorders (Zhang et al., 2003; Machado-Vieira et al., 2007; Sarandol et al., 2007), and antioxidant treatment has been shown to improve psychiatric symptoms in some clinical trials (Dakhale et al., 2005; Sivrioglu et al., 2007; Ellegaard et al., 2019). Moreover, markers of oxidative stress, including lipid peroxidation products and oxidized DNA, have been shown to be elevated in the blood of patients with psychiatric disorders. While the molecular mechanisms by which oxidative stress induces psychiatric disorders are still largely unknown, the lines of evidence strongly suggest that oxidative stress is associated with the physiology/pathology of psychiatric disorders.

Ca^{2+} channels mediate a number of neuronal functions, including neuronal excitability, neurotransmitter release, synaptic plasticity, and gene regulation. Many lines of evidence from human studies have indicated a critical contribution of voltage-dependent Ca^{2+} channels (VDCC) to the pathogenesis/pathophysiology of psychiatric disorders (Sklar et al., 2002, 2011; Ferreira et al., 2008; Ripke et al., 2011, 2014). However, since VDCCs control multiple critical physiological functions in the central nervous system (CNS), targeting VDCCs could cause complicated and uncontrollable results. Thus, targeting the "cart" that modulates Ca^{2+} signaling upon oxidative stress rather than the "horse" that governs the CNS system may be a promising approach and could reduce unwanted side effects.

Among the Ca^{2+}-permeable cation channels, transient receptor potential (TRP) channels have attracted attention as unique sensors of a wide variety of stresses, including oxidative stress (Mori et al., 2016). Given that redox-sensitive TRP channels induce Ca^{2+} influx upon oxidative stress, these channels could be mediators that link oxidative stress to dysregulated Ca^{2+} signaling in the pathogenesis/pathophysiology of psychiatric disorders.

In the first part of this review, we introduce the literature that examines oxidative stress in psychiatric disorders. In the second part, we review the significance of Ca^{2+} signaling in psychiatric disorders and provide current insights into the roles of redox-sensitive TRP channels in the functioning of neurons and glia as well as the development of neuronal connectivity and the function of the higher brain.

OXIDATIVE STRESS IN PSYCHIATRIC DISORDERS

Cellular redox status is determined by the balance between the levels of intracellular antioxidants and redox reactive species, including reactive oxygen species (ROS) and other electrophilic molecules, which can cause oxidative damage to membrane lipids, proteins, and DNA (**Figure 1A**). While a decrease in antioxidant enzymes shifts the cellular redox status to oxidative conditions, intriguingly, the expression of antioxidant enzymes is induced upon oxidative stress via oxidant defense transcription factors, such as NRF2 and NF-κB; therefore, both enhanced and suppressed antioxidant expression can be associated with oxidative stress (**Figure 1B**).

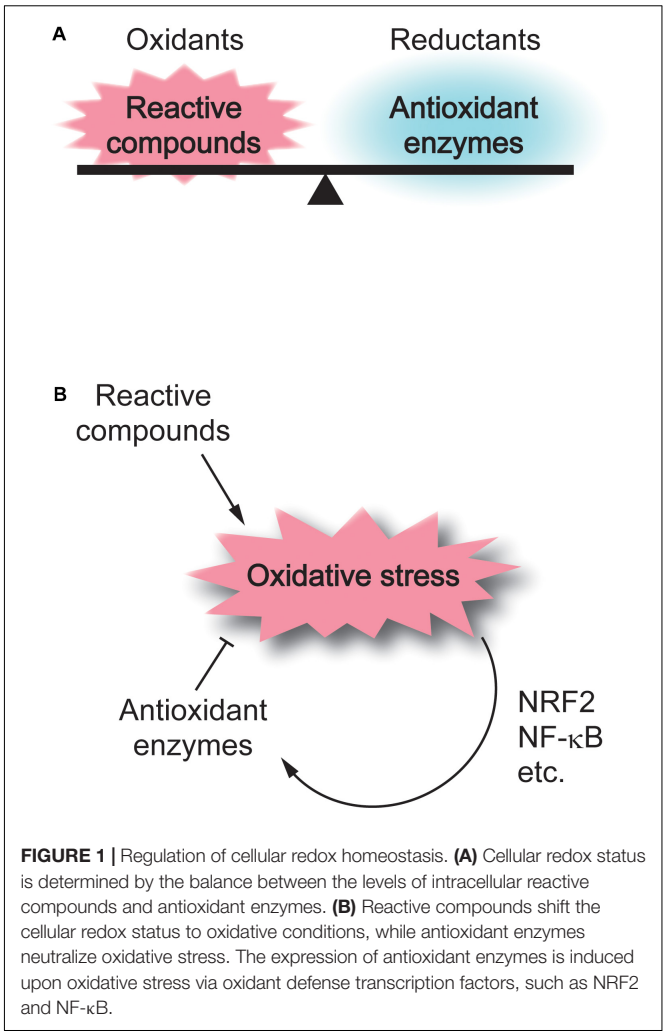

FIGURE 1 | Regulation of cellular redox homeostasis. **(A)** Cellular redox status is determined by the balance between the levels of intracellular reactive compounds and antioxidant enzymes. **(B)** Reactive compounds shift the cellular redox status to oxidative conditions, while antioxidant enzymes neutralize oxidative stress. The expression of antioxidant enzymes is induced upon oxidative stress via oxidant defense transcription factors, such as NRF2 and NF-κB.

It is highly controversial whether the antioxidant system is upregulated or downregulated in psychiatric disorders. In bipolar disorder, the activities of superoxide dismutases (SODs) and catalase (CAT) have been found to be decreased in the blood (Ranjekar et al., 2003), while the activity of glutathione peroxidases (GPXs) has been shown to be comparable between bipolar patients and healthy individuals (Kuloglu et al., 2002c; Ranjekar et al., 2003).

In contrast, several studies have demonstrated increased SOD activities in bipolar disorder (Abdalla et al., 1986; Kuloglu et al., 2002c). In major depression and anxiety disorders, such as obsessive-compulsive disorder, panic disorder, and social phobia, the activity of SODs alone or SODs and GPXs has been shown to be increased (Kuloglu et al., 2002a,b; Khanzode et al., 2003; Atmaca et al., 2004; Sarandol et al., 2007). In schizophrenia, higher activities of SODs (Abdalla et al., 1986; Kuloglu et al., 2002c) and GPXs have been reported in the blood of patients (Kuloglu et al., 2002c), while several other studies have reported

that the activities of SODs, CAT, and GPXs are lower in schizophrenic patients than in healthy controls (Ranjekar et al., 2003; Ben Othmen et al., 2008).

Some studies have reported a correlation between antioxidant activities in the blood and the degree of severity of psychiatric disorders. In a finding which suggests that antioxidant level is an indicator of disease severity and that lithium, a primary treatment for bipolar disorder, directly or indirectly normalizes redox status, one study that compared healthy controls with either unmedicated or lithium-treated patients in manic episodes of bipolar disorder indicates that, while SOD activity was significantly higher in manic patients compared with controls, it was indistinguishable between the lithium-treated group and the control group (Machado-Vieira et al., 2007). In patients with major depressive disorder, a significant positive correlation was found between the severity of the disease and SOD activity (Sarandol et al., 2007). Interestingly, serotonin reuptake inhibitors (SSRIs), which are antidepressant drugs, have been shown to decrease SOD activity (Bilici et al., 2001; Khanzode et al., 2003); however, SSRI treatment has also been reported to increase SOD activity in patients with major depressive disorder (Herken et al., 2007). In schizophrenic patients, SOD activity is significantly increased in the serum compared to control subjects and is decreased after treatment with atypical antipsychotics, such as clozapine, risperidone, olanzapine, quetiapine, and ziprasidone, in schizophrenic patients (Dakhale et al., 2004). It has also been reported that treatment with risperidone, an atypical antipsychotic, significantly decreased the blood SOD levels in schizophrenic patients and that decreased SOD levels may correlate with an improvement in symptoms (Zhang et al., 2003).

In contrast to the controversy on the effects of antioxidant activity on psychiatric disorders, overall, markers of oxidative stress, such as lipid peroxidation and oxidized DNA, have been shown to be elevated in patients with psychiatric disorders. An increase in one lipid peroxidation product, malondialdehyde (MDA), was reported in both bipolar disorder and schizophrenia (Kuloglu et al., 2002c). The level of thiobarbituric acid-reactive substances, another marker of lipid peroxidation, has also been reported to be increased in schizophrenia (Herken et al., 2001; Akyol et al., 2002). In major depression, a number of factors that maintain or disturb redox homeostasis are altered: depletion of ω-3 fatty acids, which suppress lipid peroxidation (Peet et al., 1998), elevation of MDA (Bilici et al., 2001; Khanzode et al., 2003; Sarandol et al., 2007), and increases in 8-hydroxy-2′-deoxyguanosine, a marker of oxidized DNA damage (Forlenza and Miller, 2006). In anxiety disorders, elevated MDA levels have been reported in obsessive-compulsive disorder (Kuloglu et al., 2002a; Ersan et al., 2006), panic disorder (Kuloglu et al., 2002b), and social phobia (Atmaca et al., 2004). These results provide evidence that oxidative stress is induced in psychiatric disorders and suggest both that the antioxidant system is upregulated through oxidant defense mechanisms and that decreased antioxidant activities lead to enhanced oxidative stress in some types of psychiatric disorders (**Figure 1**).

The potential significance of oxidative stress in psychiatric disorders has been demonstrated by some clinical trials that reported an improvement in symptoms through antioxidant treatment (**Table 1**). In patients with bipolar disorder, a randomized, double-blind, multicenter, placebo-controlled study of individuals (n = 75) showed that 24-week treatment with *N*-acetyl cysteine (NAC) significantly improved depressive symptoms (Berk et al., 2008), while a three-arm, 16-week, double-blind, randomized, placebo-controlled NAC treatment trial (n = 181) with depressive symptoms in bipolar disorder provided overall negative results, with no significant differences between groups detected at the primary outcome but some positive secondary signals were detected (Berk et al., 2019), suggesting that longer-term supplementation of NAC is required for the amelioration. In patients with major depressive disorder, a double-blind, randomized, placebo-controlled, clinical trial was performed with NAC as an adjunctive treatment in 269 participants, and the results exhibited limited but significant effects of adjunctive NAC treatment in reducing depressive symptoms in patients with major depressive disorder (Berk et al., 2014). Notably, meta-analysis was carried out to aggregate the data on double-blind, randomized, placebo-controlled trials evaluating the effect of NAC treatment on depressive symptoms in a total of 574 participants, of whom 291 were randomized to receive NAC and 283 received a placebo, regardless of the main psychiatric conditions. The results demonstrated that treatment with NAC significantly improved depressive symptoms in patients with bipolar and major depressive disorders and in individuals with trichotillomania or heavy smoking (Fernandes et al., 2016). In patients with schizophrenia, a 12-week, double-blind, randomized, placebo-controlled, clinical trial has recently been performed to assess the effectiveness of NAC as an adjunctive treatment with conventional antipsychotic medications in 84 patients (Sepehrmanesh et al., 2018). This clinical trial demonstrated that NAC-treated patients showed significantly improved cognitive functions (Sepehrmanesh et al., 2018). In addition to NAC supplementation, co-treatment of antipsychotic drugs with both ω-3 fatty acids and vitamins E and C for 4 months improved psychotic symptoms in schizophrenic patients (Arvindakshan et al., 2003). There results suggest that oxidative stress is involved in the pathophysiology of psychiatric disorders and that antioxidant supplementation may have a suppressive effect on symptoms.

Ca^{2+} SIGNALING IN PSYCHIATRIC DISORDERS

In the CNS, Ca^{2+} signaling is pivotal for numerous cellular events, including neuronal excitability, neurotransmitter release, synaptic plasticity, and Ca^{2+}-induced gene regulation (Catterall, 2011; Sawamura et al., 2017). Due to the fundamental role of Ca^{2+} signaling in the CNS, Ca^{2+} channels are thought to be a prime target for the pathogenesis of psychiatric disorders. Indeed, genes encoding Ca^{2+} channels have been shown to be associated with psychiatric disorders (McQuillin et al., 2006; Ripke et al., 2014; Griesi-Oliveira et al., 2015).

Among the Ca^{2+} channels, *CACNA1C*-encoded $Ca_V1.2$, an L-type VDCC, is strongly associated with psychiatric disorders

TABLE 1 | Clinical trials for antioxidant treatment in patients with schizophrenia, bipolar disorder, and major depressive disorder.

Schizophrenia			
Antioxidant	**Trial design → Outcomes**	**Sample size**	
NAC (Sepehrmanesh et al., 2018)	Adjunctive NAC 1,200 mg/day supplements 12 weeks double-blind treatment → Improvement in PANSS, MMSE, Digit Span Test, and SCWT scores	NAC: Placebo:	42 42
Vitamin C/E and ω-3 fatty acids (Sivrioglu et al., 2007)	Adjunctive vitamin C 1,000 mg/day, vitamin E 800 IU/day, EPA 360 mg/day, and DHA 240 mg/day supplements 4 months open-label treatment → Improvement in BPRS, BARS, SAS scores, and SANS total score	Vitamin C/E and ω-3 fatty acids:	17
Vitamin C (Dakhale et al., 2005)	Adjunctive vitamin C 500 mg/day supplements 8 weeks double-blind treatment → Improvement in BPRS score	Vitamin C: Placebo:	20 20
Vitamin C/E and ω-3 fatty acids (Arvindakshan et al., 2003)	Adjunctive vitamin C 1,000 mg/day, vitamin E 800 IU/day, EPA 360 mg/day, and DHA 240 mg/day supplements 4 months open-labeled treatment and 4 months follow up → 8 months; Improvement in BPRS-total, positive-PANSS, general psychopathology-PANSS, and PANSS-total scores	Vitamin C/E and ω-3 fatty acids:	33
Bipolar disorder			
Antioxidant	**Trial design → Outcomes**	**Sample size**	
NAC (Berk et al., 2019)	Adjunctive NAC 2,000 mg/day and cocktail of 16 nutrient agents supplements 16 weeks double-blind treatment and 4 weeks follow up → 16 weeks; No significant change 20 weeks; Improvement in MADRS, BDRS, SOFAS, and LIFE-RIFT scores (Placebo v.s. NAC + nutrient agents)	NAC + nutrient agents: NAC: Placebo:	61 59 61
NAC (Ellegaard et al., 2019)	Adjunctive NAC 3,000 mg/day supplements 20 weeks double-blind treatment and 4 weeks follow up → 20 weeks; Improvement in YMRS score	NAC: Placebo:	40 40
NAC (Berk et al., 2008)	Adjunctive NAC 2000 mg/day supplements 24 weeks double-blind treatment and 4 weeks follow up → 24 weeks; Improvement in MADRS, BDRS, CGI-S-BP, Q-LES-Q, LIFE-RIFT, SLICE-LIFE, GAF, and SOFAS scores	NAC: Placebo:	38 37
Major depressive disorder			
Antioxidant	**Trial design → Outcomes**	**Sample size**	
NAC (Berk et al., 2014)	Adjunctive NAC 2,000 mg/day supplements 12 weeks double-blind treatment and 4 weeks follow up → 16 weeks; Improvement in MADRS, CGI-S, SLICE-LIFE, and LIFE-RIFT scores	NAC: Placebo:	135 134

BARS, Barnes Akathisia Rating Scale; BDRS, Bipolar Depression Rating Scale; BPRS, Brief Psychiatric Rating Scale; CGI-S, Clinical Global Impressions-Severity of illness; CGI-S-BP, Clinical Global Impression Severity scale for Bipolar disorder; GAF, Global Assessment of Functioning Scale; LIFE-RIFT, Longitudinal Interval Follow-Up Evaluation-Range of Impaired Functioning Tool; MADRS, Montgomery-Åsberg Depression Rating Scale; MMSE, Mini-Mental State Examination; PANSS, Positive And Negative Syndrome Scale; Q-LES-Q, Quality of Life Enjoyment and Satisfaction Questionnaire; SANS, Scale for the Assessment of Negative Symptoms; SAS, Simpson-Angus Scale; SCWT, Stroop Color and Word test; SLICE-LIFE, Streamlined Longitudinal Interview Clinical Evaluation from the Longitudinal Interval Follow-up Evaluation; SOFAS, Social and Occupational Functioning Assessment Scale; YMRS, Young Mania Rating Scale.

(Sklar et al., 2002, 2011; Ferreira et al., 2008; Ripke et al., 2011, 2014). In humans, a risk-associated single nucleotide polymorphism (rs1006737) in *CACNA1C* predicts both increased hippocampal activity during emotional processing and higher prefrontal activity during executive cognition (Bigos et al., 2010). In rodents, conditional deletion of *Cacna1c* in the hippocampus and cortex results in severe impairment of hippocampus-dependent spatial memory based on the Morris water maze test (Moosmang et al., 2005; White et al., 2008). Acute pharmacological blockade of $Ca_V1.2$, but not chronic genetic inactivation, impairs the acquisition of fear learning (Langwieser et al., 2010), while anterior cingulate cortex-limited deletion of *Cacna1c* in mice impairs observational fear learning (Jeon et al., 2010). Constitutive *Cacna1c* heterozygous knockout (KO) mice, forebrain-specific conditional *Cacna1c* KO mice, and prefrontal cortex-specific *Cacna1c* knockdown mice show increased anxiety-like behavior in the elevated-plus maze test (Lee et al., 2012). The behavioral phenotypes in mice with genetic or pharmacological inhibition of $Ca_V1.2$ are summarized in the previous literature (Nakao et al., 2015).

Clinical studies with $Ca_V1.2$ blockers for psychiatric disorders have been summarized in a previous review paper (Kabir et al., 2017). While some clinical trials using $Ca_V1.2$ blockers have demonstrated improved symptoms of psychiatric disorders, the effect remains controversial (Kabir et al., 2017). Notably, since $Ca_V1.2$ controls multiple critical physiological functions in various organs and tissues, including the vascular system, the sinoatrial node, cardiomyocytes, pancreatic islets, adrenal medulla, and intestinal smooth muscle (Zamponi et al., 2015), the use of $Ca_V1.2$ blockers could cause highly complicated and uncontrollable results.

REDOX-SENSITIVE TRP CHANNELS

Among the Ca^{2+}-permeable channels, a class of TRP channels is activated by oxidative stress (so-called redox-sensitive TRP channels). Given the critical role of oxidative stress and aberrant Ca^{2+} signaling in psychiatry disorders, it is possible that redox-sensitive TRP channels serve as a cue for understanding the molecular pathogenesis of these disorders.

The *trp* was originally identified through the genetic studies of the *Drosophila* phototransduction mutant (Montell and Rubin, 1989). The term "TRP" is derived from "transient receptor potential" as the *trp* mutant photoreceptors fail to generate the Ca^{2+}-dependent sustained phase of receptor potential and therefore fail to induce subsequent Ca^{2+}-dependent adaptation to light in *Drosophila*. In mammals, 28 TRP homologs have been identified since the cloning of the *Drosophila trp* gene (Montell and Rubin, 1989). They are divided into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) groups, according to the amino acid homology (Clapham, 2003; Nilius et al., 2007). TRP channels are putative six-transmembrane polypeptide subunits and assemble as tetramers to form a variety of Ca^{2+}-permeable cation channels. Because of their distinct activation mechanisms and biophysical properties, TRP channels are highly suited to function in sensory receptor cells, either as molecular sensors for environmental or endogenous stimuli or as modulators of signal transduction cascades downstream of metabotropic receptors. Indeed, TRP channels play crucial roles in many types of senses, including touch, taste, and smell in mammals (Clapham, 2003; Voets et al., 2005; Moran et al., 2011). Thus, in marked contrast to VDCCs and fast ligand-gated Ca^{2+}-permeable channels that participate in specialized cellular functions, TRP channels play divergent roles in cell physiology and pathophysiology.

Among TRP channels, one particular group of TRP channels, including TRPM2, TRPC5, TRPV1–V4, and TRPA1 channels, function as exquisite sensors of redox status. The redox-sensitive TRP channels induce Ca^{2+} entry into the cells in response to cellular redox perturbation and can convert oxidative stress information into Ca^{2+} signals. Details of redox-sensitive TRP channels have been summarized in several review papers from our group (Takahashi and Mori, 2011; Takahashi et al., 2011a; Kozai et al., 2013; Sakaguchi and Mori, 2020).

TRPM2 channel, the first redox-sensitive TRP channel to be identified, is activated by H_2O_2 through the production of nicotinamide adenine dinucleotide and its metabolites, ADP-ribose and cyclic ADP-ribose (Hara et al., 2002; Perraud et al., 2005). A later study proposed that oxidation of the methionine located in the ADP-ribose binding region of TRPM2 sensitizes the channel activities (Kashio et al., 2012). H_2O_2-activated Ca^{2+} or cation influx through TRPM2 induces cell death (Hara et al., 2002) and insulin secretion in pancreatic β-cells (Togashi et al., 2006; Uchida et al., 2011). Furthermore, studies using *Trpm2* KO mice have shown that TRPM2 widely contributes to the innate immune system in monocytes, macrophages, neutrophils, and natural killer cells (Yamamoto et al., 2008; Knowles et al., 2011; Di et al., 2012; Hiroi et al., 2013; Rah et al., 2015).

In addition to the indirect redox-sensing mechanism via TRPM2, direct redox-sensing mechanisms involving cysteine (Cys) modification have emerged as mechanisms that underly the activation of various TRP channels (Yoshida et al., 2006; Takahashi and Mori, 2011; Takahashi et al., 2011b). TRPC5 channel, for example, is activated by nitric oxide (NO), reactive disulfides, and H_2O_2 via the oxidative modification of Cys residues (Cys553 and Cys558) located on the N-terminal side of the pore-forming region between S5 and S6 transmembrane helices in mouse TRPC5 (Yoshida et al., 2006). Interestingly, TRPC5 is also activated by the reducing agent dithiothreitol and extracellular-reduced thioredoxin (Xu et al., 2008). Both thermosensor channels (TRPV1, TRPV3, and TRPV4) and the closest relatives of TRPC5 (TRPC1 and TRPC4) have Cys residues corresponding to Cys553 and Cys558 on the TRPC5 protein and are activated by NO, reactive disulfides, and H_2O_2 (Yoshida et al., 2006). The mustard oil or wasabi sensor TRPA1 channel, which is predominantly expressed in sensory and vagal neurons, is also activated via modification of Cys residues located in the N-terminal ankyrin repeat domain (Hinman et al., 2006; Macpherson et al., 2007; Takahashi et al., 2008) and has the highest oxidation sensitivity among all TRP channel family members (Takahashi et al., 2011b).

Notably, all the redox-sensitive TRP channels are expressed to some extent in the CNS and affect neuronal and glial functions. Given this fact, together with the evidence that both Ca^{2+} signals and oxidative stress are critical mediators in psychiatric disorders, it is possible that redox-sensitive TRP channels could be a target for delineating the pathogenesis or pathophysiology of psychiatric disorders.

NEURONAL AND GLIAL FUNCTIONS OF REDOX-SENSITIVE TRP CHANNELS

In neuronal circuits, excitation-inhibition balance is considered a framework for understanding the mechanisms of psychiatric disorders (Sohal and Rubenstein, 2019). TRPM2 channel is abundantly expressed in the brain (Hara et al., 2002; Kraft et al., 2004; Fonfria et al., 2006) and H_2O_2-induced Ca^{2+} influx via TRPM2 in neurons was initially identified to induce cell death (Kaneko et al., 2006). Later studies have suggested several important roles for TRPM2 in the physiology of the CNS. TRPM2 activation by ADPR has been suggested as a regulator of VDCC and NMDAR via a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured hippocampal pyramidal neurons (Olah et al., 2009). In CA3-CA1 synapses, the loss of TRPM2 selectively impairs NMDAR-dependent long-term depression (LTD), while it does not affect long-term potentiation (LTP) (Xie et al., 2011). The impaired LTD in *Trpm2* KO mice is rescued by dopamine D2 receptor stimulation through the reduction of phosphorylated GSK-3β, suggesting that TRPM2 plays a key role in hippocampal synaptic plasticity via the GSK-3β signaling pathway. In guinea pig midbrain slices, TRPM2 has been shown to be required for NMDA-induced burst firing and to contribute to H_2O_2-dependent modulation of substantia nigra pars reticulata (SNr) GABAergic neurons (Lee et al., 2013). Given that an increase in burst firing in SNr GABAergic neurons is observed in Parkinson's disease and that oxidative stress is linked to Parkinson's disease, TRPM2 could be associated with the pathophysiology of Parkinson's disease.

Quantitative post-mortem investigations have convincingly demonstrated abnormalities in the glial cells of patients with psychiatric disorders (Cotter et al., 2001). In addition to neurons and immune cells, TRPM2 has been shown to be functionally expressed in glial cells, such as the microglia and astrocytes (Kraft et al., 2004; Kaneko et al., 2006; Bond and Greenfield, 2007). Oxidative stress induced by the inhibition of intracellular GSH biosynthesis with D,L-buthionine-*S,R*-sulfoximine (BSO) elicits Ca^{2+} influx potentially via TRPM2 in astrocytes isolated from humans (Lee et al., 2010). In rat cultured astrocytes, *tert*-butyl hydroperoxide-induced oxidative stress upregulates TRPM2 mRNA within 1 h – the transcripts were peaked at 4 h and were still apparent at 24 h post-stress (Bond and Greenfield, 2007), suggesting that glial TRPM2 expression is enhanced upon oxidative stress in disease conditions, including psychiatric disorders, and mediates the pathophysiology of disease.

TRPC5 is predominantly expressed in the brain, where it can form heterotetrameric complexes with TRPC1 and TRPC4 channel subunits. Recently, TRPC1, TRPC4, and TRPC5 have been demonstrated to assemble into heteromultimers in mouse brains, but not with other TRP family members, based on quantitative high-resolution mass spectrometry (Bröker-Lai et al., 2017). Brain slices prepared from *Trpc5* KO and *Trpc4* KO mice exhibit significant reductions in the synaptic responses that are mediated by the activation of $G_{q/11}$ protein-coupled receptors (e.g., group I metabotropic glutamate receptors and cholecystokinin [CCK] 2 receptors) in the amygdala (Riccio et al., 2009, 2014). As a consequence, deficiency of *Trpc5* or *Trpc4* induces anxiolytic behavior in mice (Riccio et al., 2009, 2014) (see "Redox-Sensitive TRP Channels in Higher Brain Functions" section). In hippocampal neurons from *Trpc1*/*Trpc4*/*Trpc5* triple KO mice, action potential-triggered excitatory postsynaptic currents (EPSCs) are significantly reduced, whereas the frequency, amplitude, and kinetics of quantal miniature EPSC signaling remains unchanged (Bröker-Lai et al., 2017). Consistent with this observation, evoked postsynaptic responses in hippocampal slice recordings and transient potentiation after tetanic stimulation have been reported to be decreased in triple KO mice (Bröker-Lai et al., 2017). In layer III pyramidal neurons in the entorhinal cortex, enhancement of neuronal excitability induced by CCK, one of the most abundant neuropeptides in the brain, is significantly inhibited by intracellular application of the antibody to TRPC5, suggesting the involvement of TRPC5 channels in neuropeptide-mediated neuronal excitability (Wang et al., 2011). TRPC5 has been also demonstrated to mediate muscarinic receptor-induced slowing after depolarization, which is observed in the pyramidal cells of the cerebral cortex (Yan et al., 2009). Interestingly, TRPC1, TRPC4, and TRPC5 channels are activated by leptin, a hormone that plays a key role in appetite, overeating, and obesity, via the Jak2-PI3 kinase-PLCγ1 pathway in hypothalamic proopiomelanocortin neurons (Qiu et al., 2010). Taken together, these lines of evidence suggest that TRPC1, TRPC4, and TRPC5 channels are involved in various physiological processes, including memory and appetite, by regulating neuronal functions.

TRPC1, TRPC4, and TRPC5 channels have also been shown to be expressed in astrocytes (Song et al., 2005; Malarkey et al., 2008). In the results suggesting that the TRPC1, TRPC4, and TRPC5 channels are functionally expressed in astrocytes and also modulate neuronal circuits, high-resolution imaging revealed that store-operated Ca^{2+} entry (SOCE) mediated by TRPC1 is linked not only functionally but also structurally to the ER Ca^{2+} stores in mouse astrocytes (Golovina, 2005). TRPC1-mediated Ca^{2+} entry in response to mechanical and pharmacological stimulation has been shown to contribute to glutamate release from rat astrocytes (Malarkey et al., 2008).

TRPV1 is broadly expressed in the brain regions, including the olfactory nuclei, cerebral cortex, dentate gyrus, thalamus, hypothalamus, periaqueductal gray, superior colliculus, locus coeruleus, and cerebellar cortex (Roberts et al., 2004). TRPV1 is involved in various forms of synaptic plasticity, which have been well-summarized in previous review papers (Kauer and Gibson, 2009; Ramírez-Barrantes et al., 2016; Sawamura et al., 2017). In the hippocampus, TRPV1 mediates LTD at excitatory synapses onto CA1 interneurons (Gibson et al., 2008). The excitatory

synapses of the hippocampal CA1 interneurons are depressed by TRPV1 agonist capsaicin and the potent endogenous TRPV1 activator 12-(S)-HPETE (Gibson et al., 2008). TRPV1 activation by capsaicin or by endocannabinoid anandamide depressed somatic but not dendritic inhibitory inputs onto dentate granule cells, suggesting that TRPV1 modulates GABAergic synaptic transmission in a compartment-specific manner (Chávez et al., 2014). Interestingly, acute inescapable stress on an elevated platform suppressed LTP and facilitated LTD at the hippocampal CA3-CA1 synapse, but the TRPV1 agonist capsaicin effectively prevented these effects, suggesting that TRPV1 contributes to stress-induced impairment of spatial memory (Li et al., 2008) (see "Redox-Sensitive TRP Channels in Higher Brain Functions" section).

TRPA1, the channel that has the highest oxidation sensitivity among the TRP channels, is expressed in the brain (Stokes et al., 2006), including in the hippocampus (Lee et al., 2016), the dentate gyrus (Koch et al., 2011), the supraoptic nucleus (Yokoyama et al., 2011), the nucleus tractus solitarii (Sun et al., 2009), and the somatosensory cortex (Kheradpezhouh et al., 2017). TRPA1 is also expressed in astrocytes and regulates basal $[Ca^{2+}]_i$ (Shigetomi et al., 2012, 2013; Takizawa et al., 2018). TRPA1 inhibition, which decreases resting $[Ca^{2+}]_i$ in astrocytes, impairs interneuron inhibitory synapse efficacy by reducing GAT-3-mediated GABA transport, resulting in the elevation of extracellular GABA (Shigetomi et al., 2012). Furthermore, astrocyte TRPA1 channels are required for constitutive D-serine release into the extracellular space, which contributes to NMDA receptor-dependent LTP in CA3-CA1 synapses (Shigetomi et al., 2013). In rat astrocyte cell line C6 cells and primary cultured astrocytes, a TRPA1-mediated spontaneous rise of $[Ca^{2+}]_i$ modulated the spontaneous release of peptide hormones from astrocytes (Takizawa et al., 2018). Recently, our group reported that TRPA1 acts as an acute hypoxia sensor in brainstem astrocytes through O_2-dependent protein translocation and triggers ATP release from astrocytes, which potentiates respiratory center activity (Uchiyama et al., 2020). Interestingly, vascular endothelial cell-specific *Trpa1* KO exacerbates cerebral infarcts following permanent middle cerebral artery occlusion in mice (Pires and Earley, 2018), suggesting that TRPA1 in cerebral artery endothelial cells is activated by oxidative stress under ischemia and protects from cerebral ischemia through the secretion of vasodilators, including NO. Taken together, TRPA1 is expressed in diverse cell types within the brain, including glial and endothelial cells, and promotes the secretion of factors that modulate neuronal circuits and vascular tonus upon oxidative stress in disease conditions.

REDOX-SENSITIVE TRP CHANNELS IN THE ESTABLISHMENT OF NEURONAL CONNECTIVITY

Abnormalities in neurites are considered to be involved in the pathogenesis of psychiatric disorders because they can cause impaired neuronal connectivity (Penzes et al., 2011). TRPC1 positively regulates netrin-1-induced growth-cone turning in cultured *Xenopus* spinal neurons (Wang and Poo, 2005; Shim et al., 2009), while it is not involved in brain-derived neurotrophic factor (BDNF)-induced growth-cone turning in cultured rat cerebellar granule cells (Li et al., 2005). Both TRPC1 and TRPC3 channels are required for leptin-sensitive current and leptin-induced spine formation in cultured hippocampal neurons (Dhar et al., 2014). Interestingly, SOCE mediated by TRPC1 is required for the proliferation of adult hippocampal neural progenitor cells (Li et al., 2012). Activation of TRPC4 induces inhibition and arborization of neurite growth in cultured hippocampal neurons (Jeon et al., 2013). TRPC5 that interacts with the growth-cone-enriched protein stathmin 2 has been shown to be a negative regulator of neurite extension and growth-cone morphology (Greka et al., 2003). In semaphorin 3A-induced neuronal growth-cone collapse, calpain has been demonstrated to cleave and activate TRPC5 channels, suggesting that TRPC5 acts downstream of the semaphorin signaling that causes changes in neuronal growth-cone morphology (Kaczmarek et al., 2012). Neurotrophin-3-induced Ca^{2+} influx via TRPC5 inhibits neuronal dendritic growth through activation of Ca^{2+}/calmodulin kinase (CaMK) IIα (He et al., 2012). TRPC5 channels have also been shown to induce the activation of Ca^{2+}/calmodulin kinase kinase (CaMKK) and the γ-isoform of CaMKI (CaMKIγ), followed by the promotion of axon formation in cultured hippocampal neurons (Davare et al., 2009). In the cerebellar cortex, *Trpc5* deficiency causes the formation of long and highly branched granule neuron dendrites with impaired dendritic claw differentiation, resulting in gait and motor coordination deficits (Puram et al., 2011). Mechanistically, TRPC5 forms a complex specifically with CaMKIIβ and induces the CaMKIIβ-dependent phosphorylation of ubiquitin ligase Cdc20-APC at the centrosome, suggesting the significance of the TRPC5/CaMKIIβ/Cdc20-APC signaling axis in the regulation of dendritic morphology (Puram et al., 2011). Thus, the TRPC1, TRPC4, and TRPC5 channels are key regulators for proper establishment of neuronal connectivity.

In cultured cortical neurons, pharmacological perturbation of TRPM2 markedly increases the axonal growth, and the neurons isolated from *Trpm2* KO mice have significantly longer neurites and a greater number of spines than those isolated from control mice (Jang et al., 2014). In addition, the blockage of TRPM2 reverses lysophosphatidic acid induced suppression of neurite growth (Jang et al., 2014), suggesting that TRPM2 is a negative regulator of neurite outgrowth.

REDOX-SENSITIVE TRP CHANNELS IN HIGHER BRAIN FUNCTIONS

Genome scanning for 47 bipolar disorder families has shown that there is a susceptibility locus near the telomere on chromosome 21q (Straub et al., 1994). Fine mapping linkage analysis identified that *TRPM2* on chromosome 21q is associated with bipolar disorder (McQuillin et al., 2006). Two SNPs of *TRPM2* were found in the analysis: a base pair change in exon 11 of *TRPM2*, which causes an amino acid substitution (aspartic acid to glutamic acid: D543E), and a non-conservative change from

TABLE 2 | Behavioral phenotypes in mice with genetic or pharmacological inactivation of redox-sensitive TRP channels.

	Anxiety	Memory	Other behavioral phenotype
TRPM2 KO mice (Jang et al., 2015)	**Increased anxiety-like behavior** Decreased time spent in open arms in EP Increased latency to Light part in LD	N/D	Decreased interaction time in social interaction test
TRPM2 KO mice (Andoh et al., 2019)	Normal in LD and EP Normal in social interaction test	Normal in radial maze and Y-maze tests Decreased flexibility in Barnes maze test	Normal in rotarod, forced swim, tail suspension, and prepulse inhibition tests Increased resiliency to repeated social defeat stress
Middle-aged TRPM2 KO mice (Kakae et al., 2019) Middle-aged: 12–16 months old Aged: 20–24 months old	N/D	**Enhanced spatial working memory** Middle-aged TRPM2 KO mice show increased alternation behavior compared with middle-aged WT in Y-maze test. **Enhanced spatial reference memory** Middle-aged TRPM2 KO mice show increased exploratory preference compared with middle-aged WT in novel object recognition test. Aged TRPM2 KO mice show increased exploratory preference compared with aged WT in novel location recognition test.	N/D
Stressed TRPM2 KO mice (Ko et al., 2019)	**Decreased anxiety-like behavior** Stressed TRPM2 KO mice show more sucrose consumption compared with stressed WT in sucrose consumption test.	N/D	N/D
Stressed	Stressed TRPM2 KO mice show less escaped latency compared with stressed WT in learned helplessness test. Stressed TRPM2 KO mice show less latency to feed compared with stressed WT in novelty suppressed feeding test. Stressed TRPM2 KO mice show less immobility time compared with stressed WT in forced swim test.		
Normal	**Decreased anxiety-like behavior** Non-stressed TRPM2 KO mice show less latency to feed compared with non-stressed WT in novelty suppressed feeding test. Non-stressed TRPM2 KO mice show less immobility time compared with non-stressed WT in forced swim test.	N/D	N/D
TRPC5 KO mice (Riccio et al., 2009)	**Decreased anxiety-like behavior** Increased entries in open arms in EP in center region in OF (first 5 min) Increased number of nose contacts in social interaction test	Normal in fear conditioning test	Decrease tendency in acoustic startle response Normal in novelty-suppressed feeding test Normal in locomotor activity in OF

(Continued)

TABLE 2 | Continued

	Anxiety	Memory	Other behavioral phenotype
TRPC4 KO mice (Riccio et al., 2014)	**Decreased anxiety-like behavior** Increased entry and time spent in open arms in EP in center region in OF	Normal in auditory and contextual fear conditioning tests	Normal in acoustic startle, tail suspension, and beam walking tests Normal in gait analysis Normal in total distance in OF (red light) Increased total distance in OF
TRPC1 KO mice (Lepannetier et al., 2018)	Normal in OF and EP	**Impaired fear memory** Decreased freezing response in contextual and trace fear conditioning tests **Impaired spatial working memory** Decreased alternance in Y-maze tests Decreased correct arm entry in spatial novelty preference test (modified Y-maze) Normal in Morris water maze and radial maze tests	Increased distance traveled in OF in total 2 days
TRPC1,4,5 KO mice (Bröker-Lai et al., 2017)	N/D	**Impaired spatial working memory** Increased number of errors in T-maze and radial maze tests Normal spatial reference memory in Morris water maze test	N/D
TRPC4/TRPC5 channel inhibitor (M084) (Yang et al., 2015)	**M084 decreased anxiety-like behavior** Increased number of entries in light part in the LD Increased number of entries and time spent in open arms in EP	N/D	Normal in locomotor activity (no difference among control, CUS, and CUS + M084) M084 rescues the immobility which is increased by CUS in forced swim test M084 rescues the latency to feed which is increased by CUS in novelty suppressed feeding test
TRPC4/TRPC5 channel inhibitor (HC-070) (Just et al., 2018)	**HC-070 decreased anxiety-like behavior** HC-070 rescues open arm entries decreased by CCK-4 in EP Decreased number of buried marble in marble burying test	Rescued the freezing time which is increased by CSD in auditory and contextual freezing tests (HC-070 inhibited formation of trauma.)	Decreased immobility time in tail suspension and forced swim tests Normal locomotor activity in OF
TRPV1 KO mice (Marsch et al., 2007)	**Decreased anxiety-like behavior** Increased total distance moved in light part in LD Increased time and entries in open arms in EP Normal in OF	**Impaired fear memory** Decreased freezing response in auditory and contextual fear conditioning tests	Normal in auditory response
TRPA1 KO mice (Lee et al., 2017)	**Decreased anxiety-like behavior** Increased time spent in open arms in EP	**Enhanced fear memory** Increased freezing in auditory fear conditioning test **Enhanced spatial reference memory** Increased cross numbers in probe trial in Morris water maze test Increased discrimination index in novel location recognition	Enhanced social recognition behavior

(Continued)

TABLE 2 | Continued

	Anxiety	Memory	Other behavioral phenotype
Aged TRPA1 KO mice (Borbély et al., 2019) Young: 3–4 months old. Old: 18 months old	**Decreased anxiety-like behavior** Young TRPA1 KO mice show increased number of entries in center region compared with young WT in OF.	**Enhanced reference memory** Old TRPA1 KO mice show higher discrimination index compared with old WT in novel object recognition test. **Enhanced spatial working memory** Young TRPA1 KO mice show less reference memory error compared with young WT in radial maze test. Old TRPA1 KO mice show less exploration time compared with WT in radial maze test.	Young TRPA1 KO mice show higher velocity compared with young WT in radial maze test.

CSD, chronic social defeat; CUS, chronic unpredictable stress; EP, elevated plus-maze test; LD, light-dark transition test; N/D, not determined; OF, open field test

arginine to cysteine (R755C). Notably, the D543E mutation induces loss of function of TRPM2 activities, and *Trpm2* KO mice exhibit bipolar disorder-related behaviors, such as mood disturbances and impairments in social cognition (Jang et al., 2015) (**Table 2**). Interestingly, disruption of *Trpm2* increases phosphorylation of GSK-3, which is implicated as one of the main targets of lithium (Xie et al., 2011; Jang et al., 2015). Although the significance of GSK-3 in the pathology of bipolar disorder is still debated (Alda, 2015), these observations suggest that dysregulation of GSK-3 could, at least in part, account for the bipolar disorder-related behaviors induced by hypoactive TRPM2 mutations. Furthermore, a SNP of *TRPM2* has recently been identified as a risk factor for bipolar disorder in a Japanese population (Mahmuda et al., 2020). These lines of evidence suggest a link between defects in TRPM2 activities and the pathology of bipolar disorder.

In addition to the connection between TRPM2 and bipolar disorder, accumulating evidence has indicated the impact of TRPM2 on major depressive disorder. In a mouse model of depression via chronic unpredictable stress, disruption of *Trpm2* produced antidepressant-like behaviors accompanied by reduced ROS, suppressed ROS-induced calpain activation, and enhanced phosphorylation of two Cdk5 targets, synapsin 1 and histone deacetylase 5, which are linked to synaptic function and gene expression, respectively (Ko et al., 2019). Notably, while middle-aged (12–16 months) and aged (20–24 months) wild-type (WT) mice exhibited memory dysfunction compared with young (2–3 months) WT mice, these characteristics were undetectable in *Trpm2* KO mice (Kakae et al., 2019) (**Table 2**), potentially owing to defects in the sensing of oxidative stress, whose levels are increased by aging (Tan et al., 2018). *Trpm2* deficiency also attenuates social avoidance induced by repeated social defeat stress in mice (Andoh et al., 2019). Under physiological conditions, a comprehensive behavioral test battery has shown that *Trpm2* KO mice exhibit no behavioral phenotypes in light/dark transition, rotarod, elevated plus maze, social interaction, prepulse inhibition, Y-maze, forced swim, cued and contextual fear conditioning, and tail suspension tests (Andoh et al., 2019), whereas *Trpm2* KO mice were also reported to exhibit bipolar disorder-related behaviors (Jang et al., 2015) (**Table 2**). Differences in the genetic backgrounds of mice or the animal facilities and equipment used in the behavioral analyses could explain the inconsistency of the results. Collectively, these findings suggest that disruption of *Trpm2* potentially induces behavioral phenotypes in pathological conditions that could be related to oxidative stress.

TRPC4 and TRPC5 are highly expressed in the cortex and amygdala, the regions thought to be crucial in regulating anxiety (Riccio et al., 2009, 2014). Importantly, previous works with transgenic mice suggest that TRPC4 and TRPC5 play important roles in amygdala function, and thus inhibition of TRPC4 and TRPC5 have anxiolytic effects (Riccio et al., 2009, 2014). This implicates the significance of both TRPC4 and TRPC5 in innate fear function, which represents a key response to environmental stress. *Trpc5* KO mice exhibited diminished innate fear levels in response to innately aversive stimuli (Riccio et al., 2009) (**Table 2**). *Trpc4* KO mice also showed

suppressed $G_{\alpha\ q/11}$-dependent responses in the amygdala, which regulate fear-related behavioral processes, and decreased anxiety-like behaviors (Riccio et al., 2014) (see "Neuronal and Glial Functions of Redox-Sensitive TRP Channels" section) (**Table 2**). *Trpc4* knockdown in the lateral amygdala via lentivirus-mediated gene delivery of RNAi mirrored the behavioral phenotype of constitutive *Trpc4* KO mice. Given that the animal model for the analysis of innate fear responses is limited, *Trpc4* and *Trpc5* KO mice may be useful animal models for investigating the mechanisms underlying the regulation of innate fear responses. It is worth noting that there is a caveat regarding interpretation of the behavioral data because *Trpc5* KO mice have deficits in gait and motor coordination (Puram et al., 2011).

In contrast to *Trpc4* and *Trpc5* KO mice, deletion of the *Trpc1* gene impairs fear memory formation but not innate fear behavior (Lepannetier et al., 2018), suggesting that TRPC1 regulates fear-related behavioral processes through distinct mechanisms from the TRPC5- and TRPC4-mediated pathways. In hippocampal functions, deletion of *Trpc1* impaired functioning in the Y-maze task, and the triple deficiency of *Trpc1*/*Trpc4*/*Trpc5* induced reduced performance in the T-maze and radial maze tests, while both *Trpc1* single KO and *Trpc1*/*Trpc4*/*Trpc5* triple KO mice showed normal acquisition in the Morris water maze task (Bröker-Lai et al., 2017; Lepannetier et al., 2018), suggesting that TRPC1 and possibly both TRPC4 and TRPC5 regulate spatial working memory but not spatial reference memory.

Pharmacological analysis in rats revealed that the TRPV1 antagonist, capsazepine, attenuates anxiety-like behaviors in the elevated-plus maze test (Kasckow et al., 2004). Consistent with this observation, *Trpv1* KO mice exhibited less anxiety-related behaviors in the elevated-plus maze test and in the light/dark test compared to their WT littermates (Marsch et al., 2007) (**Table 2**). Furthermore, *Trpv1* KO mice showed less freezing in auditory and contextual fear conditioning tests, suggesting that TRPV1 regulates fear memory formation (Marsch et al., 2007). Under stressful conditions, *in vivo* activation of TRPV1 by intrahippocampal or intragastrical infusion of capsaicin prevented the impairing spatial memory retrieval caused by acute inescapable stress by placing mice on an elevated platform (Li et al., 2008), suggesting that activation of TRPV1 induces anti-stress effects.

Trpa1 KO mice displayed decreased anxiety-like behaviors in elevated-plus maze, better performance in the fear conditioning and novel location recognition tests, and enhanced social

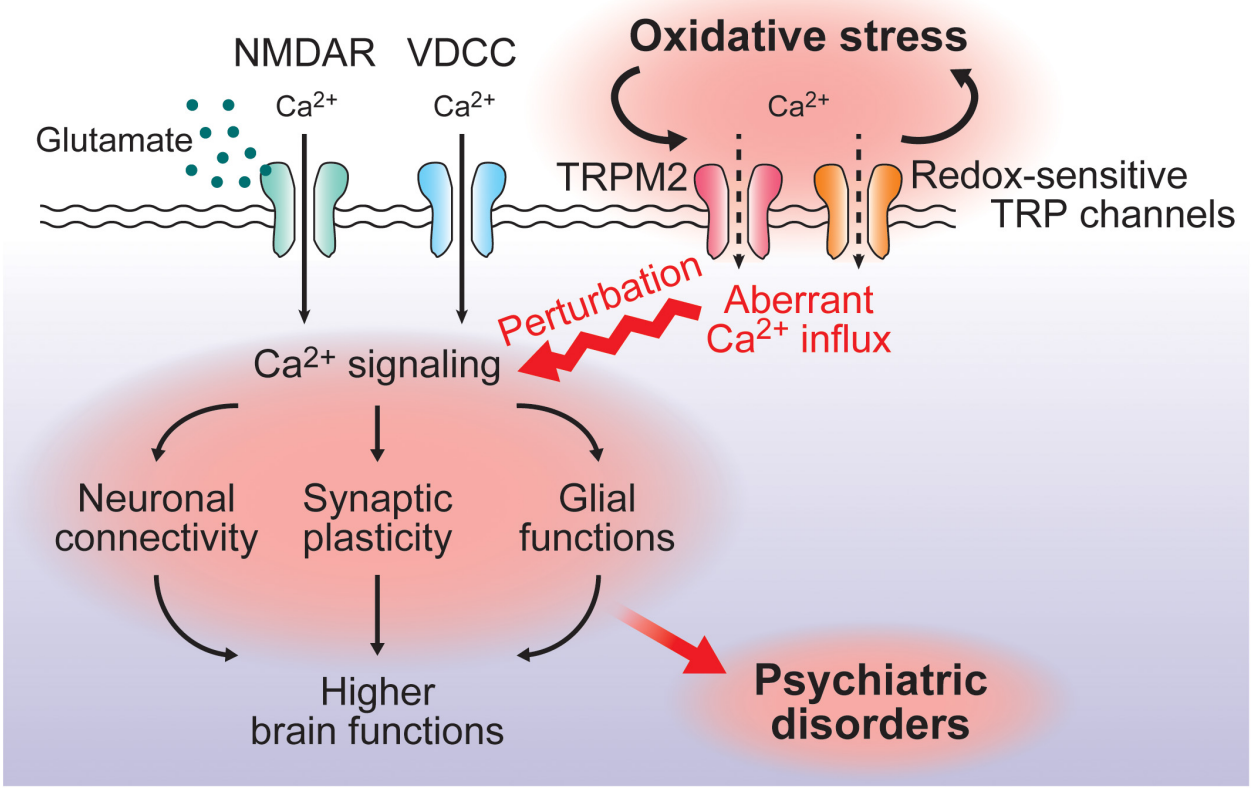

FIGURE 2 | A putative model for the impact of redox-sensitive TRP channels in the pathogenesis/pathophysiology of psychiatric disorders. VDCC- or NMDAR-mediated Ca^{2+} signaling plays fundamental roles in neural functions such as neuronal connectivity, synaptic plasticity, and glial functions, contributing to higher brain functions. Oxidative stress alters expression and activities of redox-sensitive TRP channels, which further enhances oxidative stress and induces a perturbation of the Ca^{2+} signaling mediated by VDCC or NMDAR. This could be associated with the pathogenesis/pathophysiology of psychiatric disorders. Notably, SNPs of *TRPM2* have been identified as a risk factor for bipolar disorder.

recognition behavior (Lee et al., 2017) (**Table 2**), possibly due, at least in part, to the evidence that TRPA1 negatively regulates hippocampal functions potentially through the suppression of neurite outgrowth (Lee et al., 2017). However, there is a caveat regarding the interpretation of the behavioral data because *Trpa1* KO mice are suggested to exhibit impaired motor function through axonal bundle fragmentation, downregulation of myelin basic protein, and decreased mature oligodendrocyte population in the brain (Lee et al., 2017); therefore, the observed behavioral phenotypes could be merely due to impaired motor functions rather than hippocampal functions. Interestingly, TRPA1 in astrocytes is implicated to partially contribute to cuprizone-induced demyelination, which is frequently used for the animal model of multiple sclerosis (Kriszta et al., 2020), raising the possibility that there is a connection between TRPA1 expression or function and psychiatric disorders. Notably, another behavioral analysis using aged (18-month-old) *Trpa1* KO mice supports the notion that TRPA1 negatively regulates hippocampal functions in an age-dependent manner (Borbély et al., 2019) (**Table 2**). Thus, these lines of evidence suggest that TRPA1 is a negative regulator of brain functions, raising the possibility that there is a connection between TRPA1 expression or function and psychiatric disorders; however, further studies are required to validate this hypothesis.

PERSPECTIVE

As described above, accumulating evidence has indicated that most redox-sensitive TRP channels have significant impacts on higher brain functions even though the role of oxidative stress as the activation trigger of the channels in this context is not fully understood. Comprehensive analysis of gene expression in normal brains and the brains of patients with psychiatric disorders would delineate the significance of redox-sensitive TRP channels in these disorders, where notably, TRPM2 expression is enhanced in hippocampal tissue samples from patients with major depressive disorder (Ko et al., 2019), and, furthermore, TRPA1 is upregulated in astrocytes of the hippocampus in the mouse model of Alzheimer's disease, in which oxidative stress is deeply involved (Lee et al., 2016). Given that oxidative stress is associated with psychiatric disorders and that the expression of redox-sensitive TRP channels is induced by oxidative stress partly via the activation of NRF2, an oxidant defense transcription factor (Takahashi et al., 2018), it is possible that upregulated redox-sensitive TRP channels are involved in the pathogenesis or pathophysiology of psychiatric disorders (**Figures 1**, **2**).

The concept of excitation-inhibition balance in neuropsychiatric disorders has been considered as a framework for investigating their mechanisms (Sohal and Rubenstein, 2019). Given that TRPA1 has the highest oxidation sensitivity among the TRP channels (Takahashi et al., 2011b; Mori et al., 2016), it is likely that TRPA1 senses oxidative stress acutely and moderates the excitation-inhibition balance by regulating astrocyte resting $[Ca^{2+}]_i$, which affects inhibitory synapse efficacy through the reduction of GABA transport (Shigetomi et al., 2012). Recently, several studies have shown that treatment with novel TRPC4/TRPC5 channel inhibitors produce antidepressant and anxiolytic-like effects in mice, suggesting that TRPC4 and TRPC5 channels are novel molecular candidates for the treatment of psychiatric symptoms (Yang et al., 2015; Just et al., 2018) (**Table 2**). Thus, the investigation of redox-sensitive TRP channels in the pathogenesis/pathophysiology of psychiatric disorders may lead to the development of novel therapeutic strategies for the treatment of these disorders.

AUTHOR CONTRIBUTIONS

AN and NT generated the first draft of the manuscript. All authors contributed to the revisions.

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REVIEW
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TRPM3 in Brain (Patho)Physiology

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Already for centuries, humankind is driven to understand the physiological and pathological mechanisms that occur in our brains. Today, we know that ion channels play an essential role in the regulation of neural processes and control many functions of the central nervous system. Ion channels present a diverse group of membrane-spanning proteins that allow ions to penetrate the insulating cell membrane upon opening of their channel pores. This regulated ion permeation results in different electrical and chemical signals that are necessary to maintain physiological excitatory and inhibitory processes in the brain. Therefore, it is no surprise that disturbances in the functions of cerebral ion channels can result in a plethora of neurological disorders, which present a tremendous health care burden for our current society. The identification of ion channel-related brain disorders also fuel the research into the roles of ion channel proteins in various brain states. In the last decade, mounting evidence has been collected that indicates a pivotal role for transient receptor potential (TRP) ion channels in the development and various physiological functions of the central nervous system. For instance, TRP channels modulate neurite growth, synaptic plasticity and integration, and are required for neuronal survival. Moreover, TRP channels are involved in numerous neurological disorders. TRPM3 belongs to the melastatin subfamily of TRP channels and represents a non-selective cation channel that can be activated by several different stimuli, including the neurosteroid pregnenolone sulfate, osmotic pressures and heat. The channel is best known as a peripheral nociceptive ion channel that participates in heat sensation. However, recent research identifies TRPM3 as an emerging new player in the brain. In this review, we summarize the available data regarding the roles of TRPM3 in the brain, and correlate these data with the neuropathological processes in which this ion channel may be involved.

Keywords: ion channels, channelopathies, transient receptor potential melastatin 3 channel, pregnenolone sulfate, brain, neurological disorders, gating pore current

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INTRODUCTION

The brain forms the control center of our body and is responsible for processing tremendous amounts of data to monitor and regulate our bodily functions at day and night. Such a precise control needs to be fast and accurate and requires highly sophisticated information processing. Our body accomplishes this task via neuronal cells, which form a complex connectome within the nervous system (Purves, 2004). Within the nervous system, information is delivered and

processed in form of electrical signals and synaptic events. Electrical signals occur, propagate and get transduced by neurons due to a diverse set of ion channels present in the membranes of the nerve cells (Hille, 2001). Ion channel proteins allow the permeation of ions over the otherwise ion-impermeable cell membrane, which results in a potential difference between the extracellular space and the intracellular side of the cell known as the membrane potential. Practically, changes in membrane potential serve as the information bearing electrical signals and are strictly controlled by ion channels (Hille, 2001; Purves, 2004). Therefore, genetic or acquired alterations in function of ion channels can result in severe disturbances of the electrical signaling in our nervous system causing several neurological or psychiatric diseases. For this reason, ion channels are primary targets for pharmacological interventions to treat such diseases (Kullmann, 2002; Kumar et al., 2016).

Among the hundreds of ion channels expressed in the human brain, Transient Receptor Potential (TRP) channels form a diverse group of poly-modally activated cation channels and they are generally considered as molecular sensors of external and internal stimuli (Clapham, 2003; Voets et al., 2005; Nilius, 2012). In total, 28 different TRP channels exist in mammals, which are divided into seven subfamilies according to their sequence homology. Functionally active TRP channels are composed of four individual subunits (Clapham et al., 2005) as recently evidenced by high resolution structural models (Liao et al., 2013; Yin et al., 2018; Cao, 2020; Huang et al., 2020) (**Figure 1**). Once activated, TRP channels allow the influx of cations into the intracellular space resulting in the depolarization of the plasma membrane and possible subsequent modulation of voltage-gated ion channels (Clapham et al., 2005; Gees et al., 2010; Wu et al., 2010; Nilius and Szallasi, 2014). Importantly, most TRP channels possess a substantial permeability for calcium, which is a vital signaling molecule throughout several cellular and molecular processes and therefore, plays a leading role in brain homeostasis and excitability (McBurney and Neering, 1987; Zündorf and Reiser, 2011). It is generally believed that TRP channels can play a substantial role in fine-tuning the membrane potential and neuronal excitability (Sawamura et al., 2017).

Some members of the TRP channel family are functionally expressed in various brain regions and their involvement in diverse physiological and pathological processes of the brain has already been described (Moran et al., 2004; Nilius, 2012; Reboreda, 2012; Sawamura et al., 2017). Among them, the TRP **c**anonical (TRPC) channel subfamily presents probably the most-studied TRP channel subfamily in the brain (Sawamura et al., 2017). TRPCs were reported in various brain regions (Riccio et al., 2002; Huang et al., 2011), where they were implicated in excitatory post-synaptic conduction (Kim et al., 2003), neuronal cell death and survival (Jia et al., 2007; Narayanan et al., 2008), and dendritic growth and spine formation (Amaral and Pozzo-Miller, 2007; Tai et al., 2008), among other things. Another well-studied TRP channel in the brain is TRP **v**anilloid 1 (TRPV1), which is of importance in descending modulatory pain pathways and seems to play an extended role in other brain functions, by enhancing glutamatergic neurotransmission (Martins et al., 2014).

The TRP **m**elastatin (TRPM) channel subfamily represents an especially heterogeneous group within the TRP superfamily and includes eight members in total (TRPM1-8). Most of them are non-selective calcium-permeable cation channels. As an exception, TRPM4 and TRPM5 are calcium-impermeable channels activated by intracellular calcium. As such, all TRPM family members can evoke calcium increases in cells, either by conducting calcium ions through their pores or by regulating the membrane potential, thereby activating/modulating calcium channels or transporters (Clapham et al., 2005; Wu et al., 2010; Huang et al., 2020). Moreover, three members (TRPM2, TRPM6 and TRPM7) are so-called "chanzymes," bearing an enzyme (kinase or hydrolase) domain in addition to their ion channel pore (Nilius and Owsianik, 2011; Huang et al., 2020). Furthermore, several TRPM channels are also thermosensitive, covering a wide range of physiological temperatures (TRPM2, TRPM3, TRPM4, TRPM5 and TRPM8) (Talavera et al., 2005; Dhaka et al., 2007; Vriens et al., 2011; Tan and McNaughton, 2018; Vandewauw et al., 2018). The diverse properties of the TRPM family explain their involvement in a multitude of biological processes. They are regulating sensory processes, including vision (TRPM1) (Morgans et al., 2009), taste (TRPM5) (Pérez et al., 2002; Talavera et al., 2005), temperature sensation and nociception (TRPM2, TRPM3, TRPM8) (Dhaka et al., 2007; Voets et al., 2007; Vriens et al., 2011; Held et al., 2015b; Tan and McNaughton, 2018; Vandewauw et al., 2018). Moreover, they play important roles in ion homeostasis (Mg^{2+} uptake and reabsorption by TRPM6 and TRPM7) (Schlingmann et al., 2007), and modulate secretory processes (TRPM2, TRPM3, TRPM4, TRPM5) in various cells all over the body (Cheng et al., 2007; Wagner et al., 2008; Brixel et al., 2010; Mathar et al., 2010; Uchida et al., 2011; Held et al., 2015a; Philippaert et al., 2017).

TRPM2 represents the best characterized member in the brain, where it exhibits an ubiquitous expression pattern (Allen Mouse Brain Atlas, 2004; Fonfria et al., 2006; Lein et al., 2007). It is intensively studied with regards to its role in the response to oxidative stress, which occurs during aging and neurodegenerative diseases (Sita et al., 2018). Recently, TRPM2 was also described as a hypothalamic heat sensor involved

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CA1, Cornu Ammonis 1; DEE, developmental and epileptic encephalopathies; DHEA, dehydroepiandrosterone; DRG, dorsal root ganglia; GABA, γ-aminobutyric acid; Gβγ, G beta-gamma complex; GIRK, G-protein-coupled inward rectifier potassium channel; ICFR, *r*egion *i*ndispensable for *c*hannel *f*unctions in TRPM3; *I-V* plot, current-voltage plot; KS, Kabuki syndrome; K_v, voltage-gated potassium channel; MCA/MR, multiple congenital anomaly/mental retardation syndrome; MITF, microphthalmia/melanogenesis-associated transcription factor; N.at-K_v3.2, flatworm voltage-gated potassium channel 3; Na_v, voltage-gated sodium channel; NFATc1, nuclear factor of activated T cells type c1; NMDA, N-methyl-D-aspartate; Pax6, paired-box 6 transcription factor; PD, pore domain; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PregS, pregnenolone sulfate; RANKL, receptor activator of nuclear factor kappa-B ligand; S1-6, transmembrane segments 1-6; STS, steroid sulfatase; TRPM, transient receptor potential channel melastatin; TRPC, transient receptor potential channel canonical; TRPV, transient receptor potential channel vanilloid; VM/PQ mutation, human TRPM3 DEE point mutations with amino acid substitutions valine to methionine (VM) and proline to glutamine (PQ); VSD, voltage-sensing domain; WT, wild type.

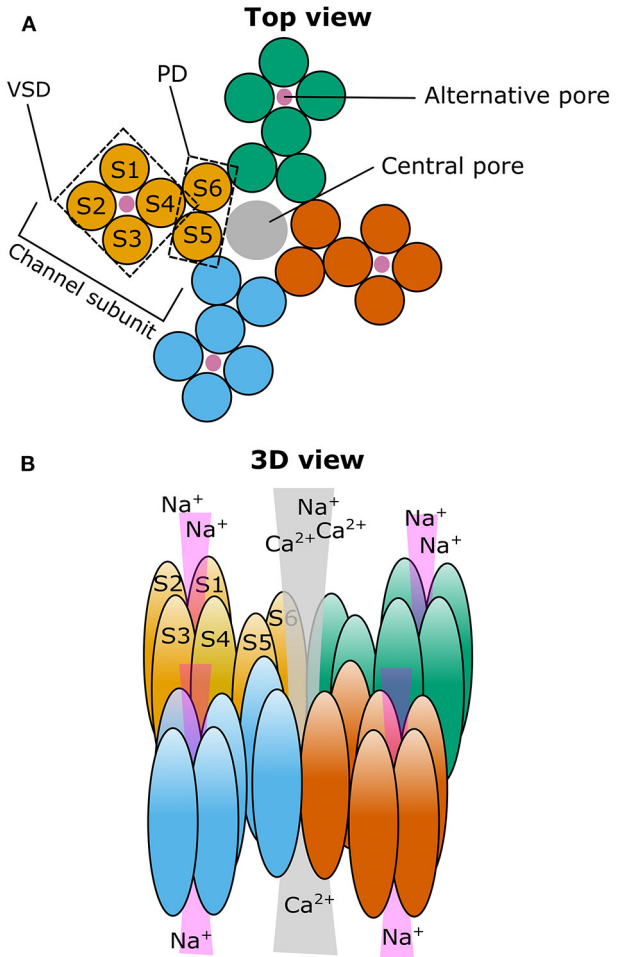

FIGURE 1 | Cartoon illustrating the putative localization of the central and alternative ion pore in TRPM3. **(A)** Top view on a representation of the four TRPM3 channel subunits (individually colored) that come together to form a functional ion channel. The voltage-sensing domain (VSD) and the pore domain (PD) are indicated in one of the subunits as well as the path of the central pore (gray circle) and the putative path(s) for the alternative ion pore(s) (four magenta circles). **(B)** Same as in **(A)** but in a three-dimensional representation looking from the diagonal top on TRPM3. S1–6: Transmembrane segment 1–6.

in central thermoregulation (Song et al., 2016). Furthermore, TRPM7 is widely expressed in the brain, and was shown to be involved in cell growth (Turlova et al., 2016) and cell death after ischemic and hypoxic brain injuries (Aarts et al., 2003; Sun et al., 2009; Chen et al., 2015; Sun, 2017). TRPM4 was also reported to be involved in cognitive functions, as well as in (patho)physiological processes, such as hippocampal plasticity (Menigoz et al., 2016; Bovet-Carmona et al., 2018) or development of trauma induced brain edema (Gerzanich et al., 2019; Woo et al., 2020).

TRPM3, a less studied member of the melastatin subfamily (Grimm et al., 2003; Lee et al., 2003), recently came into the focus of attention due to its involvement in human brain pathologies. In fact, TRPM3 is best characterized in the peripheral nervous system, where it functions as a noxious heat sensor in somatosensory neurons (Vriens et al., 2011; Vriens and Voets, 2018). Although several groups had indicated an abundant expression of TRPM3 in the brain in the past (Oberwinkler and Philipp, 2014), only few studies investigated its roles in the central nervous system, showing its functional presence in cerebellar Purkinje cells (Zamudio-Bulcock et al., 2011) and oligodendrocytes (Hoffmann et al., 2010). The more surprising were two recent publications that reported *de novo* mutations in TRPM3 as the cause of developmental and epileptic encephalopathies (DEEs) in a total of nine patients (Dyment et al., 2019; de Sainte Agathe et al., 2020). These findings motivated further functional studies on these mutant channels (Van Hoeymissen et al., 2020; Zhao et al., 2020).

In this review, we summarize our current knowledge and knowledge gaps related to TRPM3, focusing on channel properties that are relevant to understand its role in brain function and pathology.

ION CHANNEL PROPERTIES AND FUNCTIONS OF TRPM3—LESSONS FROM THE PERIPHERAL NERVOUS SYSTEM AND NON-NEURAL TISSUES

General Properties of TRPM3 Splice Variants

TRPM3 was identified <20 years ago as a functional ion channel-forming TRP protein (Grimm et al., 2003; Lee et al., 2003). In 2007, it was even labeled as an enigmatic channel (Oberwinkler, 2007), based on the fact that the *Trpm3* gene appears to encode the highest number of channel isoforms reported within the TRP family (Oberwinkler and Philipp, 2014; Shiels, 2020). The different isoforms arise from alternative splicing at the N terminal part of the channel and from alternative splicing of exons spread all over the gene. The mouse isoforms are classified into TRPM3α and TRPM3β groups depending on the start exon. TRPM3α isoforms start with exon 1 and do not express exon 2, while TRPM3β isoforms start with exon 2 (Oberwinkler et al., 2005; Oberwinkler, 2007; Oberwinkler and Philipp, 2014). Recently, the novel splice variants, TRPM3γ2 and TRPM3γ3 were identified, which start with exon2 and have a truncation in exon 28 (Uchida et al., 2019). As in mouse, several splice variants exist in humans, labeled as TRPM3a-f following their relative abundance (Lee et al., 2003). Even more transcript variants and predicted protein sequences are deposited in the public NCBI reference sequence (RefSeq) database (Shiels, 2020). The splicing pattern seems to be well-conserved between species (Oberwinkler and Phillipp, 2007; Oberwinkler and Philipp, 2014). However, most of the reported isoforms are not yet functionally characterized and their roles are largely unknown. Whether they form functional cation channels seems to depend on a specific *r*egion *i*ndispensable for *c*hannel *f*unctions (ICFR), which was identified to be essential for the channel formation. Isoforms lacking ICFR, like TRPM3α7, do not form functional ion channels, probably due to a disturbed tetrameric channel complex formation and a decreased plasma membrane expression. Therefore, when co-expressed with the

functional TRPM3α2, TRPM3α7 acts as a dominant negative regulator of the channel activity (Frühwald et al., 2012). Although TRPM3α7 transcripts were detected and estimated to form about 15% of the total TRPM3 transcripts in the brain (Frühwald et al., 2012), its impact on brain-specific function is yet to be discovered.

Upon cloning, both human and mouse TRPM3 were identified as Ca^{2+} entry channels (Grimm et al., 2003; Lee et al., 2003), but their permeability and functional features can vary tremendously between different isoforms. At this point, only a few of the TRPM3 isoforms have been functionally characterized in detail, with the most comprehensive analyses comparing the isoforms TRPM3α1 and TRPM3α2 (Oberwinkler et al., 2005; Held et al., 2020), which differ only in the presence (TRPM3α1) or absence (TRPM3α2) of a 12 amino acid-long sequence insertion into the pore-forming loop. This seemingly minor difference results in a massive change in their biophysical characteristics, which dramatically affects their permeability. Homotetrameric ion channels formed by TRPM3α2 subunits are permeable for Ca^{2+}, Mg^{2+}, and even for Zn^{2+} and other divalent cations. In contrast, TRPM3α1 displays strongly reduced permeability for divalent cations and high selectivity toward monovalent cations (Oberwinkler et al., 2005; Wagner et al., 2010). Similar to other TRP channels, both isoforms are strongly inhibited by intracellular Mg^{2+} and show reduced ionic currents in the presence of extracellular divalent cations. The permeability of TRPM3α2, but not TRPM3α1, is also markedly reduced by extracellular monovalent cations (Oberwinkler et al., 2005). In addition to differences in selectivity, there are also marked differences in the pharmacological properties of the short and long pore loop variants. For instance, the long pore loop variant TRPM3α1 is insensitive to pregnenolone sulfate (PregS), a well-characterized agonist of the short pore loop variants TRPM3α2-6, and the isoforms show different sensitivities to several other agonists and antagonists, as well (Held et al., 2020), as detailed later in this review. The recently described isoforms TRPM3γ2 and TRPM3γ3 (Uchida et al., 2019) exhibit biophysical and pharmacological characteristics that appear very similar to those of TRPM3α2, although they show a generally decreased channel activity.

The high diversity amongst TRPM3 isoforms raises the physiologically highly relevant question to what extent do they contribute to the formation of the native TRPM3 channels in the various tissues. Although the answer is uncertain, the data discussed below suggest that most native TRPM3 channels exhibit biophysical and pharmacological properties that are similar to those of TRPM3α2. Indeed, publicly available transcriptome data from mouse tissues demonstrate that the short pore loop isoforms generally dominate, although to varying degrees in different tissues (Held et al., 2020). The transcripts of the recently described γ isoforms are also highly expressed in dorsal root ganglia (DRGs), but their expression in the brain is not known. Moreover, these γ isoforms were suggested not to interact with TRPM3α2 and not to alter pharmacological properties of TRPM3α2 when co-expressed in a recombinant system (Uchida et al., 2019).

The most abundant and characterized human isoform TRPM3a also shares the short pore loop with TRPM3α2 (Oberwinkler and Phillipp, 2007) and both their pharmacological properties and functional features are very similar (Badheka et al., 2015, 2017; Held et al., 2015a). Native human TRPM3 expressed in the sensory ganglia also seems to be functionally similar to the mouse TRPM3α2 channel (Vangeel et al., 2020). Cumulatively, these data indicate that the mouse channel, especially the TRPM3α2 variant, is a highly relevant model to study the function of (native) human TRPM3. Therefore, in the following parts we use the term TRPM3 when referring to the TRPM3α2 variant or to native channels, and specify distinct other variants when relevant.

It is important to mention that, besides the various isoforms, the *Trpm3* gene also codes a microRNA, miR-204, in intron 9 in both humans and mice. It is often co-expressed with the TRPM3 ion channel and should be considered in certain situations when investigating TRPM3 functions, especially when analyzing gene-deleted animal models (Oberwinkler and Philipp, 2014; Shiels, 2020). TRPM3 and miR-204 are co-expressed in pancreatic beta cells and affect insulin production and secretion (Wagner et al., 2008; Thiel et al., 2013; Xu et al., 2013). They are also highly co-expressed in several cells of the eye and seem to be regulated by the same promoter and transcription factors, including paired-box 6 transcription factor (Pax6) and microphthalmia/melanogenesis-associated transcription factor (MITF). These results suggest a possible synergism between their function in eye development and the onset of some ocular diseases (Karali et al., 2007; Adijanto et al., 2012; Xie et al., 2014; Shiels, 2020). Interestingly, TRPM1, the closest relative of TRPM3, also hosts a microRNA, miR-211, which belongs to the same microRNA family as miR-204 and also plays a significant role in the eye (Shiels, 2020). Moreover, in clear cell renal cell carcinoma, TRPM3 and miR-204 were found to play an antagonistic role in the control of oncogenic autophagy (Hall et al., 2014).

(Patho)physiological Roles of TRPM3 in the Periphery

TRPM3 was originally described as a constitutively active Ca^{2+} entry channel in the plasma membrane, which can contribute to store-operated Ca^{2+} entry in certain conditions (Lee et al., 2003), although later studies suggested that store depletion is not significant in regulating TRPM3 activity (Grimm et al., 2003, 2005; Oberwinkler and Phillipp, 2007). The activity of recombinant TRPM3 expressed in HEK293 cells can be stimulated by hypotonic solutions (Grimm et al., 2003; Held et al., 2020), although it remains unclear whether TRPM3 is directly activated by hypotonicity-related membrane stretching or the hypoosmotic environment activates the channel indirectly (Oberwinkler and Phillipp, 2007). TRPM3 was reported to mediate the effects of hypotonic stress in different context: decreased serum osmolarity stimulated the constriction of ductus arteriosus via activation of TRPM3 (Aoki et al., 2014) and hypotonic stress-induced upregulation of the receptor activator of nuclear factor kappa-B ligand (RANKL) and the nuclear factor

of activated T cells type c1 (NFATc1) was mediated by both TRPM3 and TRPV4 in periodontal ligament cells and osteoblasts (Son et al., 2015, 2018). These results suggest that TRPM3 can contribute to the mechanical stress-induced bone remodeling. Oppositely, inhibition of TRPM3 by hypertonic solutions may contribute to hypertonicity-induced gene expression in ciliated renal epithelial cells. However, conclusions of this study may be regarded with some caution, as the expression of TRPM3 was confirmed with antibodies and at RNA level, but TRPM3-mediated currents were not reported. Furthermore, the pharmacological effect of the TRPM3 agonist PregS on the hypertonic (500 mosM NaCl) solution-induced gene expression was only very partial, although it could be reversed by the TRPM3 antagonist isosakuranetin (Siroky et al., 2017). Another study, using pharmacological tools, suggested the presence of TRPP2-TRPM3 heteromeric channels in renal primary cilia (Kleene et al., 2019). In contrast to the most widespread variant, the long pore loop isoform TRPM3α1 is not sensitive to hypotonic clues (Held et al., 2020).

TRPM3 is also expressed in vascular smooth muscle cells, where its activation evoked smooth muscle contraction resulting in vasoconstriction, and inhibited IL-6 secretion (Naylor et al., 2010). However, activation of TRPM3 in the perivascular nerves resulted in vasodilation via release of vasoactive substances from perivascular nerve endings (Alonso-Carbajo et al., 2019). Stimulation of TRPM3 also inhibited secretory activity in fibroblast-like synoviocytes from rheumatoid arthritis patients, as TRPM3 agonist inhibited hyaloronan release (Ciurtin et al., 2010). The pharmacological activation of TRPM3 channels expressed in pancreatic beta cells induced insulin secretion (Wagner et al., 2008; Colsoul et al., 2011; Becker et al., 2020). Upon its activation, TRPM3 stimulated the opening of voltage-gated Ca^{2+} channels and initiated complex signaling pathways, upregulating the expression of different transcription factors (Mayer et al., 2011; Müller et al., 2011; Thiel et al., 2013; Becker et al., 2020). The opening of TRPM3 also serves as a regulated Zn^{2+} entry pathway in beta cells (Wagner et al., 2010), where zinc plays a relevant role in the biosynthesis and storage of insulin (Dunn, 2005). Interestingly, extracellular zinc entering via TRPM3 or voltage-gated Ca^{2+} channels can inhibit gene expression initiated by Ca^{2+} entry via the same channels. Therefore, the zinc release accompanying insulin release is hypothesized to act as a negative feedback on exocytosis (Loviscach et al., 2020). Zinc plays an important role in the central nervous system as well, by regulating excitability of ion channels and it can be released together with glutamate (Frederickson et al., 2005; Sensi et al., 2009), but until today, the role of TRPM3 in zinc-related signaling in the brain remains elusive.

TRPM3 is intensely studied in the somatosensory neurons of DRGs and trigeminal ganglia. In 2011, it was introduced as a new member of the thermosensitive TRP channels activated by warming. Elevation of temperature from room temperature to 33°C potentiates agonist induced TRPM3 activation, but its sensitivity toward warming is more dominant in the noxious heat range (>42°C) (Vriens et al., 2011, 2014b; Voets, 2012; Held et al., 2015b). Interestingly, heat sensitivity is completely lost in the long pore loop variant TRPM3α1 (Held et al., 2020). Compared to the well-characterized heat sensor TRPV1, the current–temperature relationship curve of TRPM3 is shifted slightly toward higher temperatures and its temperature-dependent increase in open probability is less steep (Vriens et al., 2011, 2014b; Voets, 2012; Held et al., 2015b). In good accordance with its thermosensitivity and expression by small-sized somatosensory neurons, TRPM3 plays a role in noxious heat sensation together with TRPV1 and TRPA1 (Vriens et al., 2011; Vandewauw et al., 2018; Vriens and Voets, 2018, 2019). TRPM3 activation results in neuropeptide release from the sensory terminals (Held et al., 2015a) and the channel is sensitized by inflammatory conditions, which may contribute to inflammatory hyperalgesia (Vriens et al., 2011; Mulier et al., 2020). In contrast to TRPV1, TRPM3 does not to appear to play a role in central thermoregulation and neither agonists nor antagonists induce noticeable changes in core body temperature (Vriens et al., 2011; Straub et al., 2013a). It selectively mediates pain, and is not involved in itch evoked by pruritic mediators such as histamine or serotonin (Kelemen et al., 2021), which are known to signal via TRPV1 (Shim et al., 2007) and TRPA1 (Morita et al., 2015), respectively.

In addition to somatosensory afferents, TRPM3 is also functional in the vagal afferents of the nodose ganglion (Staaf et al., 2010; Fenwick et al., 2014; Wu et al., 2014; Ragozzino et al., 2020). These channels contribute to basal and temperature-driven spontaneous glutamate release from the central terminals in the nucleus tractus solitarii, while not affecting the synchronous or asynchronous glutamate release (Ragozzino et al., 2020).

Regulation of the Channel Activity and Pharmacology of TRPM3

Intrinsic Regulation by Signaling Molecules

Until today, only a few intracellular signaling molecules were reported to interact with TRPM3. Like other TRP channels, TRPM3 activity is reduced by intracellular Mg^{2+} (Oberwinkler et al., 2005) and Ca^{2+} (Przibilla et al., 2018), and potentiated by phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and other phosphoinositides, among which PtdIns(3,4,5)P_3 was found to be the most effective (Badheka and Rohacs, 2015; Badheka et al., 2015; Tóth et al., 2015, 2016; Uchida et al., 2016). Multiple Ca^{2+}-calmodulin binding sites on the N-terminus were identified, which may interact with PtdIns(4,5)P_2, and S100A protein (Holakovska et al., 2012; Holendova et al., 2012; Przibilla et al., 2018). In good accordance, signaling pathways decreasing endogenous PtdIns(4,5)P_2, like phospholipase C activation evoked by M1 or M3 muscarinic acetylcholine receptors, inhibited both recombinant and native TRPM3 (Badheka et al., 2015; Tóth et al., 2015). $G_{\beta\gamma}$ subunits of trimeric G-proteins were also shown to be negatively coupled to TRPM3 activity, which underlies inhibition of TRPM3 upon stimulation of several G-protein-coupled receptors, including G_q-coupled M1 muscarinic acetylcholine, B2 bradykinin receptors and G_i-coupled M2 muscarinic acetylcholine, D2 dopamine, $GABA_B$, neuropeptide Y, μ-opioid receptors and G_s-coupled EP-2 prostaglandin, and

A2B adenosine receptors or receptors of somatostatin (Badheka et al., 2017; Dembla et al., 2017; Quallo et al., 2017; Alkhatib et al., 2019). A 10-amino-acid-long domain in TRPM3 was identified that interacted with $G_{\beta\gamma}$ proteins. This domain is encoded in an alternatively spliced exon, and is absent in the TRPM3α4 and TRPM3α5 variants, rendering them insensitive to μ-opioid receptor activation or overexpressed $G\beta_1\gamma_2$ subunits. X-ray crystallographic analysis of the corresponding peptide bound to $G\beta_1\gamma_2$ revealed that this domain of TRPM3 interacts exclusively with the $G\beta_1$ (and not $G\gamma_2$) subunit, and amino acids on both the TRPM3 peptide and the $G\beta_1$-proteins that mediate the interaction were identified. Interestingly, the interacting residues in $G\beta_1$ only partially overlap with those involved in the inhibitory interaction with G-protein-coupled inward rectifier K^+ (GIRK) channels (Behrendt et al., 2020). These results suggest that pharmacological targeting of TRPM3 may have a great potential to influence several signaling pathways relevant in various brain functions and in peripheral pain sensation.

TRPM3 Is a Steroid Regulated Channel

The first chemical activator of TRPM3 to be identified was D-erythro sphingosine, which was known from earlier studies to inhibit other ion channels. Two structural analogs, dihydro-D-erythro-sphingosine and N,N-dimethyl-D-erythro-sphingosine, although less effectively, also activated TRPM3, while neither ceramide and 1-sphingosine-phosphate (other significant signaling lipids of the sphingolipid pathway), nor arachidonic acid, anandamide, linoleic acid, linolenic acid, and diacylglycerol analogs affected the channel (Grimm et al., 2005). However, the TRPM3 specificity of D-erythro sphingosine was questioned later (Wagner et al., 2008). The L-type Ca^{2+} channel blocker nifedipine also activated TRPM3 in a reversible way (Wagner et al., 2008), but the long pore loop variant TRPM3α1 was found to be insensitive for nifedipine (Held et al., 2020).

The best characterized and most widely used endogenous TRPM3 agonist is the steroid compound PregS. It activates the channel by shifting its current-voltage activation curve toward more negative membrane potentials and also potentiates temperature-induced activation (Wagner et al., 2008; Vriens et al., 2011; Held et al., 2018). These mechanisms of action are typical features of the agonist-evoked activation of thermosensitive TRP channels in general (Voets et al., 2004). Although PregS activated TRPM3 only in supraphysiological concentrations in most of the experiments carried out at room temperature, a marked activation was evoked by only 100 nM PregS at 37°C, which is in the range of the physiological plasma concentrations (Vriens et al., 2011). Therefore, PregS can be considered as an endogenous activator of TRPM3, even in physiological circumstances. Due to the presence of the negatively charged sulfate group, pregS is a quite lipophobic substance and it behaves as a membrane-impermeable ligand that activates the channel only if applied to the extracellular side (Wagner et al., 2008). This finding suggests that the steroid-binding pocket of TRPM3 is located on the extracellular surface. The steroid (PregS) sensitivity is conserved in the short pore loop (TRPM3α2-α6) variants but completely lost in the long pore loop (TRPM3α1) variant, arguing for the importance of the pore region in the steroid activation of the channel (Held et al., 2020). Analysis of the structure-activity relationship of the steroid ligands revealed that the natural PregS is more effective than its enantiomer and the position and orientation of the sulfate group is also very important to preserve TRPM3 activation (Majeed et al., 2010; Drews et al., 2014). A few other, structurally similar steroid compounds [pregnenolone, dehydroepiandrosterone (DHEA) and DHEA-sulfate] also evoked a moderate activation of TRPM3 (Wagner et al., 2008).

Other steroids were also tested for activity toward the channel. In contrast to PregS, dihydrotestosterone, 17β-estradiol, and progesterone and its metabolites inhibited TRPM3 activation. The inhibitory effect of progesterone was independent of the used TRPM3 activator, whereas dihydrotestosterone behaved as competitive antagonist of PregS (Majeed et al., 2012).

Opening of a Non-canonical Pore and Its Significance

We found that activation by PregS is strongly potentiated by the co-application of the antifungal clotrimazole or its structural analogs TRAM34, senicapoc, and tamoxifen. Importantly, clotrimazole did not only potentiate the outwardly rectifying PregS evoked currents flowing through the well-established central pore of the channel, but also evoked monovalent-selective inwardly rectifying currents at negative membrane potentials (Vriens et al., 2014a). The biophysical characteristics of these currents resemble the so-called omega currents or gating pore currents described earlier in mutated voltage-gated Na^+ and K^+ channels (Sokolov et al., 2005, 2007; Tombola et al., 2005, 2007). The existence of an alternative ion permeation pathway conducting "omega-like" currents in TRPM3 is supported by several lines of evidence. Among others, the voltage sensitivity and permeability of this alternative ion permeation pathway strikingly differs from the main pore, and, in contrast to the main pore, it is resistant to Ca^{2+}-induced desensitization, as well as to classical pore blockers like La^{3+}. Importantly, mutations in the pore domain disrupting the channel's permeability did not affect the alternative pore current, but several mutations generated in the voltage-sensing domain affected the gating pore current, underlining the fundamental role of the voltage sensor domain in forming the non-canonical pore (**Figure 1**). Furthermore, introducing an arginine residue (Trp982Arg) into the S4 segment of the voltage sensor domain prevented PregS and clotrimazole from activating the alternative pore current without inhibiting the main pore-related conductance (Vriens et al., 2014a; Held et al., 2018). Indeed, arginine residues are essential components of the S4 segment of voltage-gated ion channels, and eliminating these positively charged residues results in the appearance of the gating pore current in the above mentioned mutated K^+ and Na^+ channels (Sokolov et al., 2005; Tombola et al., 2005; Held et al., 2016). Based on our best knowledge, until today, the opening of non-canonical pores was revealed only in three naturally occurring wild type ion channels: a flatworm K_v3 channel (N.at-$K_v3.2$), the H_v proton channel, which even does not possess a classical pore domain (Ramsey et al., 2006, 2010; Sasaki et al., 2006; Okamura et al., 2015), and the mammalian TRPM3 (Vriens et al., 2014a). Alignment of the S4 segment of these channels to other voltage-gated ion channels reveals that some of the arginine

residues are substituted by uncharged or negatively charged amino acid residues, thereby further highlighting that the loss of positively charged residues from the S4 plays a crucial role in the appearance of an alternative ion permeation pathway [For a comparative review about non-canonical pores in ion channels, we refer to our recent work (Held et al., 2016)]. Recently, we also identified CIM0216 as the currently available most potent and highly effective exogenous activator of TRPM3. Application of CIM0216 alone (i.e., without any other agonist) results in the simultaneous opening of both the classical and the non-canonical pore of the channel (Held et al., 2015a). However, until now, no endogenous ligand was identified that opens this alternative permeation pathway through TRPM3. Interestingly, clotrimazole applied on its own activated the PregS-insensitive long pore loop variant TRPM3α1, which resulted in a linear *I-V* relationship, including large inward currents at negative membrane potentials. The clotrimazole-evoked currents were found to be largely resistant to the classical pore blocker La^{3+} and to Ca^{2+} desensitization, suggesting a potential contribution of the non-canonical pore (Held et al., 2020).

Although, the physiological circumstances that may open the non-canonical pore of TRPM3 remain elusive, it may have important pathological significance. Compared to the exclusive activation of the main pore conductance, the additional opening of the non-canonical pore in TRPM3 increases the discharge rate of somatosensory neurons and exacerbates TRPM3-mediated nociception (Vriens et al., 2014a; Held et al., 2015a). Mutations in the voltage-sensing domain of Na_v and Ca_v channels that result in the appearance of ion conducting non-canonical pores are known to cause muscle (periodic paralysis) and heart diseases (Mixed Arrhythmias and Dilated Cardiomyopathy). Some mutations in the voltage sensor of $K_v7.2$ and $K_v7.3$ subunits also result in the development of non-canonical pore currents. These currents can contribute to the hyperexcitability of neurons and are associated with benign familial neonatal seizures. Recently, two mutations in TRPM3 were found in patients with developmental and epileptic encephalopathies (DEE) (Dyment et al., 2019; de Sainte Agathe et al., 2020). Notably, the mutations were characterized as gain of function mutations (Van Hoeymissen et al., 2020; Zhao et al., 2020), and in one of them the natural ligand PregS activated the gating pore currents (Van Hoeymissen et al., 2020).

Antagonists of TRPM3

Only few blockers of TRPM3 have been described. As nifedipine was recognized as an agonist of the channel, other dihydropyridines were also tested and found to inhibit TRPM3 (Drews et al., 2014). 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of the IP_3 receptor, interacts with several TRP channels. It activates the warm sensitive TRPV1-3, and inhibits several other members of the family, including TRPM3 (Xu et al., 2005). The non-steroidal anti-inflammatory fenamates also inhibited multiple TRP channels, but only mefenamic acid was found to be selective for TRPM3 (Klose et al., 2011). The phospholipase C inhibitor compound U73122 is also suggested to inhibit TRPM3 activation (Leitner et al., 2016).

TRPM3 is inhibited by ononetin, a deoxybenzoin from the plant Ononis spinosa (spiny restharrow), which belongs to the *Fabaceae*. The channel is also blocked by the citrus fruit flavanones hesperetin, naringenin, eriodictyol, liquiritigenin, and isosakuranetin, among which isosakuranetin is the most potent blocker reported until today (Straub et al., 2013a,b). Isosakuranetin and related compounds were also shown to inhibit acute thermal nociception (Straub et al., 2013a) and neuropathic pain (Jia et al., 2017). Another non-steroidal anti-inflammatory drug, diclofenac was also characterized as a TRPM3 blocker, inhibiting agonist-induced currents (Suzuki et al., 2016). The anticonvulsant primidone and the tetracyclic antidepressant maprotiline are also effective blockers of TRPM3. Primidone was found to block the main pore currents evoked by heat, Nifedipine, and PregS, as well as the alternative pore current induced by PregS and clotrimazole. Primidone also attenuated TRPM3-mediated acute thermal pain and heat hyperalgesia. Importantly the IC_{50} value of primidone is in the range of its therapeutic plasma concentrations (Krügel et al., 2017). Recently, we demonstrated that volatile anesthetics can also inhibit TRPM3 in slightly higher concentrations than reached in the plasma during general anesthesia (Kelemen et al., 2020). Although antidepressants, anticonvulsants and volatile anesthetics probably target ion channels in the central nervous system, the putative role of TRPM3 in their therapeutic effect remains to be elucidated.

Interestingly, primidone and volatile anesthetics inhibited both the classical and the non-canonical pore mediated currents, which suggests that these inhibitors do not act as classical pore-blockers but rather inhibit a more general conformational change in the proteins (Krügel et al., 2017; Kelemen et al., 2020).

ROLE OF TRPM3 IN THE BRAIN

Expression of TRPM3 in Various Brain Regions

The brain represents, next to the kidney, one of the tissues with the highest indicated TRPM3 expression. High levels of TRPM3 mRNA were found in several studies of whole brain tissues from rodent and human (Fantozzi et al., 2003; Grimm et al., 2003; Lee et al., 2003; Oberwinkler et al., 2005; Fonfria et al., 2006; Inoue et al., 2006; Wagner et al., 2008; Gilliam and Wensel, 2011; Jang et al., 2012). More detailed expression analysis showed a high abundance of TRPM3 in the choroid plexus, the cerebellum, the forebrain and the hippocampus (dentate gyrus), among others (Lee et al., 2003; Oberwinkler et al., 2005; Kunert-Keil et al., 2006; Hasselblatt et al., 2009; Hoffmann et al., 2010; Zamudio-Bulcock et al., 2011; Oberwinkler and Philipp, 2014) (**Table 1**). Likewise, TRPM3 also displays a diverse distribution pattern over several different cell types within the brain. TRPM3 expression was found in neuronal cells, epithelial cells as well as in oligodendrocytes (Hasselblatt et al., 2009; Hoffmann et al., 2010; Zamudio-Bulcock et al., 2011), but on a functional level it was only confirmed in cerebellar Purkinje neurons (Zamudio-Bulcock et al., 2011) and in oligodendrocytes isolated from whole brain tissue (Hoffmann et al., 2010) (**Table 1**). Actually, to date

TABLE 1 | Overview of reported brain regions expressing TRPM3.

Brain region	Cell type	Tested expression level
Whole brain	Not specified (n.s.)	mRNA Protein
	Oligodendrocytes	mRNA Protein Functional
Basal ganglia	n.s.	mRNA
Substantia nigra	n.s.	mRNA
Brain stem	Neuronal cells	Protein
	Oligodendrocytes	Protein
Cerebrum	n.s.	mRNA
Corpus callosum	Oligodendrocytes	Protein
Choroid plexus	n.s.	mRNA
	Epithelial cells	mRNA
Cerebellum	n.s.	mRNA Protein
	Purkinje cell	Protein Functional
Cortex	Neuronal cells	protein
Fimbria hippocampi	Oligodendrocytes	protein
Forebrain	n.s.	mRNA
Hippocampus	n.s.	mRNA
Dentate gyrus	n.s.	mRNA
Hypothalamus	n.s.	mRNA
Locus coeruleus	n.s.	mRNA
Tenia tecta	n.s.	mRNA
-	Oli-neu/OLN-93 cells	Protein Functional

Brain regions, cell types and the levels of the reported expression for TRPM3 are summarized; n.s., not specified.

no structured analysis of the TRPM3 expression was carried out in the different brain areas and the variety of used detection techniques makes it almost impossible to compare the TRPM3 expression between different brain regions (Oberwinkler and Philipp, 2014).

It is interesting to note that several different splice variants of TRPM3 were described to be expressed in the mouse brain, with a total of seven alpha (Oberwinkler et al., 2005; Frühwald et al., 2012) and 17 beta variants (Frühwald et al., 2012; Oberwinkler and Philipp, 2014). Although it was reported that certain splice variant mRNA levels are tissue- and development-dependent in the brain (Hoffmann et al., 2010; Held et al., 2020), it remains unknown whether these different splice variants are functionally expressed in the reported tissues and whether they may be involved in mechanisms regulating TRPM3 activity in a cell-specific manner.

Function of TRPM3 in the Brain

Since TRPM3 is a non-selective, calcium-permeable cation channel, TRPM3 activity will result in a depolarization of the neuronal membrane. So far, the only studies that showed functional TRPM3 in brain cells were performed in primary oligodendrocytes isolated from the whole brain (Hoffmann et al., 2010) and in cerebellar Purkinje neurons in brain slices (Zamudio-Bulcock et al., 2011). Furthermore, a functional role of TRPM3 in the choroid plexus was suggested (Millar and Brown, 2006; Millar et al., 2007). These studies provided molecular evidence of TRPM3 activity, using allegedly TRPM3-specific pharmacology or a dominant-negative TRPM3 protein block. Despite this molecular evidence, no further efforts have been made to investigate potential effects of TRPM3 activation on a behavioral or (patho)physiological level in these or other studies. Given the expression of TRPM3 in the brain areas mentioned above, certain assumptions can be made concerning the potential physiological roles of TRPM3 in the brain. For instance, the high mRNA expression and functional activity of TRPM3 in cerebellar Purkinje neurons suggests a role of TRPM3 in the coordination of movement (Beckstead, 1996; Purves, 2004). Furthermore, the high expression of TRPM3 in the choroid plexus (Oberwinkler et al., 2005; Millar and Brown, 2006; Millar et al., 2007), may indicate a potential role of TRPM3 in the ion homoeostasis that is necessary for the production of the cerebrospinal fluid (Damkier et al., 2013). Moreover, high levels of TRPM3 in the hippocampus (Oberwinkler et al., 2005; Kunert-Keil et al., 2006; Hoffmann et al., 2010) hint at a potential role in memory formation and consolidation (Andersen, 2007). Fittingly, some endogenous TRPM3 (ant)agonists are known to influence synaptic signaling and memory functions. For instance, the endogenous TRPM3 activator PregS was reported to increase long-term potentiation at hippocampal CA1 synapses (Sabeti et al., 2007), which may lead to memory-improving effects (Dastgheib et al., 2015). Although these actions of PregS were often attributed to effects on NMDA and $GABA_A$ receptors (Paul and Purdy, 1992; Mayo et al., 1993; Akk et al., 2001; Horak, 2004), it is equally plausible that effects of PregS were partially mediated via TRPM3 channel activation (Wagner et al., 2008). Similarly, estradiol, a reported TRPM3 antagonist, was shown to enhance memory consolidation mediated via the dorsal hippocampus (Tuscher et al., 2019). In addition, three recent studies reported in parallel on the modulation of TRPM3 by $G_{\beta\gamma}$-proteins via a signaling cascade with GPCRs, such as $GABA_B$-, μ-opioid- and NPY receptors (Badheka et al., 2017; Dembla et al., 2017; Quallo et al., 2017). Of note, all of these receptors can be found abundantly in the brain (Hill and Bowery, 1981; Delfs et al., 1994; Reichmann and Holzer, 2016), and the inhibitory effects of endogenous, brain-relevant GPCR ligands such as somatostatin (Martel et al., 2012) and morphine (Beltrán-Campos et al., 2015) on TRPM3 currents were illustrated in these studies (Badheka et al., 2017; Dembla et al., 2017; Quallo et al., 2017). However, all of these studies were performed in heterologous expression systems or peripheral sensory neurons and no brain tissue was used to confirm similar actions. Nevertheless, it can be hypothesized that these mechanisms are not exclusive to peripheral nerves, and that receptor-mediated modulation of TRPM3 may also occur in brain tissue. Undoubtedly, the identification of several brain-relevant receptors and ligands that are either directly or indirectly modulating TRPM3 strengthens

the evidence that TRPM3 might present an important player in various brain functions. However, such assumptions still have to be confirmed in experiments specifically designed to investigate the here hypothesized or other functions.

TRPM3 in Brain Disorders

The first link between TRPM3 and brain pathologies was reported in 2009, when Kuniba et al. (2009) performed molecular karyotyping in 17 patients and mutation screening in 41 patients with Kabuki syndrome (KS), a multiple congenital anomaly/mental retardation syndrome (MCA/MR). They identified a chromosomal region that also contains the *TRPM3* gene as a potentially contributing factor in the manifestation of KS. A few years later, Pagnamenta et al. reported a rare *TRPM3* exon deletion in a family with autism and proposed this deletion to contribute to the autism phenotype that was observed in these patients (Pagnamenta et al., 2011). Additionally, TRPM3 overexpression was observed in benign and malignant choroid plexus tumors (Hasselblatt et al., 2009; Japp et al., 2015, 2016). Very recently, TRPM3 was suggested to be involved in mood and anxiety disorders, as an interesting potential player in post-partum mood disorders (Thippeswamy and Davies, 2020). This suggestion was based on the facts that the PregS level-regulating steroid sulfatase (STS) enzyme deficiency is positively linked to mental health conditions and depression in human patients (Cavenagh et al., 2019) and PregS levels were reportedly increased in STS-deficient patients (Sánchez-Guijo et al., 2016). Fittingly, it was also shown that TRPM3 expression was altered in a mouse model of bipolar disorder due to serotonin depletion (Maddaloni et al., 2018), thereby supporting the idea that TRPM3 may regulate mood conditions. However, no hard evidence was provided yet for the mechanistic involvement of TRPM3 in the development and/or manifestation of the above-studied diseases. Considering that several other genes were found to be altered in these patients, conclusions concerning the role of TRPM3 in the investigated pathologies should be regarded with caution.

Interestingly, more evidence for a role of TRPM3 in brain pathologies was provided by a Ca^{2+} influx assay-based drug screening study that identified the clinically approved and commonly used anticonvulsant drug primidone and the anti-depressant maprotiline as potent and relatively selective TRPM3 inhibitors (Krügel et al., 2017). Both drugs were able to completely block the channel activity with IC_{50} values of $\sim$0.6 μM for primidone and $\sim$1.3 μM for maprotiline. However, despite this promising pharmacological profile of maprotiline, reported plasma concentrations during patient treatment are not reaching the concentration ranges necessary to inhibit TRPM3. In contrast, the plasma concentrations of primidone that are obtained in patients treated for epilepsy are in the range of concentrations needed to induce a full block of the PregS-induced TRPM3 currents in a HEK cell culture model (Krügel et al., 2017). Given that there is no consensus on the mechanism for the anticonvulsant action of primidone, TRPM3 represents a potential novel target of this anti-epileptic drug. It can be speculated that a downregulation of the TRPM3 activity might stabilize neuronal membrane potential and/or decrease presynaptic calcium release, thereby rendering the cells less susceptible to damaging overexcitation.

Finally, a recent case study reported two *de novo* mutations in the *TRPM3* gene to be the cause of DEE in a total of eight patients between the age of 4 and 38 (Dyment et al., 2019). All eight patients in this study were heterozygous for a *TRPM3* mutation and the majority of them were male (six out of eight). From all eight patients, seven carried an identical point mutation in the linker region between transmembrane segments 4 and 5. This mutation resulted in the substitution of a valine with a methionine (VM mutation) (**Figure 2A**). The remaining patient carried a proline to glutamine substitution (PQ mutation) at the boundary of the TRPM3 pore-forming loop (Dyment et al., 2019) (**Figure 2A**). More supporting evidence of these findings was given last year by de Sainte Agathe et al. (2020) who reported about another female DEE patient carrying the VM TRPM3 mutation. Interestingly, this patient did not have epilepsy at the moment of assessment, although it could not be excluded that this might occur in a later stage of life, as was observed in one patient described in Dyment et al. (2019) Although both studies did not further investigate the molecular mechanisms surrounding the disease phenotype, two other research teams performed thorough biophysical characterizations of the two reported TRPM3 disease mutations in *in vitro* cell systems (Van Hoeymissen et al., 2020; Zhao et al., 2020). Both studies concluded that the mutations are causing a gain-of-function in TRPM3, which results in an increased basal channel activity with elevated calcium concentrations at rest, a leftward shift of the concentration-response curve for the endogenous agonist PregS and an increased sensitivity to heat stimuli (**Figures 2B–D**). Of note, it was shown that the VM mutation additionally results in the opening of the earlier described alternative ion permeation pathway (Vriens et al., 2014a; Held et al., 2015a, 2016) in TRPM3 upon sole application of PregS (Van Hoeymissen et al., 2020). This causes a dramatic increase of the inward currents at physiological resting membrane potentials (**Figure 2E**). Despite these detailed biophysical characterizations, it remains uncertain how a gain-of-function in the TRPM3 protein causes the observed DEE disease phenotype. Considering the essential role of calcium as a signaling molecule in several neuronal pre- and post-synaptic mechanisms, such as vesicle release, cell depolarization, receptor (de)phosphorylation and internalization as well as in the expressional regulation of proteins (Beattie et al., 2000; Brini et al., 2014), it is not unlikely that the recently studied TRPM3 DEE mutants may have detrimental effects in disease-carrying patients. High calcium levels in the pre- or post-synaptic site of excitatory synapses, which can be caused by an elevated basal channel activity (**Figure 2B**) or an increased neurosteroid-induced activity (PregS) (**Figures 2C,E**), might lead to an elevated firing frequency of excitatory post-synaptic neurons. This may subsequently result in hyper-excitable neuronal cells and explain the observed epileptic phenotype (Badawy et al., 2009). Moreover, the patients show initial developmental deficits prior to the epileptic phenotype (Dyment et al., 2019; de Sainte Agathe et al., 2020). It is hard to predict the exact mechanisms that lead to such developmental abnormalities, but it can be speculated that high calcium levels in mutant TRPM3-expressing

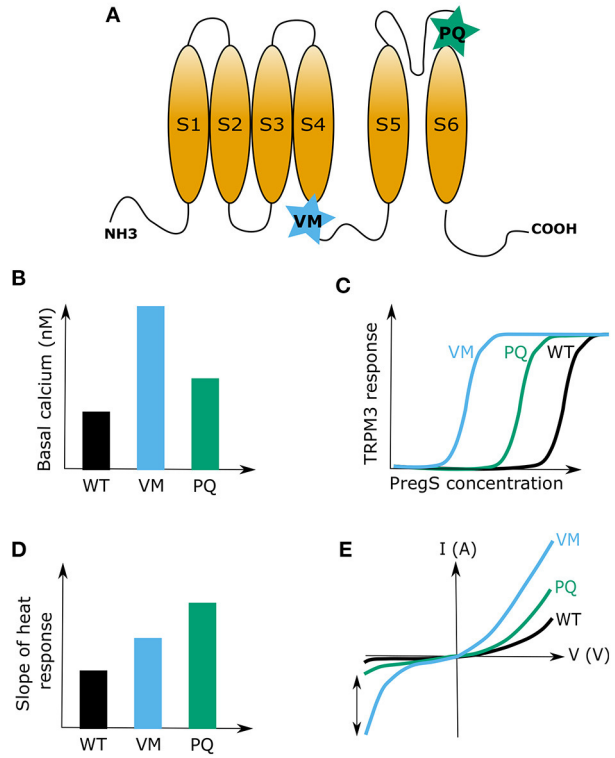

FIGURE 2 | Summary of the two reported developmental and epileptogenic encephalopathy (DEE) disease mutations in TRPM3. **(A)** Cartoon of a TRPM3 channel subunit indicating the positions of the valine to methionine (VM) and proline to glutamine (PQ) substitution mutations. **(B)** Representative graph indicating differences in basal calcium levels between HEK cells that express the TRPM3 wild type channel or the mutant channels VM or PQ. **(C)** Representative graph showing the leftward shift of the concentration-response curve of pregnenolone sulfate (PregS) for the two TRPM3 mutations (VM and PQ) causing developmental and epileptic encephalopathies (DEE). **(D)** Illustration of the heat sensitivity increase for the two DEE mutations compared to wild type (WT) TRPM3. **(E)** Typical examples of current-voltage (I-V) plots for wild type TRPM3 and the DEE mutants VM and PQ during activation with PregS. Note the increase in inward currents for both channel mutants. The two-sided arrow is indicating the high increase in inward currents for the VM mutant, which was attributed to the opening of the alternative ion pore in TRPM3.

brain cells may cause neuronal cell death (Toescu, 1998) or that mutated TRPM3 is abundantly expressed in certain inhibitory neurons, which could lead to abnormal synaptic plasticity (Baroncelli et al., 2011). It is definitely intriguing that the sensitivity of TRPM3 to hormonal clues is increased in the TRPM3 DEE mutant channels (**Figures 2C,E**) (Van Hoeymissen et al., 2020; Zhao et al., 2020), as hormones are known to be centrally involved in developmental regulations (McEwen, 1988, 1992). However, at this point, our knowledge regarding the functional roles of TRPM3 in the brain is very confined, and any kind of mechanistic speculation is rather premature. To illuminate the disease-causing processes in the brain, it first seems necessary to understand, which exact brain areas are affected in the disease-carrying patients, and subsequently confirm and

TABLE 2 | Overview of reported diseases that were linked with *TRPM3* alterations.

Disease	TRPM3 related changes	References
Autism	Deletion of exons 1-9 of *TRPM3*	Pagnamenta et al., 2011
Choroid plexus tumors	Up-regulation of *TRPM3* expression	Hasselblatt et al., 2009; Japp et al., 2015, 2016
Developmental and epileptic encephalopathies (DEE)	*De novo* substitutions in TRPM3 (V837M and P937Q)	Dyment et al., 2019; de Sainte Agathe et al., 2020
Kabuki syndrome	Deletion of chromosomal region that encodes *TRPM3* (9q21.11-q21.12)	Kuniba et al., 2009

For each disease the reported gene or protein alterations of TRPM3 are presented together with the according case studies.

determine specific TRPM3 functions in the various cell types of these brain regions. Clearly, a genetic animal disease-model would be of high scientific value to address such questions, and to shed light on the disease-causing mechanisms of TRPM3 mutations in the brain.

DISCUSSION

Several lines of evidence suggest TRPM3 as an emerging interesting novel player in brain physiology and pathology. First, TRPM3 was shown to be abundantly expressed and functionally active in different brain regions (Oberwinkler and Philipp, 2014). Furthermore, TRPM3 can be modulated by several endogenous brain ligands and receptors (Held et al., 2015b; Badheka et al., 2017; Csanády, 2017; Dembla et al., 2017; Quallo et al., 2017). In addition, TRPM3 was also shown to be targeted by a commonly used anti-convulsion drug, primidone (Krügel et al., 2017), a compound of which the exact molecular actions are up until today still illusive. However, a brain-specific interaction of these drugs and receptors with TRPM3, and the resulting consequences for brain functions still have to be demonstrated. Finally, various genetic alterations in the *TRPM3* gene were linked to several neurological disorders in human patients (Kuniba et al., 2009; Pagnamenta et al., 2011; Japp et al., 2016; Dyment et al., 2019; de Sainte Agathe et al., 2020) (**Table 2**). It is interesting to note that almost all of the neuropathologies that were linked to the *TRPM3* gene resulted in a state of intellectual disability in the affected patients. This may suggest a vital role of TRPM3 in neuronal development and could indicate that TRPM3 is of particular importance in defined brain regions. However, so far there are no studies that investigated genetic *Trpm3* alterations in a systematic way, by linking molecular TRPM3 functions directly to the *in vivo* phenotypes that are caused by these alterations. Clearly, there is an urgent need for more detailed functional studies of TRPM3 in the brain.

As TRPM3 represents a non-selective, cation permeable ion channel with a high permeability for calcium, it is very likely that TRPM3 has an impact on neuronal functions

and development by affecting electrical and chemical signals in brain regions where it is expressed. Therefore, thorough morphological, molecular and electrophysiological assessments of different brain areas, circuits, and cells that were shown to express TRPM3 RNA or protein, are warranted in the future in healthy and diseased brains. Additionally, behavioral screening experiments to specifically address selected brain functions linked to the investigated areas, in combination with (brain region-specific) pharmacological targeting or genetic alterations in animal models would be of immense value for our future quest to explore the role(s) of TRPM3 in the brain. Considering the broad hormonal regulation profile of TRPM3 (as discussed in section TRPM3 Is a Steroid Regulated Channel), it would be of further scientific value to apply behavioral tests to animals of different gender and different developmental stages. Such experiments will not only provide insights into the physiological roles of TRPM3 in the brain but could also illuminate the mechanisms of disease in selected TRPM3 animal disease models.

Obviously, more research is required to investigate TRPM3 expression, regulation and function in different brain regions and cell types, and to validate its role in brain (patho)physiology. Nevertheless, given our current knowledge about the molecular and biophysical properties of TRPM3 and its recent genetic links to brain pathologies, it is tempting to imagine TRPM3 as an attractive potential new target for future drug interventions in neurological diseases such as epilepsy or autism spectrum disorders.

AUTHOR CONTRIBUTIONS

KH and BIT drafted, corrected, and wrote this article. BIT was responsible for the funding acquisition. All listed authors qualify for authorship and all authors qualifying for authorship are listed above.

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MINI REVIEW
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Functional Importance of Transient Receptor Potential (TRP) Channels in Neurological Disorders

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Transient receptor potential (TRP) channels are transmembrane protein complexes that play important roles in the physiology and pathophysiology of both the central nervous system (CNS) and the peripheral nerve system (PNS). TRP channels function as non-selective cation channels that are activated by several chemical, mechanical, and thermal stimuli as well as by pH, osmolarity, and several endogenous or exogenous ligands, second messengers, and signaling molecules. On the pathophysiological side, these channels have been shown to play essential roles in the reproductive system, kidney, pancreas, lung, bone, intestine, as well as in neuropathic pain in both the CNS and PNS. In this context, TRP channels have been implicated in several neurological disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and epilepsy. Herein, we focus on the latest involvement of TRP channels, with a special emphasis on the recently identified functional roles of TRP channels in neurological disorders related to the disruption in calcium ion homeostasis.

Keywords: TRP channels, neurological disorders, calcium homeostasis, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy

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INTRODUCTION

TRP Channel Subfamily

Transient receptor potential (TRP) channels are classified into 28 members that function as a group of unique non-selective cation channels in mammals. TRP channels are conserved in yeast, invertebrates, and vertebrates and share a common three-dimensional structure with six transmembrane helical segments (S1–S6), two variable and intracellular amino (-NH_2) and a carboxy (-COOH) terminal cytosolic domain, and the channel pore formed by S5 and S6, which allow transport of various ions including sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium ions (Mg^{2+}). Based on significant sequence homology and a common structure, TRP channels are divided into six subfamilies: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin). TRP subfamilies are divided into Group 1 (TRPC, TRPM, TRPV, and TRPA) and Group 2 (TRPP and TRPML) according to differences in their sequence and topology. Subfamilies of TRP channels are divided into groups and subtypes as represented in the phylogenetic tree in **Supplementary Figure 1**.

Transient receptor potential channels are ubiquitously expressed in many cell types (especially neurons and non-neuronal cells in the central nervous system, CNS) and tissues, including brain, kidney, pancreas, lung, bone, intestine, reproductive system as well as dorsal root ganglia (DRG) neurons in the peripheral nervous system (PNS). In addition, TRP channels are primarily expressed in plasma membranes that play critical roles in stimulus perception (i.e., thermosensation, mechanosensation, and chemosensation) and ion homeostasis (Nishida et al., 2006; Nilius and Owsianik, 2011).

Initially, TRP channels were shown to regulate cellular Ca^{2+} influx through the so-called store-operated channels (Nilius, 2004; Ramsey et al., 2006; Yazgan and Naziroglu, 2017). Several studies have shown that TRP channels regulate neuronal excitability, intracellular Ca^{2+} and Mg^{2+} homeostasis, as well as cell proliferation and differentiation (Nilius, 2004).

In addition to their physiological functions, TRP channels are known to contribute to various pathophysiological roles in neurological disorders of the CNS (Nilius, 2007; Colsoul et al., 2013; Takada et al., 2013).

Neurological Disorders

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) and epilepsy, collectively known as "neurological disorders," have distinct pathologies and represent a significant medical burden in the modern world.

Alzheimer's disease is the most common neurodegenerative disease in the world and is characterized by the accumulation of beta-amyloid (Aβ) plaques from amyloid precursor protein (APP) and hyperphosphorylated tau protein (Iqbal et al., 2010; Murphy and Levine, 2010). PD is also a common brain disorder, primarily characterized by a resting tremor, postural instability, rigidity, and bradykinesia caused by dopaminergic (DA) neuronal loss in the substantia nigra (SN) pars compacta (SNpc) (Michel et al., 2013; Kalia and Lang, 2015). HD is an inherited neurodegenerative disorder that causes cognitive deficits, emotional imbalance, and uncontrolled excessive motor movements caused by a CAG trinucleotide repeat expansion within the Huntingtin gene that leads to the synthesis of polyglutamine tracts (Kremer et al., 1994). ALS, also known as Lou Gehrig's disease, is another fatal type of neurodegenerative motor disease characterized by the deterioration of motor neurons in the motor cortex, brainstem, and spinal cord that leads to impairments in voluntary movement (Guatteo et al., 2007). Finally, epilepsy is a neurological disorder characterized by recurrent epileptic seizures, abnormal brain activity, and unusual behavior.

Over the past few decades, enormous efforts have been made to unveil the pathogenesis of neurological disorders. For example, endoplasmic reticulum (ER) stress, also known as oxidative stress, which is caused by misfolded proteins and abnormal Ca^{2+} homeostasis, neuroinflammation, and mitochondrial dysfunction have been shown to lead to neuronal cell death. Most notably, Ca^{2+} regulation, which is involved in normal physiological functions such as neuronal survival, proliferation, differentiation, gene transcription, and exocytosis at synapses, has been shown to be dysregulated in various neurological disorders (Bojarski et al., 2008; Bezprozvanny, 2009; Grosskreutz et al., 2010; Surmeier et al., 2010; Wu et al., 2011; Nikoletopoulou and Tavernarakis, 2012).

Interestingly, several studies have reported a correlation between intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and other pathogenic mechanisms, including the imbalance between antioxidant function and reactive oxygen species (ROS) production (Gorlach et al., 2015) as well as mitochondrial dysfunction (Contreras et al., 2010; Pivovarova and Andrews, 2010).

In fact, exposure of neuronal cells to Aβ peptides, induces an elevation of $[Ca^{2+}]_i$ that leads to cell death as observed in *in vitro* experiments (Adhya and Sharma, 2019). Aggregation of α-synuclein, which is associated with the pathology of PD, can also induce neuronal cell death via the disruption of cellular Ca^{2+} homeostasis (Fonfria et al., 2005; Danzer et al., 2007). Furthermore, the polyglutamine-expanded huntingtin protein and mutant superoxide dismutase-1 (SOD1), which are implicated in the pathogenesis of HD and ALS, respectively, also disrupt cellular Ca^{2+} homeostasis (Giacomello et al., 2013; Barrett et al., 2014). The disruption of intracellular Ca^{2+} concentration in epilepsy induces ROS production, apoptosis, and caspase activation through mitochondrial oxidative stress (Yilmaz et al., 2011; Naziroglu and Ovey, 2015). Therefore, alleviating disturbances in Ca^{2+} homeostasis may represent a potential therapeutic target for the treatment of neurological disorders (Nilius, 2007; Colsoul et al., 2013; Takada et al., 2013).

TRP CHANNELS IN NEUROLOGICAL DISORDERS

TRP Channels in AD

Importantly, a strong correlation between the pathological hallmarks of AD (Aβ accumulation and neurofibrillary tangles) and perturbed cellular Ca^{2+} homeostasis have been reported in AD patients as well as in animal and cell culture models of AD (Mattson and Chan, 2001). To date, TRPC1, TRPC3, TRPC6, TRPM2, TRPM7, TRPV1, TRPV4, TRPA1, and TRPML1 have been shown to be involved in AD (**Figure 1A**). TRPC1 is a member of the most prevalent TRPC channels in the brain and is linked to the store-operated Ca^{2+} channel-mediated Ca^{2+} entry (SOCE) channels. Interestingly, SOCE was reduced by downregulation of TRPC1 in astrocytes in APP knockout (KO) mice (Linde et al., 2011). Additionally, the alteration of the brain-derived neurotrophic factor-tropomyosin receptor kinase B-TRPC3 (BDNF-TrkB-TRPC3) signaling pathway led to hyperphosphorylation of tau protein caused by increased $[Ca^{2+}]_i$ levels in AD (Elliott and Ginzburg, 2006). Moreover, it has been reported that TRPC1 and TRPC3 are associated with caveolin-1, which is the main component of the plasma membrane caveolae that interacts with APP (Ikezu et al., 1998). Several studies have reported that TRPC6 in neurons promotes neuronal survival (Jia et al., 2007), synaptogenesis, and learning and memory (Zhou J. et al., 2008). Furthermore, in pharmacological studies using hyperforin, one of the

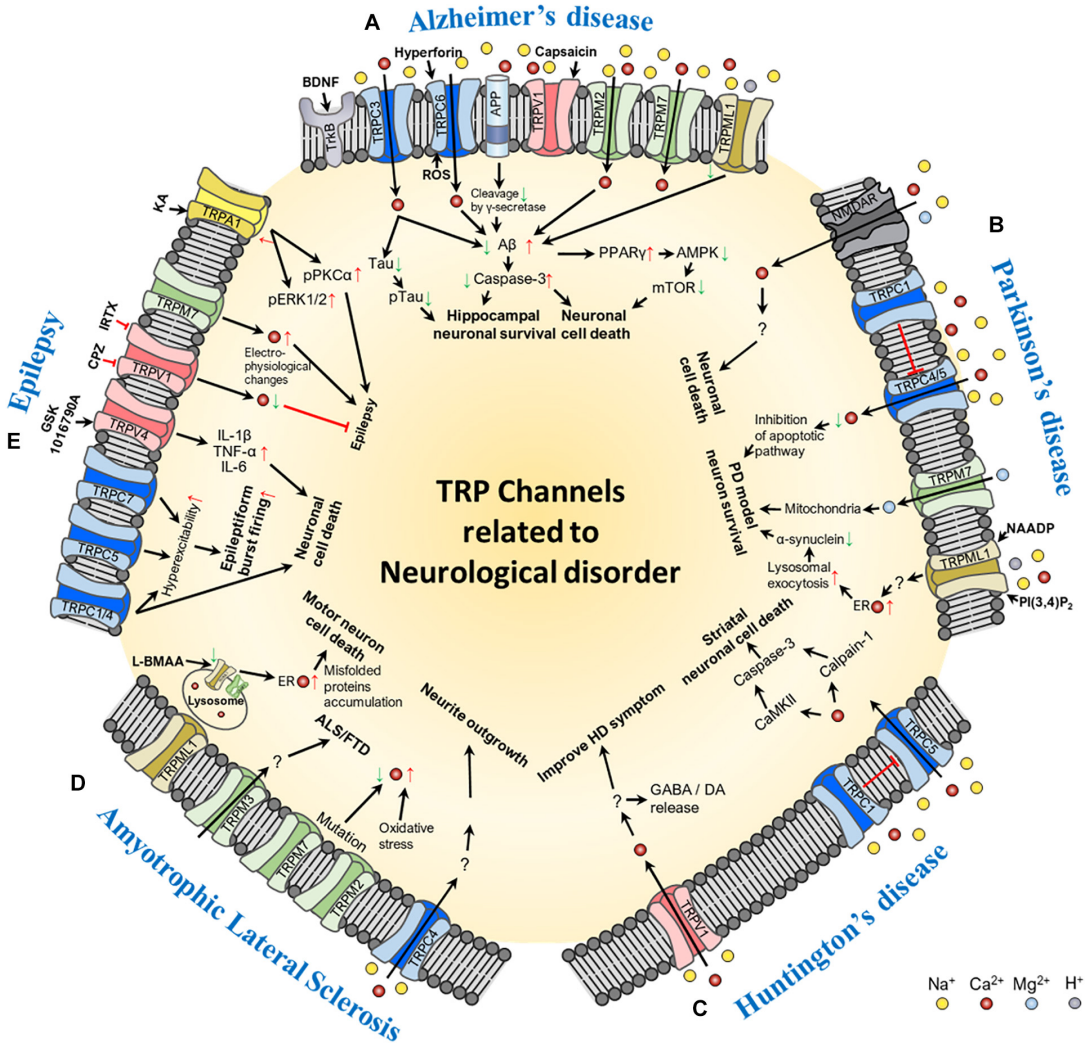

FIGURE 1 | Schematic of the molecular mechanism of TRP channel-mediated pathogenesis of neurological disorders: **(A)** Alzheimer's disease, **(B)** Parkinson's disease, **(C)** Huntington's disease, **(D)** Amyotrophic lateral sclerosis, and **(E)** epilepsy. In the figure, red arrows represent increase or up-regulation; green arrows, decrease or down-regulation; red thick bar, inhibition of the signaling pathway; and black arrows, activation of the signaling pathway. Ion channels involved in each of the neurological disorders and their transporting ions, channel agonists, and antagonists are also shown in the figure. *Abbreviations*: BDNF, brain-derived neurotrophic factor; ROS, reactive oxygen species; Aβ, beta-amyloid; PPAR, peroxisome proliferator-activated receptor gamma; AMPK, 5′ adenosine monophosphate-activated protein kinase; mTOR, mechanistic target of rapamycin; NMDAR, N-methyl-D-aspartate receptor; NAADP, Nicotinic acid adenine dinucleotide phosphate; PI(3,4)P_2, phosphatidylinositol (3,4)-bisphosphate; PD, Parkinson's disease; ER, endoplasmic reticulum; GABA, gamma-aminobutyric acid; DA, dopamine; ALS/FTD, amyotrophic lateral sclerosis patients with frontotemporal dementia; L-BMAA, L-beta-methylamino-L-alanine; IL, interleukin; TNF-α, tumor necrosis factor alpha; CPZ, capsazepine; IRTX, 5′-Iodoresiniferatoxin; KA, kainic acid; pPKCα, phospho-protein kinase C alpha; pERK1/2, phospho-extracellular signal-regulated kinase 1/2.

main natural compounds of the medicinal plant Saint John's wort that acts as an antidepressant drug (Zanoli, 2004) and TRPC6 activator (Tu et al., 2010), or tetrahydrohyperforin, a stable semisynthetic compound derived from hyperforin (Rozio et al., 2005), TRPC6 was shown to play a potential role in AD through the reduction of Aβ accumulation due to increased cerebrovascular P-glycoprotein (Brenn et al., 2014) and increased adult hippocampal neurogenesis and long-term spatial memory (Abbott et al., 2013), respectively. In contrast, AD-linked presenilin (PS)-2 mutants influenced TRPC6-mediated neurotoxic Ca^{2+} entry (Lessard et al., 2005), whereas TRPC6 was shown to be neuroprotective against AD through interaction with the cleavage of APP (Wang et al., 2015). In fact, a recent study observed that hyperforin induced activation of TRPC6, reduced Aβ levels, and improved mild cognitive impairment in AD models and that TRPC6 mRNA levels in the blood cells were reduced in AD patients (Lu et al., 2018).

While APP/PS1 transgenic (Tg) mice were observed to have age-dependent spatial memory deficits through TRPM2 channel activation by toxic oligomeric Aβ, genetic elimination of TRPM2 in APP/PS1 Tg mice ameliorated the synaptic loss and spatial memory deficits (Ostapchenko et al., 2015). It has been suggested

that the TRPM2 channel may be a possible therapeutic agent of neuronal toxicity and memory impairment in AD. In contrast, TRPM7, which is another TRPM channel, plays an important role in not only inducing anoxic neuronal cell death by disrupting Ca^{2+} and Mg^{2+} homeostasis but also increasing susceptibility to degenerative processes (Aarts et al., 2003).

In recent studies of TRPV channels, the activity of TRPV1 was reported to reduce oxidative/nitrosative stress (Hofrichter et al., 2013; Jayant et al., 2016), rescue Aβ-induced degradation of hippocampal neurons (Balleza-Tapia et al., 2018), and increase levels of presynaptic synapsin I and postsynaptic density 93 (PSD93). In contrast, increased TRPV4 expression has been observed in the brain of aged rats (Lee and Choe, 2014), which is known to increase neuronal cell death via increased Ca^{2+} influx and ROS production (Hong et al., 2016). In APP/PS1 Tg/TRPA1 KO mice, the genetic loss of TRPA1 has been shown to exacerbate spatial learning and memory deficits, increase Aβ deposition, promote the release of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-4, IL-6, and IL-10, and inhibit the activities of transcriptional factors NF-κB and nuclear factor of activated T cells (Lee et al., 2016).

Lastly, it has been shown that overexpression of TRPML1 is sufficient to rescue memory and cognitive deficits by diminishing neuronal apoptosis in APP/PS1 Tg mice (Zhang et al., 2017).

TRP Channels in PD

TRPC1, TRPC3, TRPM2, TRPM7, and TRPV1 have been shown to be involved in PD (**Figure 1B**). The activation of TRPC1 channels has been reported to induce neuroprotection against apoptosis in SH-SY5Y neuroblastoma cells (Bollimuntha et al., 2006), as well as to regulate SOCE channels and reduce DA neuronal cell death in the SN of TRPC1 KO mice (Selvaraj et al., 2009, 2012). Consistent with these reports, decreased levels of TRPC1 have been detected in brain lysates from the SNpc of PD patients (Sun et al., 2017). In contrast to TRPC1 levels, TRPC3 levels are not altered in SNpc DA neurons in PD patients (Sun et al., 2017) although their levels are increased by the compensatory effect of decreased TRPC1 in 1-methyl-4-phenyl-1,2,3,6-tetrahyrdropyridine (MPTP)-induced PD-like conditions (Selvaraj et al., 2009). Constitutively active TRPC3 channels are known to express and regulate firing intensity and pattern in GABAergic neurons of the SN pars reticulata (SNpr) (Zhou F. W. et al., 2008). Therefore, this channel may also be involved in different brain subregions or pathophysiological mechanisms of PD.

Additionally, 1-methyl-4-phenylpyridinium ion (MPP^+)-induced oxidative stress has been shown to increase intracellular Ca^{2+} influx via TRPM2 channel activity and promote DA neuronal cell death in the SNpc (Sun et al., 2018). Importantly, TRPM7 channels regulate Mg^{2+} homeostasis in cells, and increased concentrations of Mg^{2+} significantly inhibit MPP + -induced neurotoxicity by reducing the number of DA neurons and ameliorating the length of DA neurites (Hashimoto et al., 2008; Paravicini et al., 2012).

The activation of TRPV1 has also been shown to induce cell death in DA neurons, increase Ca^{2+} influx, and mediate mitochondrial disruption (Kim et al., 2005; Nam et al., 2015).

Furthermore, activation of TRPML1 evokes global ER Ca^{2+} release and Ca^{2+} influx (Kilpatrick et al., 2016) and results in upregulated lysosomal exocytosis, thus preventing α-synuclein accumulation in DA neurons (Tsunemi et al., 2019).

TRP Channels in HD

TRPC1, TRPC5, and TRPV1 have been shown to be involved in HD (**Figure 1C**). Recently, it was shown that the expression level of endogenous TRPC1 was decreased in Q111 HD striatal cells compared to wild-type (Q7) cells (Hong et al., 2015). Furthermore, increased glutathionylation of TRPC5, activated by oxidants, leads to Ca^{2+}-induced apoptosis of the striatal neurons in HD Tg mice (Hong et al., 2015). However, knockdown by siTRPC5 and inhibition of TRPC5 with ML204, a selective TRPC4 blocker produced by the Molecular Libraries Probe, produces a protective effect against oxidative stress in Q111 HD striatal cells and improves motor behavior in HD Tg mice (Miller et al., 2011; Hong et al., 2015, 2020a).

Moreover, it has been shown that administration of N-arachidonoylphenolamine (AM404), an inhibitor of endocannabinoid reuptake, has potential antihyperkinetic effects via the TRPV1 receptor using the 3-nitropropionic acid-induced HD model, suggesting that the activity of the TRPV1 channel may contribute to the motor dysfunction in HD patients (Lastres-Becker et al., 2003).

TRP Channels in ALS

TRPC4, TRPM2, TRPM3, TRPM7, and TRPML1 have been shown to be involved in ALS (**Figure 1D**). The clinical symptoms of ALS overlap with those of Parkinsonism dementia complex (PDC), a neurodegenerative disorder characterized by symptoms of PD and dementia (Garruto, 2006; Hermosura and Garruto, 2007). While the pathogenesis of ALS/PDC is not fully understood, it is thought to be caused by two potential scenarios: (i) low levels of Ca^{2+} and Mg^{2+}, which cause excess ROS production and cell death, and (ii) excess exposure to putative neurotoxin β-methylamino-L-alanine (L-BMAA), derived from the cycad plant, which causes an increase of $[Ca^{2+}]_i$ (Brownson et al., 2002).

A previous study demonstrated that the expression of TRPC4 is increased by nerve growth factor and dibutyryl-cAMP treatment in cultured DRG neurons. Conversely, inhibition of TRPC4 using a selective siRNA approach reduces neurite outgrowth in cultured DRG neurons (Wu et al., 2008). In ALS, reactive astrocytes accelerate nerve growth factor production (Pehar et al., 2004). In addition, a recent study demonstrated that ALS-resistant motor neurons from mutant SOD1 ALS models upregulate axonal outgrowth and dendritic branching (Osking et al., 2019). Taken together, these findings suggest that TRPC4 regulates DRG differentiation and plays a pivotal role in ALS.

In contrast, mutations in both $TRPM2^{P1018L}$ and $TRPM7^{T1482I}$ have been found in Guamanian ALS/PDC patients. Importantly, the $TRPM2^{P1018L}$ variant was shown to attenuate oxidative stress-induced Ca^{2+} influx through inactivation of the channel (Hermosura et al., 2008). In contrast, the $TRPM7^{T1482I}$ variant promotes an imbalance in Ca^{2+} and Mg^{2+} homeostasis

(Hermosura and Garruto, 2007). TRPM3, which is in the same group as TRPM2, has also been considered as a possible candidate gene involved in the pathogenesis of ALS with frontotemporal dementia (Lee et al., 2003).

TRPML1 was expressed in cell lysosomes or in the endosomal membrane and in the main Ca^{2+}-releasing channel used as a regulator of lysosomal storage via phosphatidylinositol 3,5-biphosphate ($PI(3,5)P_2$) (Cheng et al., 2010; Li et al., 2013). In addition, TRPML1 also regulates the maintenance of lysosome homeostasis and accumulation of autophagy (Cheng et al., 2010; Curcio-Morelli et al., 2010; Morgan et al., 2011). Interestingly, $PI(3,5)P_2$ levels are significantly impaired in some forms of ALS (Chow et al., 2009; Osmanovic et al., 2017). Moreover, in an experimental model of ALS/PDC, the expression of TRPML1 was shown to be reduced through autophagy leading to the loss of motor neurons. Additionally, with an L-BMAA-induced ALS mouse model, it was discovered that TRPML1 is downregulated, and autophagy is impaired in primary motor neurons, which leads to ER stress and neuronal cell death (Tedeschi et al., 2019).

TRP Channels in Epilepsy

TRPC1, TRPC3-7, TRPM2, TRPM7, TRPV1, TRPV4, and TRPA1 have been shown to be involved in epilepsy (**Figure 1E**). As mentioned above, TRPC channels are not only known to play an important role in neuronal outgrowth and survival during brain development but are also believed to play a pivotal role in various epileptogenic processes. For example, the expression of TRPC1 is increased in cortical lesions of epilepsy patients and regulated by the mediation of astrocyte-induced epilepsy (Zang et al., 2015). Using a pilocarpine (muscarinic agonist)-induced status epilepticus (PISE) model, studies have shown that the genetic elimination of TRPC3 reduces the susceptibility of seizures to pilocarpine, while enhancing the expression of TRPC3 induces hyperexcitability and increases susceptibility to epileptiform activity in the cortex (Zhou and Roper, 2014; Phelan et al., 2017). In contrast, the expression of TRPC6 has been shown to be down-regulated in chronic epileptic rats, whereas the genetic ablation by siTRPC6 increases seizure susceptibility and seizure-induced neuronal damage in the dentate gyrus but not in CA1 and CA3 neurons of the hippocampus (Kim and Kang, 2015). In addition, in other members of the TRPC family, the genetic deletion of TRPC1/4 reduces seizure-induced neuronal cell death. Furthermore, TRPC5 KO mice exhibit significantly reduced seizures as well as minimal seizure-induced neuronal cell death in the CA1 and CA3 areas of the hippocampus (Phelan et al., 2013). Conversely, the TRPC7 channel plays an important role in spontaneous epileptiform bursting in the CA3, the reduction of which is correlated with a reduction in PISE in TRPC7 KO mice (Phelan et al., 2014).

The TRPM family is also involved in the pathogenesis of epilepsy. For example, TRPM2 channels are co-expressed with the EF-hand domain-containing protein 1 gene, which is related to an increased susceptibility to juvenile myoclonic epilepsy, and are regulated in the hippocampal neurons (Katano et al., 2012). Moreover, TRPM7 has been shown to be activated during epilepsy (Aarts and Tymianski, 2005). Importantly, genetic ablation of TRPM7 blocks the activation of a cation current, which is produced by oxygen-glucose deprivation (I_{OGD}), and prevents ROS-mediated I_{OGD} activation (Aarts et al., 2003).

Although the TRPV1 channel is believed to play an essential role in the development of neurogenic pain and inflammation in the sensory neurons (Caterina and Julius, 2001; Julius and Basbaum, 2001), it is also expressed in other brain regions, including the cerebral cortex, hippocampus, cerebellum, thalamus, hypothalamus, striatum, midbrain, and amygdala (Cristino et al., 2006). Furthermore, increased expression of TRPV1 has been found in the hippocampus of rats and the dentate gyrus of mice with temporal lobe epilepsy as well as in the cortex of patients with temporal lobe epilepsy (Bhaskaran and Smith, 2010; Sun et al., 2013; Saffarzadeh et al., 2015). In fact, a recent study suggested that the activation of the TRPV1 channel may play a key role in the development of epilepsy (Naziroglu and Ovey, 2015). Using capsazepine, 5′-iodoresiniferatoxin, and resolvins, the authors showed that inhibition of the TRPV1 channel induced protective effects against epilepsy and epilepsy-induced Ca^{2+} entry in the hippocampal and DRG neurons (Naziroglu and Ovey, 2015).

Activation of TRPV3 by eugenol was shown to suppress epileptiform field potentials and decrease the amplitude of field postsynaptic potentials evoked in CA1 neurons of the hippocampus and the third layer of the neocortex (Muller et al., 2006). Another study using the PISE model of epilepsy found that activation of TRPV4 by the specific agonist GSK1016790A increased pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), while the inhibition of TRPV4 by HC-067047, a selective TRPV4 antagonist, significantly increased cell survival post status epilepticus (Wang et al., 2019).

Using a kainic acid-induced seizure model, TRPA1 was found to be upregulated while TRPV4 was not, which is contradictory to the earlier findings concerning TRPV4 (Hunt et al., 2012; Wang et al., 2019).

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we described the functional importance of TRP channels in the regulation of Ca^{2+} and oxidative stress responses as well as their contributions to neurological disorders, including AD, PD, HD, ALS, and epilepsy.

Overall, in addition to playing a broad range of physiological roles throughout the CNS and PNS, TRP channels also contribute to pathophysiology across a wide range of diseases and disorders through abnormalities in Ca^{2+} homeostasis. In the CNS, TRP channels are expressed in several brain regions (including the spinal cord) and have been shown to be key regulatory proteins involved in lipid metabolism, glucose homeostasis (Liu et al., 2009; Zhu et al., 2011), and the pathobiology of aforementioned neurological disorders.

Apart from their important role in neurological disorders of the CNS, TRP channels are also expressed in the neurons of the DRG, trigeminal ganglion, and sympathetic ganglion, and contribute to both normal and pathological sensory processing in

the PNS (Lee et al., 2019). For example, TRP channels are known to be involved in diabetic peripheral neuropathy, chemotherapy-induced peripheral neuropathy, and autonomic neuropathy.

In light of the physiological and pathophysiological functions of TRP channels in both the CNS and PNS, we believe that they represent potential therapeutic targets for treating neurological disorders of the CNS as well as neuropathic pain in the PNS.

AUTHOR CONTRIBUTIONS

KL, YYJ, YHK, and C-KP contributed to the conception and design. KL and YYJ drafted the manuscript. KL, GC, JHJ, YHK, and C-KP revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.611773/full#supplementary-material

Supplementary Figure 1 | Summary of TRP subfamilies-mediated studies related to neurological disorders [modified from Nilius (2007), Hong et al. (2020b), Thapak et al. (2020)]. Different colors are used in the table for better classification and the gray boxes in the "Related Disease" column represent the roles of specific TRP subfamilies in each neurological disorders as reported in the published literature. *Abbreviations*: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AMG, amygdala; APP, amyloid precursor protein; BBB, blood-brain barrier; BG, basal ganglia; Bs, brainstem; CB, cerebellum; CC, corpus callosum; Cd, caudate; Cg, cingulate gyrus; CNS, central nervous system; CTX, cortex; DA, dopamine; DG, dentate gyrus; DRG, dorsal root ganglion; ER, endoplasmic reticulum; FB, forebrain; fCTX, frontal cortex; GABA, gamma-aminobutyric acid; GP, globus pallidus; HD, Huntington's disease; HPC, hippocampus; HT, hypothalamus; IC, inferior colliculus; JME, juvenile myoclonic epilepsy; KA, kainic acid; LS, lateral septum; MB, midbrain; mFBb, medial forebrain bundle; mPFC, medial prefrontal cortex; mTOR, mechanistic target of rapamycin; n.d., not determined; OB, olfactory bulb; PD, Parkinson's disease; PISE, pilocarpine-induced status epilepticus; PNS, peripheral nervous system; Pm, putamen; PS1, presenilin 1; RCh, retrochiasmatic area; SC, spinal cord; Sep, septum; SN, substantia nigra; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; SOCE, store-operated calcium entry; Str, striatum; TH, thalamus; vS, ventral subiculum.

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REVIEW
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TRP Channels in Brain Tumors

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Malignant glioma including glioblastoma (GBM) is the most common group of primary brain tumors. Despite standard optimized treatment consisting of extensive resection followed by radiotherapy/concomitant and adjuvant therapy, GBM remains one of the most aggressive human cancers. GBM is a typical example of intra-heterogeneity modeled by different micro-environmental situations, one of the main causes of resistance to conventional treatments. The resistance to treatment is associated with angiogenesis, hypoxic and necrotic tumor areas while heterogeneity would accumulate during glioma cell invasion, supporting recurrence. These complex mechanisms require a focus on potential new molecular actors to consider new treatment options for gliomas. Among emerging and underexplored targets, transient receptor potential (TRP) channels belonging to a superfamily of non-selective cation channels which play critical roles in the responses to a number of external stimuli from the external environment were found to be related to cancer development, including glioma. Here, we discuss the potential as biological markers of diagnosis and prognosis of TRPC6, TRPM8, TRPV4, or TRPV1/V2 being associated with glioma patient overall survival. TRPs-inducing common or distinct mechanisms associated with their Ca^{2+}-channel permeability and/or kinase function were detailed as involving miRNA or secondary effector signaling cascades in turn controlling proliferation, cell cycle, apoptotic pathways, DNA repair, resistance to treatment as well as migration/invasion. These recent observations of the key role played by TRPs such as TRPC6 in GBM growth and invasiveness, TRPV2 in proliferation and glioma-stem cell differentiation and TRPM2 as channel carriers of cytotoxic chemotherapy within glioma cells, should offer new directions for innovation in treatment strategies of high-grade glioma as GBM to overcome high resistance and recurrence.

Keywords: ion channel, TRP channel, brain tumor, glioma, glioblastoma

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INTRODUCTION

Malignant gliomas are the most prevalent group of primary brain tumors in adults, with an incidence of 8.9 cases per 100,000 persons/year in the US (Ostrom et al., 2015, 2017). Glioblastoma (GBM) remains one of the most aggressive human cancers. Glial tumors or glioma represent a wide spectrum of malignancies including grades II and III oligodendroglioma, grades II and III

astrocytoma and glioblastoma from initial classification based on anatomocytopathological criteria related to the morphotypic characteristics and numerous cytonuclear atypologies, accompanied by anaplasia for high-grade glioma (GBM, grade IV) (Louis et al., 2007; Miller and Perry, 2007). The diagnosis of GBM was based on the presence of vascular micro-proliferations signs of intense vascularization, associated with zones of necrosis delimited by a hypoxic pseudopalissadic cellular zone, evidencing important intra-tumoral heterogeneity (Karsy et al., 2012; Alifieris and Trafalis, 2015). Now, brain tumors and glioma are classified according to the histomolecular classification recently published by the World Health Organization (WHO) (Louis et al., 2016), which represents a major clinical improvement for both diagnosis and treatments, as well as patient prognosis. The aim was to implement the histopathological classification with the following molecular signatures: first including the mutation in the isocitrate dehydrogenase1/2 (IDH1/2mut) and/or the co-deletion 1p/19q associated with oligodendroglioma or mutation of TP53; second the l′α-thalassemia mental retardation syndrome X-linked (ATX) or amplification of the epidermal growth factor receptor (EGFR) were associated with GBM IDHwt; finally the hypermethylation of the O6-methyl guanine-DNA methyl transferase (MGMT) promoter constitutes an important parameter of the GBM aggressiveness (Louis et al., 2016).

Despite these molecular attempts to stratify glioma patients with the objective to allow personalization of the treatments, the median survival of GBM patients currently ranges from 15 to 17 months, despite a safe maximal surgical resection, radiation/concomitant and adjuvant alkylating-based chemotherapy by temozolomide (TMZ) (Stupp et al., 2005, 2009; Wen and Brandes, 2009). However, more than 95% of GBM recur in the margin of the resection cavity, an area in which glioma tumor cells acting as a tumor reservoir are found (Giese et al., 2003). This invasiveness associated with X-ray and/or intrinsic or acquired chemoresistance of the glioma cells and the presence of an intrinsic or acquired blood-brain barrier (BBB), limit the effectiveness and/or the delivery of anti-neoplastic agents and justify the development of new strategies. In agreement, over the last decade, despite very important advances in the field of targeted therapy, none of them, e.g., drug/antibody or combination of small molecule inhibitors, has been shown to be more effective than TMZ or capable of increasing the efficacy of standard therapy in patients with primary or recurrent GBM (Stepanenko and Chekhonin, 2018), and no curative treatment is currently identified in GBM. This failure may be explained at least in part by the intratumoral heterogeneity which is a conserved consequence of the GBM micro-environment (Prabhu et al., 2017), referring to the physico-chemical characteristics and matrices interacting with the tumor. Indeed, the GBM heterogeneity is tightly related to angiogenic and hypoxic features as well as invasive processes, thus future strategies should consider targeting mechanisms associated with resistance and invasion. In particular, Watkins et al. (2014) showed that GBM cells can thus control the regulation of vascular tone, *via* the release of K^+ through K^+ channels activated in response to Ca^{2+}, leading to an adaptation of cell volume to facilitate their invasion.

Transient receptor potential (TRP) channels are a superfamily of cationic tetrameric channels, mostly permeable to Ca^{2+}, involved in various physiological functions, and for the most part sustain calcium homeostasis and calcium signaling. Calcium-dependent mechanisms determine several aspects of brain tumor cell homeostasis including survival, proliferation, invasion or treatment resistance, making TRP channels putative potent modulators of tumorigenesis and glioma progression. Approximately 30 TRPs have been identified and are classified into TRPA (ankyrin family), TRPC (canonical family), TRPM (melastatin family), TRPN (NomPC family), TRPML (mucolipin family), TRPP (polycystin family), and TRPV (vanilloid family) (Li, 2017). These cationic channels have been shown to be gated by many physical or chemical stimuli (temperature, membrane potential, pH, hormones, vitamins ...). TRP channels are expressed in various excitable and non-excitable cell types and are present in many organs, including brain, heart, liver, lung, kidney, spleen, muscle, skin, pancreas (Venkatachalam and Montell, 2007). Since a decade, TRP channels have attracted much interest in the cancer field and tumorigenesis. Activities of TRP channels have been linked to cell growth, survival or migration, being involved in a plethora of cancers, especially for TRPC, TRPM, and TRPV (Prevarskaya et al., 2007; Gkika and Prevarskaya, 2009; Fiorio Pla and Gkika, 2013). Recent research unravels the role of some TRP channels in glioma growth and progression or glioma stem-like cell fate determination. In this review, we will mainly focus on a new class of molecular players, TRP channels emerging in gliomas and for which we will develop three aspects: (i) the expression profile and use as clinical markers; (ii) the molecular mechanisms through which they act; and (iii) their potential use in therapeutics.

TRANSIENT RECEPTOR POTENTIAL (TRP) EXPRESSION PROFILE AND PUTATIVE BIOMARKERS

Changes in expression of TRP channels have been related to cancer development and progression, thus making them valuable diagnostic and/or prognostic markers in several tumor types, including glioma. Furthermore, a strong correlation between clinical-pathological findings and mRNA and/or protein expression of different TRPs has been recently provided. For instance, mRNA encoding TRPC1, TRPC6, TRPM7, TRPM8, TRPV4, and TRPML2 appeared up-regulated in GBM tumor specimens in comparison with normal tissues and their expression was found to increase with glioma tumor grade with the highest mRNA level found in GBM patient samples (Ding et al., 2010; Alptekin et al., 2015). These findings are consistent with a pro-tumorigenic role of these channels in glioma progression and aggressiveness, as described in more detail in the next paragraph. According to a qPCR screening of 33 GBM patient tumors, additional mRNA-encoding TRP channels including TRPM2, TRPM3, TRPV1, TRPV2 showed significantly higher expression levels in GBM compared with control normal brain tissues (Alptekin et al., 2015). However, other studies reported opposite results, more consistent with an

anti-tumorigenic function of these channels, as confirmed by several experimental data (Amantini et al., 2007; Nabissi et al., 2010; Ying et al., 2013; Morelli et al., 2019). These discrepancies could be due to the relatively low number of patients considered in the first study (Alptekin et al., 2015), which may not be very representative enough while further investigations should now be reconsidered in light of the new molecular GBM patient stratification and the methylome.

Among more than 20 TRP channels investigated, TRPM8 showed the highest mRNA upregulation in GBM as compared with normal brain tissue (Alptekin et al., 2015; Zeng et al., 2019), suggesting a pivotal function of TRPM8 in gliomagenesis. Moreover, TRPM8 expression in human GBM specimens and established GBM cell lines was found to be up-regulated at both mRNA and protein level to a variable extent (Klumpp et al., 2017) and to be significantly correlated with worse patient overall survival (Zeng et al., 2019). Interestingly, it was previously reported that TRPM8 is a primary androgen-responsive gene since its promoter is located downstream an androgen response elements (AREs) to which androgen receptor (AR) may bind once activated by androgens thus promoting TRPM8 expression (Bidaux et al., 2005; Asuthkar et al., 2015). Therefore, TRPM8 overexpression in high-grade glioma might be associated with the documented upregulation of AR in GBM (Yu et al., 2015). Moreover, it has been recently found that AR may also directly regulate TRPM8 channel activity via protein-protein interaction and/or TRPM8 phosphorylation, further accentuating the close TRPM8-androgens relationship (Grolez et al., 2019a; Gkika et al., 2020). Similarly to TRPM8, TRPV4 has been shown to positively correlate with glioma progression, since high levels of TRPV4 gene and protein expression were associated with a poorer patient prognosis (Ou-yang et al., 2018). Thus, TRPM8 and TRPV4 may be currently considered promising biomarkers accompanying aggressiveness of glioma and signature of GBM while constituting potential therapeutic targets for future treatment options. TRPML2 expression was also detected in normal astrocytes and neural stem/progenitor cells and to be up-regulated at both mRNA and protein level in glioma to a variable extent, increasing with the pathological grade (Morelli et al., 2016). Such observation was linked to the up-regulation of the transcriptional activator of the TRPML-2 gene Paired box 5 (PAX5) (Valadez and Cuajungco, 2015) found in human astrocytoma and correlated with malignancy and pathological grade of glioma (Stuart et al., 1995).

Moreover, the loss of TRPM3, TRPV1, TRPV2, and TRPML1 expression has been proposed as a negative prognostic marker for GBM patients, because of their significant and progressive down-regulation as the tumor grade increases. For instance, a study focusing on the role of miR-204 in high-grade glioma cell lines has revealed a significant down-regulation of TRPM3, due to the hypermethylation of its promoter (Ying et al., 2013). Interestingly, miR-204 is an intronic miRNA located between exons 7 and 8 of the TRPM3 gene and its loss in glioma, due to the high methylation of its host gene TRPM3, is associated with an enhancement in cell migration and cellular stemness (Ying et al., 2013) questioning the direct role of TRPM3 and the indirect regulatory functions of miR-204 *via* its target genes. Consistently, restoration of miR-204 in LN382T and SNB19 cells orthotopically xenografted in the brains of nude mice suppressed tumorigenesis and invasiveness and increased animal survival (Ying et al., 2013). Taken together, these findings might suggest a potential tumor-suppressive function of TRPM3 in glioma, but further studies are required to clarify its involvement in cancer development and/or progression and to establish whether TRPM3 and miR-204 might cooperate with each other in the pathogenesis of gliomas. Concerning TRPV1 and TRPV2, the preventing role in gliomagenesis and tumor progression has been more clearly established and characterized (see next paragraph). First, TRPV1 and TRPV2 genes and protein expression appeared inversely correlated with glioma grade, showing an almost undetectable level in GBM (Amantini et al., 2007; Nabissi et al., 2010; Morelli et al., 2016). In particular, a study performed by Nabissi and coworkers has shown that GBM and glioma stem-like cells (GSC) selectively express the TRPV1 5′-untranslated region (5′UTR) variant three ($TRPV1_{v3}$), one of the four variants resulting from alternative first exon splicing (Nabissi et al., 2016). The 5′UTR can generate different transcripts encoding the same protein but characterized by different stability and translation efficacy (Audic and Hartley, 2004; Gebauer and Hentze, 2004; Hinnebusch et al., 2016) and $TRPV1_{v3}$ is the most stable TRPV1 5′UTR transcript. In GBM, the mRNA expression of the unique $TRPV1_{v3}$ variant correlates with the patient's survival, suggesting that its loss or low mRNA expression may represent a potential marker of poor prognosis in GBM patients (Nabissi et al., 2016). Similarly, the clinical relevance of the overexpression of TRPV2 in GBM was confirmed through the analysis of the TRPV2-interactome based signature using a systematic proteomics and computational analysis approach (Doñate-Macián et al., 2018), predicting GBM patient overall survival. Indeed, high TRPV2 interactome protein expression was correlated with tumor progression, recurrence, TMZ-resistance and a poor prognosis (Doñate-Macián et al., 2018). Finally, also TRPML1 might have a potential role as a negative prognostic marker for GBM patients (Morelli et al., 2019) since TRPML1 mRNA down-regulation or loss strongly correlates with reduced overall survival in GBM patients. However, additional studies are needed in order to further investigate the relationship between TRPML1 expression and lower glioma grades (Morelli et al., 2019). However, TRPML1 expression at mRNA and protein levels displayed variability within patient samples and its subcellular localization may also be distinct since TRPML1 is mainly expressed in the late endosome/lysosome of normal cells while found in endolysosomes and as dot spots in the nuclear cell compartment in glioma cells (Morelli et al., 2019). The mechanisms underlying this nuclear localization in tumor cells and the effects of this specific localization are not completely characterized, although it has been shown that TRPML1 is able to bind DNA somehow and thus, it might affect the transcription of some genes involved in tumor progression (Morelli et al., 2019).

Together, these studies highlight that some TRPM, TRPV, and/or TRPML channels overexpressed in glioma should be considered as predictive and specific biomarkers of high-grade glioma and GBM, and through changes in their permeability to cations they may play a role in GBM aggressiveness.

MOLECULAR MECHANISMS OF TRP CHANNELS ACTION

Transient receptor potential channels have revealed a direct involvement in determining many hallmarks of glioma and GBM (**Table 1**), including some typical histological cellular abnormalities (Bomben and Sontheimer, 2008), its relentless growth, and its intrinsic severe aggressiveness due to its high capability to diffuse into the non-neoplastic brain parenchyma, which contributes to treatments resistance and bad prognosis (Demuth and Berens, 2004; Schwartzbaum et al., 2006; Liu et al., 2018). TRP channels may exert both anti-tumorigenic and pro-tumorigenic functions in gliomas and the main TRPs-mediated signaling pathways associated with gliomas progression are schematically summarized in **Figure 1**. Most of the TRP channels involved in gliomagenesis and tumor progression were found to affect more than one cellular process related to carcinogenesis. In this chapter, we will therefore discuss the molecular mechanisms by which each TRP affects cancer cell behavior, subgrouping them into subfamilies.

Canonical TRPs

It has been suggested that TRPC channels-relayed mechanisms may contribute to some of the most common histopathological hallmarks of GBM such as nuclear atypia and enlarged cell shape (Bomben and Sontheimer, 2008). Glioma cell lines and surgical patient-derived tumors have revealed the expression of four TRP channels belonging to the TRP canonical subfamily that are TRPC1, TRPC3, TRPC5, and TRPC6. Further investigations on their role in glioma cells have suggested an involvement of these channels in a Ca^{2+} influx pathway impacting cellular growth. More specifically, it has been shown that TRPC channels contribute to the resting conductance of glioma cells while their acute pharmacological inhibition with SKF96365 increased membrane resistance of glioma cells and caused a transient hyperpolarization followed by a sustain depolarization of the cells' membrane (Bomben and Sontheimer, 2008). Additionally, chronic application of the TRPC inhibitor SKF96365 (from 0 to 5 days) would lead to an almost complete growth arrest at the G_2/M phase of GBM D54MG cell cycle, as revealed by FACS analysis (Bomben and Sontheimer, 2008). In most cases the blockage of the cell cycle during the G_2/M transition leads to cell death (Stark and Taylor, 2006), or in the particular case of D54MG cells, TRPC inhibition was accompanied by a continued growth exhibiting multinuclear and enlarged cells due to incomplete cytokinesis. This phenotype might render impossible a dynamic adaptation of the cell volume to invade the brain parenchyma, through the narrow extracellular brain spaces compatible with less recurrence.

Glioma cells display a depolarized resting membrane potential around −30 mV. Some TRPC channels are opened at rest and contribute to this membrane potentials (Bomben and Sontheimer, 2008). Activation of TRPC channels can also lead to membrane potentials fluctuations by various ways. First of all, TRPC channels are non-selective cation channels and they directly depolarize cells following activation. In addition, TRP channels are functionally coupled with other ion channels, so their activation can indirectly lead to depolarizations or hyperpolarizations, depending on the channels involved (Gees et al., 2010). In particular, major conductances of glioma cells are mediated by Ca^{2+}-activated K^+ channels (Ransom et al., 2001) and Cl^- channels such as ClC3 or ClC2 (Mcferrin and Sontheimer, 2006), which both are modulated by TRPC channels. Glioma cells do express many types of channels and transporters (Cuddapah and Sontheimer, 2011; Molenaar, 2011), which are sensitive to membrane potential fluctuations. Furthermore, glioma cells have been shown to display electrical activities similar to Na^+ spikes, which are sustained by TTX-sensitive voltage-gated Na^+ channels (Bordey and Sontheimer, 1998). Thus, TRPC channels may impact many voltage-dependent cellular processes and modulate electrical behavior of glioma cells.

Among the TRPCs, TRPC1, and TRPC6 are those for which the mechanism of action has been best characterized in human malignant glioma. For instance, TRPC1 was found to act on several hallmarks of cancer, including growth, cell cycle and migration. Interestingly, all the TRPC1 effects on glioma cell behavior strictly resulted from lipid regulation, e.g., in some cases the channel activity is directly affected by lipids [phosphatidylinositol-(4,5)-bisphosphate PIP_2, phosphoinositides, diacylglycerol, cholesterol, etc.] localized on the plasma membrane, in other cases, it can be by signaling pathways which lead to the production of specific lipids such as sphingolipids. It has been shown that the loss of TRPC1-mediated Ca^{2+} influx upon pharmacological inhibition or constitutive/inducible shRNA silencing, is associated with reduced cell proliferation and incomplete cell division, thus resulting in multinucleated cells similar to those found in patient biopsies (Bomben and Sontheimer, 2010). The important role of TRPC1 in glioma cell division has been also confirmed *in vivo* through a shRNA knockdown approach on a flank GBM cell tumor model: TRPC1 downregulation led to a significant decrease in tumor size, most likely impairing calcium signaling during cytokinesis (late M-phase) (Bomben and Sontheimer, 2010). TRPC1 has also revealed a role in controlling glioma cell migration. In particular, it has been shown that TRPC1 is essential for chemotactic migration in human malignant gliomas in response to chemoattractant growth factors like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) which affect TRPC1 activity through different signaling pathways (Bomben et al., 2011; Lepannetier et al., 2016). Stimulation with EGF was associated with a re-localization of TRPC1 channel at the leading edge of migrating D54MG glioma cells within lipid rafts, specialized membrane microdomains enriched in cholesterol and sphingolipids (Mollinedo and Gajate, 2015). In agreement, it has been shown that both TRPC1 channel activity and lipid raft integrity were required for gliomas chemotaxis (Bomben et al., 2011). Moreover, the disruption of lipid rafts by depletion of cholesterol not only affected chemotaxis but also impaired TRPC currents in whole-cell recordings and decreased store-operated Ca^{2+} entry (SOCE), confirming a direct interplay between lipid rafts and TRPC1 channels and localized Ca^{2+} rise in regulating the chemotactic movement of

TABLE 1 | TRP channels expression and functionality in gliomas/glioblastomas.

	Expression					
Channel	**Healthy**	**Tumor progression**	**Biological effect**	**Mechanism**	**Potential therapeutic tools**	**References**
TRPC1	Yes	↑	Cell growth (+)	↑ Cytokinesis	TRPC1/SPK/PI3K inhibitors	Bomben and Sontheimer, 2008; Bomben and Sontheimer, 2010
			Cell migration (+)	↑Chemotaxis		Bomben et al., 2011; Lepannetier et al., 2016
TRPC6	Low	↑	Cell growth (+)	→ NFAT	TRPC6/NFAT inhibitors	Chigurupati et al., 2010; Ding et al., 2010
			Cell migration (+)	→ RhoA		Chigurupati et al., 2010
			Angiogenesis (+)	→ NFAT		
			Radioresistance (+)	↑ G_2/M (Cdc25C)		Ding et al., 2010
TRPM2	Yes	↑	Cell death (+)	↑ ROS-induced Ca^{2+} influx	TRPM2 gene insertion (+ Se and DXT)	Ishii et al., 2007; Ertilav et al., 2019
TRPM3	Yes	↑/↓	n.d.	n.d.	n.d.	Ying et al., 2013; Alptekin et al., 2015
TRPM7	Yes	↑	Cell growth (+)	↑ STAT3/Notch	TRPM7/STAT3/Notch/ ALDH1 inhibitors miR-28-5p	Liu et al., 2014; Wan et al., 2019
				⊣miR-28-5p ⊣ Rap1b		
			Cell invasion (+)	⊣miR-28-5p ⊣ Rap1b		Wan et al., 2019
			Stem cell renewal and differentiation (+)	STAT3-ALDH1 ↑		Liu et al., 2014
TRPM8	Yes	↑	Cell migration (+)	↑ BK channels RTK signaling		Wondergem et al., 2008; Wondergem and Bartley, 2009; Klumpp et al., 2017
		(2 different isoforms)	Cell growth (+)	→ BK channels → CaMKII ⊣cdc25C ⊣ cdc2 MAPK signaling		Klumpp et al., 2017; Zeng et al., 2019
			Cell death (–)	MAPK signaling		Klumpp et al., 2017; Zeng et al., 2019
			Radioresistance (+)	Supporting DNA repair and cell cycle upon genotoxic stress		Klumpp et al., 2017
TRPV1	Yes	↓	Apoptosis (+)	→ p38 MAPK		Amantini et al., 2007
				↑ ER stress (ATF3)		Stock et al., 2012
TRPV2	Yes	↓	Cell proliferation (–)	ERK signaling		Nabissi et al., 2010; Morelli et al., 2012
			Apoptosis (+)	Fas signaling		Nabissi et al., 2010
			Cell differentiation (+)	↑ GFAP and β_{III}-tubulin expression ↑ Aml-1 a (PI3K/AKT pathway)	CBD	Morelli et al., 2012; Nabissi et al., 2015
			Drug sensitivity (+)	↑ drug uptake ↑ drug-mediated apoptotic pathway	CBD + TMZ/BCNU/ DOXO	Nabissi et al., 2013, 2015

(Continued)

TABLE 1 | Continued

Channel	Expression: Healthy	Expression: Tumor progression	Biological effect	Mechanism	Potential therapeutic tools	References
TRPV4	Yes	↑	Cell migration (+)	AKT/Rac1 signaling	TRPV4 inhibitors (HC-067047)	Ou-yang et al., 2018
TRPA1	n.d.	n.d.	Cell apoptosis (+)	↑ mitochondrial stress	TRPA1 activators	Deveci et al., 2019
TRPML1	Yes	↓	Cell apoptosis (+) Autophagy (+)			Morelli et al., 2019
TRPML2	Yes	↑	Cell proliferation (+) Cell apoptosis (–)	PI3K/AKT – ERK 1/2 signaling		Morelli et al., 2016

Abbreviations: +, increase; –, decrease; ↑, increment; ↓, reduction; →, activation; ⊣, inhibition. SPK, sphingosine kinase; PI3K, phosphoinositide-3 kinase; NFAT, nuclear factor of activated T-cells; Se, selenium; DXT, docetaxel; STAT3, signal transducer and activator of transcription 3; miR-28-5p, miRNA 28-5p; ALDH1, aldehyde dehydrogenase1; BK, large-conductance Ca^{2+}-activated K^+ membrane ion channels; RTK, Met receptor tyrosine kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Cdc25C, M-phase inducer phosphatase 3; Cdc2, subunit of the M phase-promoting factor; MAPK, mitogen-activated protein kinase; ATF3, transcription factor-3; CBD, cannabidiol; ERK, extracellular signal-regulated kinase 1/2; GFAP, glial fibrillary acidic protein; Aml-1 a, acute myeloid leukemia variant a; AKT, protein kinase B; TMZ, temozolomide; BCNU, carmustine; DOXO, doxorubicin. n.d., not determined.

glioma cells (Bomben et al., 2011). It must be noted that TRPC pharmacological inhibition through non-selective inhibitors caused an almost complete loss of chemotactic migration but TRPC1 knockdown through shRNA compromised directional migration but did not eliminate it and did not affect non-directional motility. This suggests TRPC1 specific implication in chemotactic migration and the potential implication of other TRPC channels in migration processes (Bomben et al., 2011). As shown by a more recent study by Lepannetier et al. (2016), lipids are not only important for the regulation of TRPC1 at the membrane level, but also through their signaling. The authors shed light on another store-independent mechanism by which TRPC1 may be activated and thus affect cell migration in GBM. In particular, they have shown that PDGF may induce the translocation of TRPC1 from the cytosolic compartment to the front of migrating cells through a mechanism requiring the phosphoinositide-3 kinase (PI3K) and at the same time induces the production of the lipid second messenger sphingosine-1-P (S1P) which in turn, activates TRPC1-mediated Ca^{2+} entry. Indeed, the PDGF-induced Ca^{2+} influx through TRPC1 can be partially inhibited by pretreatment of the cells with a specific inhibitor of the sphingosine kinase (SPK) producing S1P (Lepannetier et al., 2016). However, whether S1P directly or indirectly triggers TRPC1-mediated and store-independent entry of Ca^{2+} channel remains to be clarified (Lepannetier et al., 2016). In any case, it has been well established that both TRPC1 targeting to the leading edge of lamellipodia and its activation by S1P are essential in regulating PDGF-induced chemotaxis in U251 glioblastoma cells (Lepannetier et al., 2016).

Another member of the TRPC subfamily specifically implicated in glioma progression is TRPC6. Indeed, TRPC6 was found to affect different hallmarks of GBM including tumor growth, cell survival, invasiveness and angiogenesis (Chigurupati et al., 2010; Ding et al., 2010). More specifically, it has been demonstrated that under hypoxia, which is one of the main characteristics of GBM aggressiveness, invasion and resistance to treatment (Flynn et al., 2008), Notch1 activation consequently led to TRPC6 upregulation in primary GBM samples and cell lines (Chigurupati et al., 2010). Indeed, the inhibition/silencing of TRPC6 was associated with a reduction in glioma growth, invasion and angiogenesis. This Notch1-mediated induction of TRPC6 expression in hypoxic U373 cell line was subtype-specific, since other members of the TRPC subfamily were unaffected, indicating that TRPC6 is primarily responsible for the hypoxia-induced sustained increase in the intracellular Ca^{2+} concentration. Additionally, it has been proven that TRPC6 is essential for GBM cell survival, since its downregulation not only suppressed cell growth *in vitro* and reduced tumor volume *in vivo*, but also impaired clonogenic ability, induced cell cycle arrest at the G_2/M phase, and enhanced the antiproliferative effect of ionizing radiation.

An accelerated G_2 phase progression may lead to an impaired DNA damage checkpoint and thus to an enhanced genomic instability, explaining the TRPC6 association with enhanced glioma cell malignancy. Mechanistically, TRPC6 effects on cell proliferation, tumor growth and angiogenesis seemed to be directly mediated by the Ca^{2+}-induced activation of the

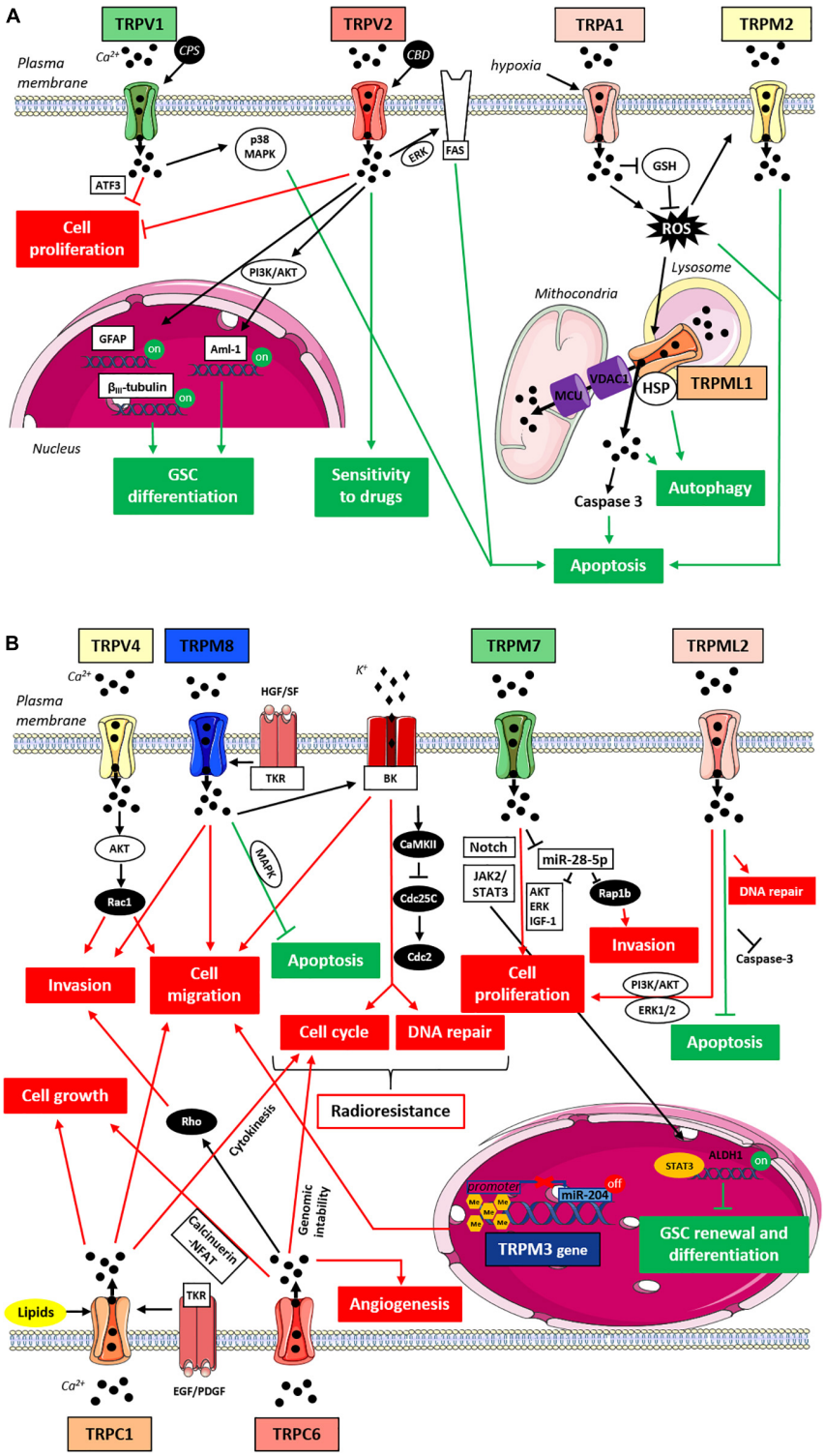

FIGURE 1 | TRPs-mediated signaling pathways in gliomas. **(A)** Anti-tumorigenic TRPs-mediated signaling pathways in gliomas. Cartoon depicting TRP channels signaling pathways suppressing gliomas progression through the inhibition of pro-tumorigenic pathways (in red) and/or the activation of anti-tumorigenic signaling pathways (in green). TRPM2 and TRPML1 upon ROS activation promote cell apoptosis through an increase in oxidative stress and intracellular Ca^{2+} concentration; due to its localization on the lysosomal membrane TRPML1 may also mediates the autophagic cell death pathway through the interaction with HSP; TRPA1 under hypoxia promotes apoptosis inducing ROS formation and inhibiting antioxidants such as GSH; TRPV1 upon stimulation with CPS triggers the apoptotic pathway through p38 MAPK and reduces glioma expansion via ER pathway in a ATF3-dependent manner; TRPV2 reduces cell proliferation and enhances the Fas-induced

(Continued)

FIGURE 1 | Continued
apoptosis in an ERK-dependent manner; TRPV2 also acts on cell differentiation since its overexpression is associated with GFAP and βIII-tubulin increased expression and its activation by CBD promotes Aml-1 up-regulation via PI3K/AKT pathway; finally, TRPV2 activation by CBD can improve cell sensitivity to chemotherapeutic agents favoring drug uptake. **(B)** Pro-tumorigenic TRPs-mediated signaling pathways in gliomas. Cartoon depicting TRP channels signaling pathways promoting gliomas progression through the activation of pro-tumorigenic pathways (in red) and/or the inhibition of anti-tumorigenic signaling pathways (in green). TRPC1 affects cell growth and cell proliferation mainly promoting cytokinesis in response to lipid activation, whereas upon stimulation with growth factors it induces chemotactic migration; TRPC6 affects cell proliferation, tumor growth and angiogenesis likely through the Ca^{2+}-induced activation of the calcineurin-NFAT pathway, whereas TRPC6 effects on cell migration might rather involve Rho activation and subsequent actin cytoskeleton rearrangements; TRPV4 promotes cell migration and tumor invasiveness through the AKT-mediated Rac1 activation; TRPM8 supports glioma progression by inhibiting apoptosis through MAPK pathway and impairing the cell cycle through the activation of BK channels and the subsequent CaMKII-mediated inhibition of phosphatases like Cdc25C and Cdc2; TRPM8 effects on cell migration/invasion are also associated with BK channels activation and the function of TRPM8 in aggressiveness and resistance to treatment may also be potentiated by TKR-mediated HGF/SF stimulation; TRPM7 increases glioma cell proliferation and invasion through the down-regulation of miR-28-5p and the subsequent up-regulation of oncogenic signaling pathways involving AKT, ERK, IGF-1 and Rap1b; TRPM7 effects on glioma proliferation may also be mediated by Notch and/or JAK2/STAT3 signaling pathways and through the activation of STAT3; TRPM7 might also be involved in GSC renewal and differentiation thanks to the up-regulation of the well-known GSC marker ALDH1; the hypermethylation of TRPM3 promoter through the down-regulation of miR-204 enhances cell migration; TRPML2 enhances cell proliferation and slows down apoptosis improving DNA repair and inhibiting Caspase 3 likely through PI3K/AKT and ERK1/2 pathways. Abbreviations: ROS, reactive oxygen species; HSP, heat shock proteins; GSH, glutathione; CPS, capsaicin; ATF3, transcription factor-3; CBD, cannabidiol; GFAP, glial fibrillary acidic protein; Aml-1, acute myeloid leukemia transcription factors; PI3K, phosphoinositide 3-kinases; AKT, protein-kinase B; HGF/SF, hepatocyte growth factor/scatter factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TKR, tyrosine kinase receptor; NFAT, nuclear factor of activated T-cells; MAPK, mitogen-activated protein kinase; BK, big potassium channels; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Cdc25C and Cdc2, cell division cycle proteins; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3; ERK, extracellular signal-regulated kinases; IGF-1, insulin-like growth factor-1; GSC, glioma stem cells; ALDH1, aldehyde dehydrogenase 1.

calcineurin-NFAT pathway (Chigurupati et al., 2010), whereas TRPC6 effects on cell migration might rather involve Rho activation and subsequent actin cytoskeleton rearrangements (Singh et al., 2007). Together, these data stress a possible role of TRPC6 as a promising therapeutic target in the treatment of human GBM (Chigurupati et al., 2010; Ding et al., 2010).

Melastatin TRPs

Four TRP melastatin subfamily members, TRPM2, TRPM3, TRPM7, and TRPM8, have been implicated in glioma cell growth, proliferation and migration. Among them, TRPM2 and TRPM3 would exert anti-tumorigenic effects, while TRPM7 and TRPM8 may contribute to glioma malignancy.

TRPM2 is known for its role as a sensor of oxidative stress and inductor of necrotic cell death upon activation by reactive oxygen species (ROS) (Naziroğlu and Lückhoff, 2008; Takahashi et al., 2011). TRPM2 expression likely induced no effect on cell proliferation, migration and invasion. But in A172 human GBM cells the transfection with TRPM2 channels increased cell death induced by H_2O_2 in a Ca^{2+}-dependent manner (Ishii et al., 2007) and in human GBM (DBTRG) cells, TRPM2 activation led to an increase in oxidative stress and intracellular Ca^{2+} concentration, thus promoting GBM cell death through apoptosis (Ertilav et al., 2019). Concerning TRPM3, which is the most recently described melastatin subfamily member, Its roles and action mechanisms were only recently investigated (Zamudio-Bulcock et al., 2011). Like TRPM2, TRPM3 may show a protective role in glioma probably via the miR-204 regulation (Ying et al., 2013), but its function needs further studies.

TRPM7 also controls glioma progression through miRNA regulation in GBM cells with subsequent effects on cell proliferation and invasion (Wan et al., 2019). More specifically, TRPM7 expression can be associated with a decreased production of miR-28-5p, a tumor suppressor inhibiting the expression of oncogenic signaling pathways involving protein-kinase B (AKT) (Xiao et al., 2018), ERK (Liu et al., 2016), and IGF-1 (Shi and Teng, 2015). Accordingly, the downregulation of miR-28-5p caused a significant increase in glioma cell proliferation and invasion. Among miR-28-5p targets, expression of Rap1b appeared as being positively correlated with TRPM7 in GBM and was up-regulated in tumor samples due to the suppression of the repressing role of miR-28-5p (Wan et al., 2019). This miR-28-5p/Rap1b axis shown in gliomagenesis is not the exclusive signaling route in which TRPM7 acts on GBM progression. Indeed, TRPM7 may also regulate the Notch pathway (Liu et al., 2014), recently shown as linked with Rap1b signaling and integrin-mediated cell adhesion in hematopoietic stem cells (Rho et al., 2019). This can be connected with the role of TRPM7 on cell proliferation, migration and invasion in glioma cells and GSCs through the upregulation of JAK2/STAT3 and/or Notch signaling pathways (Liu et al., 2014). Moreover, TRPM7 was found to activate STAT3, which in turn binds to the aldehyde dehydrogenase1 (ALDH1) promoter upregulating the expression of this well-known GSC marker involved in many pathways maintaining stem cell-like state (Rasper et al., 2010), when expanded as spheroids (Liu et al., 2014). Since ALDH1 is functionally involved in self-protection, differentiation, expansion and proliferation (Choudhary et al., 2005; Ma and Allan, 2011), this potentially means that TRPM7 is not only implicated in proliferation, migration and invasion, but also in GSC renewal and differentiation. This has to be put into the context that TRPM7 channel exhibits an intrinsic kinase activity, thus supporting that TRPM7 effects on glioma cell growth are mediated by its channel activity while cell migration and invasion required its kinase domain (Wan et al., 2019). The discovery of different cellular and molecular targets affecting gliomas development and progression through their modulation by TRPM7 provides key insights for the development of novel therapeutic agents for glioma treatments.

TRPM8 was found to affect the rate of GBM cell migration by mediating a significant increase in intracellular Ca^{2+}

concentration upon stimulation with specific agonists such as menthol and icilin (Wondergem et al., 2008; Wondergem and Bartley, 2009; Klumpp et al., 2017). It has been shown that TRPM8 activation by icilin leads to a significant increase in the migration speed and chemotaxis of GBM cells and, consistently, TRPM8 downregulation by RNA interference as well as TRPM8 inhibition by the specific channel blocker BCTC [*N*-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide] reduces cell migration rate and decreases transfilter chemotaxis (Klumpp et al., 2017). One of the possible mechanisms through which TRPM8-mediated Ca^{2+} influx may affect cell migration in glioma is by the activation of the large-conductance Ca^{2+}-activated K^+ ion channels (BK channels) (Wondergem and Bartley, 2009). BK channels contribute to maintaining the plasma membrane ionic fluxes essential to support cell shrinkage-driven cell migration (Mcferrin and Sontheimer, 2006). Interestingly, BK overexpression was detected in human glioma cells (Ransom and Sontheimer, 2001) and pharmacological inhibition of BK channels was shown to abolish the menthol-stimulated Ca^{2+} influx within the cell cytoplasm and cell migration suggesting a key role of TRPM8. TRPM8 activation by agonists has been shown to increase the open probability of single BK channels (Wondergem and Bartley, 2009; Klumpp et al., 2017). In agreement, ionizing radiation, known to induce the migration through a Ca^{2+}-mediated activation of BK channels (Steinle et al., 2011; Edalat et al., 2016), has been shown to activate and upregulate TRPM8-mediated Ca^{2+} influx in glioma cells (Klumpp et al., 2017), thus confirming a direct and reciprocal interplay between these two channel families in the control of GBM migration. The function of TRPM8 in aggressiveness and resistance to treatment was suggested by the potentiating impact of hepatocyte growth factor/scatter factor (HGF/SF), a multifunctional effector of cells expressing the Met tyrosine kinase receptor (TKR), on TRPM8-induced Ca^{2+} homeostasis and cell migration. This evidence suggests that TRPM8 might converge to a common HGF/SF and cMET, known to play a role in malignancy of solid tumors including glioma (Laterra et al., 1997; Birchmeier et al., 2003; Wondergem et al., 2008), signaling pathway leading to migration/invasion. An enhancement in the invasion rate of human GBM cells has also been associated with TRPM8 overexpression (Zeng et al., 2019). However, DBTRG cells would express two different variants of TRPM8 (Wondergem et al., 2008) as revealed in Western blot showing a molecular band at 130–140 kDa in the plasma membrane-enriched fraction and consistent with the molecular weight of TRPM8 full-length isoform (Peier et al., 2002), and a second molecular band at 95–100 kDa in microsome- and membrane-enriched fractions more consistent with a truncated TRPM8 splice variant expressed in the endoplasmic reticulum (ER) (Bidaux et al., 2007). The observed greater increase in menthol-induced Ca^{2+} influx among migrating cells compared with non-migrating cells (Wondergem et al., 2008), likely indicates that only migrating cells express full-length TRPM8 protein within the plasma membrane. However, these results have to be taken with caution in a future context of drug therapy, since TRPM8 was shown to have an anti-migratory activity in other cancers including prostate cancer and may play a role in the tumoral and tumor-derived endothelial cells (Gkika et al., 2010, 2015; Genova et al., 2017; Grolez et al., 2019b). TRPM8 contribution to GBM progression was found to go far beyond its effects on cell migration and invasion, significantly affecting other determinant processes such as cell cycle, cell survival and radioresistance (Klumpp et al., 2017; Zeng et al., 2019). Indeed, it has been proven that TRPM8 inhibition or knockdown impaired the cell cycle, triggered apoptotic cell death and attenuated DNA repair and clonogenic survival (Klumpp et al., 2017). A recent study by Zeng et al. (2019) have suggested an involvement of the mitogen-activated protein kinase (MAPK) signaling pathway in TRPM8-mediated effects on cell proliferation and apoptosis, since the expression of the channel was associated with the expression levels of important regulators of these pathways, including extracellular signal-regulated kinase (ERK), cyclin D1 and the apoptosis-related protein Bcl-2 in human glioma cells. Moreover, it has been shown that TRPM8 signaling directly regulates the cell cycle, contributing to S phase progression and mitosis. These effects on glioma's cell cycle are most likely mediated by intracellular signaling pathways involving the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and some Cdc phosphatases like Cdc25C and Cdc2, which control entry into, and progression through, various phases of the cell cycle (Klumpp et al., 2017). More specifically, the TRPM8-mediated Ca^{2+} entry, through the activation of BK channels, may increase the CaMKII activity, which in turn inhibits the Cdc2 subunit of the mitosis-promoting factor likely through the inhibitory phosphorylation of the Cdc25C phosphatase (Klumpp et al., 2017). Finally, TRPM8 channel might also modulate proliferation by dynamically control glioma resting potentials levels, which are key regulator of cell cycle. TRPM8 agonists have been shown to increase the Kir4.1 mediated membrane conductances of glioma cells (Ratto et al., 2020). Kir4.1 is an inward rectifier K^+ channel, with an altered pattern of expression in glioma. In astrocytes, it is responsible for high potassium conductance and hyperpolarized membrane potential (Olsen and Sontheimer, 2008). Overexpression of this channel in glioma cell lines D54MG reduces cell proliferation (Higashimori and Sontheimer, 2007). Thus, TRPM8 channel by regulating K^+ resting conductances of glioma cells may exert a regulation of cell cycle transitions. Moreover, TRPM8 has also been found to be a contributor to the genotoxic stress response of GBM upon treatment with ionizing radiation, restoring G_1/S transition and S phase progression to levels of unirradiated cells (Klumpp et al., 2017). Interestingly, it has been found that ionizing radiation stimulated TRPM8 availability both *in vitro* and *in vivo* and that TRPM8 played a role in the re-entry in mitosis and cell division upon radiation-induced G_2/M arrest, since its knockdown resulted in an impaired DNA repair and a decreased survival of irradiated cells (Klumpp et al., 2017). This, combined with the slowdown of apoptosis in irradiated GBM cells, may explain the enhanced radioresistance acquired by GBM cells overexpressing TRPM8, thus stressing that the key interest of targeting TRPM8 alone

or in combination with radiotherapy for future treatments of GBM.

Vanilloid TRPs

Some TRP members of the vanilloid family have been related to gliomagenesis and progression. More specifically, TRPV1 and TRPV2 have revealed a protective role in glioma cells by regulating cell proliferation and survival, stem cell differentiation and sensitivity to drugs, whereas TRPV4 was found to increase cancer cell invasiveness.

The anti-tumorigenic functional role of TRPV1 in gliomas, suggested by its marked downregulation or loss in patients with the shortest overall survival, has been investigated and several findings highlighted a role of TRPV1 in the induction of apoptotic cell death signaling in gliomas (Amantini et al., 2007; Stock et al., 2012). Upon exposure to low doses of the TRPV1 specific agonist capsaicin (CPS), the TRPV1-Ca^{2+} may sustain apoptosis in gliomas through the selective activation of p38 MAPK, but not ERK MAPK (Amantini et al., 2007). More in detail, it has been shown that CPS-mediated TRPV1 activation leads to reduced cell viability, DNA fragmentation, externalization of phosphatidylserine on the outer layer of the plasma membrane, mitochondrial transmembrane potential dissipation, and caspase 3 activation (Amantini et al., 2007). Moreover, TRPV1 translation in GBM was found to be sensitive to interferon-gamma (INF-γ) and to the well-known autophagic inducer rapamycin (Rap), suggesting a link between TRPV1 channel and autophagy often related to pro-survival in tumors including GBM (Galluzzi et al., 2015) but also in migration (Coly et al., 2017). This biological effect might be achieved by the TRPV1-mediated induction of apoptosis previously reported in GBM cells (Amantini et al., 2007).

One of the mechanisms through which the brain, especially in the juvenile phase, can protect itself against high-grade astrocytoma (HGAs) involves the activation of TRPV1 by neural precursor cells (NPCs), known to show extensive tropism for brain tumors (Mayor et al., 2001). Interestingly, NPCs accumulate at HGA especially in the context of the juvenile brain, exhibiting a high proliferative activity in the stem cell niche (Walzlein et al., 2008), and can release tumor-suppressive factors, such as endovanilloids able to activate TRPV1 expressed by HGA cells (Stock et al., 2012). The activation of the latter would trigger astrocytoma cell death through the ER pathway in a transcription factor-3 (ATF3)-dependent manner, thus reducing glioma expansion mostly in young brain (Stock et al., 2012). In light of these data, the inverse correlation between TRPV1 expression and glioma grade from I to III and the reduced or lost TRPV1 expression found in GBM patients is most likely a mechanism by which tumor cells may evade anti-proliferative and pro-apoptotic signals. This hypothesis is also supported by the finding that TRPV1 is also downregulated in GSCs (Stock et al., 2012), whose resistance to cytotoxic therapies and to pro-apoptotic signals is accepted (Bao et al., 2006a). Furthermore, the induction of GSCs differentiation was accompanied by $TRPV1_{v3}$ expression at a similar level than found in low-grade glioma, thus confirming a protective role of this channel against aggressiveness (Nabissi et al., 2016).

TRPV2 exerts its anti-tumorigenic function on gliomas through the regulation of several signaling pathways involved in cell proliferation and survival, stem cell differentiation and sensitivity to drugs. Physiologically, the triggering of TRPV2 by agonists/activators such as growth factors, hormones and cannabinoids led to TRPV2 translocation from the endosome to the plasma membrane, where it mediates several pathways associated with cell proliferation and cell death (Liberati et al., 2014). Thus, loss or alterations of TRPV2 expression in cancer cells results in an impairment of these processes, as shown in prostate tumor-derived endothelial cells (Bernardini et al., 2019) and gliomas (Liberati et al., 2014). In gliomas, it has been shown that TRPV2 reduced cell proliferation and increased cell sensitivity to Fas-induced apoptosis in an ERK-dependent manner (Nabissi et al., 2010). Consistently, enhanced cell growth and rescuing from apoptotic cell death was observed when TRPV2 was silencing in U87MG GBM cells. In contrast, TRPV2 upregulation in MZC primary glioma cells, by inducing Fas overexpression led to reduced viability and increased spontaneous as well as Fas-induced apoptosis (Nabissi et al., 2010). Similar findings were also described in GSCs, whose proliferation appeared strongly impaired by TRPV2 pharmacological inhibition or knocking down (Morelli et al., 2012). In GSCs, TRPV2 acts also on differentiation (Morelli et al., 2012; Nabissi et al., 2015). More specifically, TRPV2 overexpression was associated with glial fibrillary acidic protein (GFAP) and β_{III}-tubulin increased expression, thus promoting a glial phenotype differentiation while inhibiting GSCs proliferation both *in vitro* and *in vivo*. In agreement, TRPV2 silencing or inhibition during differentiation impaired differentiation and reduced GFAP and β_{III}-tubulin expression (Morelli et al., 2012). Moreover, TRPV2 activation through cannabidiol (CBD) was found to trigger GSCs differentiation activating the autophagic process, in addition to inhibiting GSCs proliferation and clonogenic capability (Nabissi et al., 2015). More specifically, it has been observed that CBD, through the TRPV2-mediated activation of the PI3K/AKT pathway, upregulated the expression of acute myeloid leukemia (Aml-1) transcription factors, known for their pivotal role in GBM proliferation and differentiation. Furthermore, it has also been shown that the spliced variant Aml-1 a, upregulated during GSCs differentiation, directly influenced the expression of TRPV2 by binding its gene promoter (Nabissi et al., 2015), thus establishing a positive feedback circuit, which on the whole caused glial differentiation.

Conversely to TRPV1 and TRPV2, TRPV4 has revealed a pivotal role in promoting glioma progression (Ou-yang et al., 2018). In particular, the tumorigenic potential of TRPV4 comes from its critical involvement in glioma cell migration and invasion. Indeed, it has been demonstrated that TRPV4-mediated Ca^{2+} influx upon stimulation with the selective agonist GSK1016790 A, is able to promote cell migration of glioma cells (Ou-yang et al., 2018), a similar mechanism previously reported in breast cancer (Lee et al., 2017). It was established that TRPV4 effects on cell migration are relayed by phosphorylation of AKT (P-AKT) and activation of Rac1 (Ou-yang et al., 2018), a

member of the Rho GTPases family known for its central role in cytoskeleton remodeling, cell motility and cell adhesion as well as for its involvement in the enhanced migration of several tumor types including GBM, colon, colorectal and ovarian cancer (Guo et al., 2015; Guéguinou et al., 2016; Qin et al., 2017; Zhou et al., 2018). Accordingly, TRPV4 blockade, induced by the specific TRPV4 inhibitor HC-067047, was found to decrease motility and invasiveness of U87 glioma cells through a P-AKT and Rac1 signaling pathway (Ou-yang et al., 2018).

Mucolipin TRPs

The two TRP members of mucolipin subfamily have revealed opposite effects on glioma carcinogenesis.

TRPML1 showed a protective role against glioma progression. Indeed, it has been shown that TRPML1 activation by its specific agonist MK6-83 reduced T98 and U251 cell line viability and increased caspase 3-dependent apoptosis. Accordingly, TRPML1 silencing or pharmacological inhibition restored cell viability suppressing the Ca^{2+} influx responsible for apoptosis induction. Furthermore, TRPML1 may also mediate the autophagic cell death pathway, upon cell treatment with the ROS inducer carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Morelli et al., 2019). Indeed, high ROS levels may trigger a TRPML1-mediated lysosomal Ca^{2+} release and the subsequent enhancement of autophagy (Zhang et al., 2016b). Accordingly, TRPML1 silencing or inhibition by sphingomyelin pre-treatment reverted CCCP effects (Morelli et al., 2019). In this context, it has also been demonstrated that TRPML1, through a large intraluminal loop between its first and second transmembrane domains, may interact with chaperone-mediated autophagy-related proteins such as the heat shock proteins Hsc70, and Hsp40 (Venugopal et al., 2009). Therefore, TRPML1 may exert its cytotoxic effects through two different pathways, e.g., (i) it can act as a ROS sensor on the lysosomal membrane and attenuate oxidative cell stress through an autophagy-dependent negative-feedback mechanism (Zhang et al., 2016a; Morelli et al., 2019) or (ii) it may trigger Ca^{2+} release but no ROS production upon direct activation by its specific agonist, thus inducing apoptosis (Morelli et al., 2019). Moreover, the important role of TRPML1 in controlling intracellular Ca^{2+} homeostasis has been further corroborated by the recent finding of a functional localization of TRPML1 at the so-called "mitochondria-lysosome contact sites" where it mediates a calcium flux from lysosomes to mitochondria adjuvanted by VDAC and MCU on the outer and inner mitochondrial membranes, respectively (Peng et al., 2020). Consequently, TRPML1 functions go beyond the regulation of lysosomal dynamics and function and, through the control of mitochondrial Ca^{2+} dynamics, can affect other Ca^{2+}-dependent mitochondrial functions, including oxidative phosphorylation, motility, and ROS signaling (Peng et al., 2020).

Conversely to TRPML1, the other member of the mucolipin family, TRPML2, has revealed a pro-tumorigenic function in glioma progression. Indeed, it has been shown that TRPML2 enhanced glioma cell survival and proliferation (Morelli et al., 2016). More in detail, TRPML2 suppression leads to impaired cell cycle, reduced cell viability and decreased proliferation (Morelli et al., 2016). In addition, its knocking-down was found to induce apoptosis by increasing DNA damage, Ser139 H2AX phosphorylation and caspase-3 activation (Morelli et al., 2016). TRPML2 effects on tumor progression are probably mediated by PI3K/AKT and ERK1/2 pathways, since these pathways remained inactivated in TRPML2-silenced cells (Morelli et al., 2016). Thus, TRPML2 might also be an interesting therapeutic target to control GBM cell survival and proliferation.

THERAPEUTIC TARGETING

Considering the altered expression and the great contribution given by TRP channels to the establishment and progression of glioma, they may be considered very promising new therapeutic molecular targets against which novel drug compounds must be developed. One of the main advantages provided by most TRPs is their accessibility from the extracellular side, which makes them efficient targetable sites via administration of specific TRPs inhibitors or blockers when these channels are overexpressed in high-grade glioma. For instance, some of the TRPC channels by interfering with cytokinesis pathways would be promising targets for the development of drugs able to interfere with the almost unrestrained growth of gliomas, making tumors more susceptible to surgical removal or focal radiotherapy. Moreover, the low specificity of some TRPC modulators might allow to achieve a higher antitumor effect, through the simultaneous triggering of more than one channel. The progressive understanding of the molecular mechanism underlying TRPC function in glioma has also provided opportunities and arguments in favor of small molecule targeted therapies. Through studies concerning TRPC1 and its specific pharmacological inhibition, the key option by inhibiting SPK or PI3K inhibitors is once again here confirmed to attempt controlling GBM growth and invasiveness. Among TRPC channels, great potential as a promising new candidate for GBM treatment comes from TRPC6. Among TRP, TRPC6 is to date the only one being implicated in GBM angiogenesis suggesting that specific TRPC6 inhibitors could simultaneously target both cancer progression and vascularization, thereby improving the efficacy of standard (radio-chemotherapy) options. The barely detectable TRPC6 expression in normal glial cells should limit the potential side effects on normal glial cells. But a possible impact of TRPC6 blockade on neurons must be considered in regards to the central role of TRPC6 in neuronal functions (Tai et al., 2008; Zhou et al., 2008; Kim et al., 2017). A possible strategy to overcome this problem might be the use of viral vectors as a drug delivery system toward glioma cells since adenovirus may target glioma cells more efficiently than neurons (Ding et al., 2010).

As recurrence is due to migration and invasion (and resistance to treatment), pharmacological inhibition of TRPV4 might represent a potential new therapeutic approach in GBM treatment to control migratory and invasive capabilities of GBM cells (Ou-yang et al., 2018). One of the mechanisms through which tumor cells are able to sustain a prolonged survival is by the inhibition of apoptotic pathways (Thompson, 1995). In this context, TRPA1 has been recently proposed as a new potential therapeutic target in GBM treatment. Indeed, TRPA1 is

a ROS-sensitive cation channel and can subsequently be activated by hypoxia-induced oxidative stress (Naziroğlu, 2012; Takahashi et al., 2018). It has been shown that TRPA1 activation following cobalt chloride ($CoCl_2$) treatment with the aim to mimic hypoxia, may increase apoptosis, inflammation and oxidative effects on DBTRG cells (Deveci et al., 2019). More specifically, TRPA1-mediated Ca^{2+} entry was associated with an enhancement of ROS production and mitochondrial membrane depolarization (JC-1). Moreover, TRPA1 activation leads to increased levels of Annexin V, cytokines IL-1β and IL-18, and caspase 3 and 9 and decreased levels of thiol cycle antioxidants (GSH and GSH-Px) (Deveci et al., 2019). These effects were shown attenuated by α-lipoic acid (ALA) treatment, a physiological source of energy for cells which may exert both anti- and pro-oxidant functions (Moini et al., 2002). In glioma cells under hypoxia, ALA likely acts as an antioxidant agent, upregulating GSH and GSH-Px and down-regulating mitochondrial ROS production, thus blocking TRPA1-mediated induction of apoptotic cell death (Deveci et al., 2019). This suggests that targeting and activating TRPA1 or targeting TRPV1 in glioma exhibiting such expression, can restore apoptotic signaling and might provide new insights for the development of alternative therapies against glioma progression.

Besides being potential anti-tumor targets, TRP channels should play a role as "drug carriers" in cancer therapy, facilitating *via* the central pore chemotherapy drug uptake thus improving the efficacy of cancer therapies. For instance, TRPV2 activation by CBD can sensitize GBM cells to chemotherapeutic agents currently used, e.g., TMZ, carmustine (BCNU) and doxorubicin (DOXO) (Nabissi et al., 2013, 2015). The CBD-induced TRPV2 activation was found to increase GSCs sensitivity to cytotoxic effects of alkylating agents like BCNU favoring drug uptake (Nabissi et al., 2013), in synergy with the Ca^{2+}-dependent triggering of apoptotic cell death, a mechanism not found in normal astrocytes (Nabissi et al., 2015). Specifically, by using the natural red fluorescent DOXO, it has been demonstrated that TRPV2 overexpression in MZC glioma cells markedly increased DOXO uptake in a Ca^{2+}-dependent manner, since Ca^{2+} chelation by EGTA completely inhibited the CBD-induced TRPV2-mediated increase of DOXO-positive cells (Nabissi et al., 2013). Similar findings were also observed in hepatocellular carcinoma in which TRPV2 activation by CBD or 2-APB (Aminoethoxydiphenyl borate) was found to improve DOXO permeation into tumor cells, thus corroborating an intriguing role of TRPV2 in increasing tumor cell sensitivity to chemotherapy drugs (Neumann-Raizel et al., 2019). Taking into account other evidence on the role of TRP channels as "drug carriers" thanks to the permeation of chemotherapy agents into the cell through their pore domain (Santoni and Farfariello, 2011), it is reasonable to speculate that the activation of TRPV2 channel may cause a conformational change in the pore helix structure, which allow for intracellular non-specific chemotherapy uptake (Nabissi et al., 2013), opening the route for combinatorial co-administration of TRPV2 specific agonist CBD and lower chemotherapeutic doses to overcome the high resistance of GBM and GSCs to chemotherapeutic agents. In this context, recent developments on the role of the so-called "pore turret," i.e., the region of the extracellular ring that connects the S5 helix to the pore helix, in controlling the upper gate of some TRP channels including TRPV2 (Dosey et al., 2019) have provided new insights into the role of TRPV2 as a drug target to reduce GBM chemoresistance. Indeed, the well-defined pore turret, in addition to allowing the coupling between the lower gate and the upper gate in response to intracellular stimuli stabilizing a fully open unliganded channel, represents a possible and interesting binding site for extracellular modulators through which it may affect channel activity allowing the passage through the plasma membrane of partially hydrophilic molecules which otherwise could not enter the cell (Dosey et al., 2019).

With a view to looking ahead, some data indicate that TRP channels may constitute targets of gene therapy. It has been recently shown that TRPM2 can promote cell death (Ertilav et al., 2019). Therefore, TRPM2 might represent a good candidate for gene therapy to be used for instance in combination with γ-radiation and/or chemotherapeutic agents to improve the effectiveness of GBM treatments. Preliminary observations indicate that Selenium (Se) tested on GBM cells resistant to Docetaxel (DTX) may improve the apoptotic efficacy of DTX through the activation of TRPM2 by oxidative stress (Ertilav et al., 2019). The cytotoxic effect of DTX likely comes from the formation of excessive mitochondrial ROS and Ca^{2+} influx into the cells which causes DNA damage by triggering hyperactivation of the DNA nick sensor PARP, thus leading to NAD+ and ATP depletion and subsequent apoptotic cell death (Ertilav et al., 2019). In this case, Se, in particular, stimulated oxidative stress production in the mitochondria, which in turn activated a TRPM2-mediated Ca^{2+} influx, thus supporting and enhancing the same Ca^{2+}-dependent apoptotic pathway induced by DTX and other chemotherapeutic agents, as seen also in other tumor types (Hazane-Puch et al., 2016; Çetin et al., 2017). Overall the combination and synergistic activity of Se and DTX in GBM expressing TRPM2 might offer a new option for adjuvant chemotherapy as treatment of GBM.

FUTURE PERSPECTIVES

Increasing understanding of the signaling pathways involved in tumorigenesis has made it possible to identify a wide range of molecular targets involved in self-renewal and proliferation, angiogenesis but also in invasion of GBM cells. A number of therapeutic strategies have therefore been developed during the last decade and few of them have proven to be effective, even though anti-angiogenic treatments appear to be able to provide a 6 months delay for GBM patients before recurrence. It is, therefore, necessary to identify other therapeutic targets that can be combined with anti-angiogenic, cytotoxic, DNA repair inhibitors and/or immunotherapy strategies. In this context, targeting the activity of factors or components expressed by glioma cells themselves and by other cell types of the micro-environment would also be promising. It has also to be considered that main RNAseq and/or transcriptomic databases were constituted by means of the glioma tumor

bulk composed of the different populations of GBM cells and other constituents such as endothelial cells, pericytes, reactive astrocytes, macrophages (M1 and/or M2), microglial, neurons, and potentially lymphocytes, depending on the level of heterogeneity GBM subgroup. New potential family targets, expressed at the plasma membrane and involved in survival, GSC differentiation, angiogenesis and invasion, constitute a choice option. TRP channels not systematically ubiquitously expressed, potentially playing pleiotropic mechanisms and being overexpressed in pathologic situations such as hypoxia in glioma cells deserve to be more explored, especially since they could be also expressed by other cell types belonging to the tumor micro-environment.

For instance, a direct involvement of TRP channels in vascular endothelial growth factor (VEGF) signaling pathways affecting brain neovascularization and tumor growth it has been proven. GCSs positive for CD133 (human prominin-1/AC 133) not only are capable of self-renewal and proliferation, but also possess the capability to secrete high levels of VEGF (Bao et al., 2006b; Yao et al., 2008), known to play a crucial role in endothelial cell recruitment and angiogenesis of malignant human gliomas (Fischer et al., 2005; Bian et al., 2006). In GSCs isolated from U87 cell line the production of VEGF and the angiogenic CXCL8 (chemokine interleukin-8) by tumor cells appeared to be mediated by a G protein-coupled receptor named formylpeptide receptor (FPR) which, upon activation, induces directional migration, growth and angiogenic factors production through a Ca^{2+} mobilization. Although a direct involvement of TRPs in this Ca^{2+}-mediated mechanism has not been highlighted, a GCPR-TRP axis in many signaling pathways is nowadays well established (Veldhuis et al., 2015). Moreover, an interesting crosstalk between TRPM8, TRPV1 and the VEFG receptor (VEGFR) it has been recently characterized in uveal melanoma, suggesting a good potential for TRPM8 as a pharmacological target for blocking brain neovascularization and tumor growth (Walcher et al., 2018). Indeed, it has been demonstrated that in different cell types including corneal epithelial and endothelial cells (Lucius et al., 2016) and uveal melanoma cells (Walcher et al., 2018) the activation of TRPM8 inhibits the VEGF transactivation of TRPV1 and the consequent pro-tumorigenic effects mediated by the VEGFR. These findings further highlighted the central role played by TRPs interactions with other TRP channels, other channels families like that of BK channels, and the GCPRs, in affecting signaling pathways directly involved in carcinogenesis and brain tumor progression. Moreover, they strongly sustain the possible TRPs application in anti-angiogenic therapy.

However, to date, to the best of our knowledge TRPC6 is the only TRP channel directly implicated in GBM angiogenesis. TRPC6 has been found to play a key role in promoting GBM growth, angiogenesis and invasion under hypoxia through Notch1 (Chigurupati et al., 2010). TRPC6 knockdown or NFAT inhibition has been shown to reduce the number of branch points and thus impair the ability of the hypoxic U373MG to induce endothelial cell tube formation *in vitro*, suggesting a role of TRPC6 in the "vascular mimicry" played by glioma cells (Chigurupati et al., 2010).

Other TRPs among those previously described to have a function in gliomas, such as TRPM2, TRPM7, TRPV2, and TRPV4 have also been found in brain vasculature, thus suggesting a possible double function for these channels in affecting glioma progression (Hatano et al., 2013; Pires and Earley, 2017; Ouyang et al., 2018; Luo et al., 2019) and contributing to GBM angiogenesis. Regarding TRPA1 and TRPC3 not likely described in glioma, some studies highlighted their involvement in other brain vasculature diseases, exerting a protective role against ischemic damage, controlling vasodilation in brain endothelial cells (Sullivan et al., 2016; Pires and Earley, 2018). TRPC3, when overexpressed, would lead to an increase in the BBB permeability, leading to vasogenic edema formation (Ryu et al., 2013).

CONCLUSION

Taken together all these data suggest a key role of some TRP channels in high-grade glioma development and angiogenesis. The current activators or inhibitors directed against these channels should provide lead compounds and knowledge for future research in the design of drugs targeting simultaneously glioma cells and key components of the micro-environment such as abnormal tumoral vascularization.

AUTHOR CONTRIBUTIONS

DG and HC provided a rational of the study. GC performed an in-depth analysis of the roles of TRP channels in inducing glioma behavior and generated a preliminary draft. GC, HC, OC, and DG performed the literature searches and contributed to writing and editing of the content. All the authors contributed to the article and approved the submitted version.

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Activation of TRPV1 by Capsaicin or Heat Drives Changes in 2-Acyl Glycerols and *N*-Acyl Ethanolamines in a Time, Dose, and Temperature Dependent Manner

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Endocannabinoids (eCBs) and transient receptor potential (TRP) channels are associated with thermoregulation; however, there are many gaps in the understanding of how these signaling systems work together in responding to changes in temperature. TRPV1, a calcium-permeable ion channel, is activated by capsaicin, elevated temperature, the eCB Anandamide, and over 15 additional endogenous lipids. There is also evidence for signaling crosstalk between TRPV1 and the eCB receptor, CB_1. We recently found that activation of TRPV1-HEK cells by capsaicin increases the production of the eCB, 2-arachidonoyl glycerol (2-AG), suggesting a molecular link between these receptors. Here, we tested the hypothesis that TRPV1 activation by capsaicin drives regulation of a wider-range of lipid signaling molecules and is time and dose-dependent. We also tested the hypothesis that changes in temperature that drive changes in calcium mobilization in TRPV1-HEK will likewise drive similar changes in lipid signaling molecule regulation. Lipid analysis was conducted by partial purification of methanolic extracts on C18 solid phase extraction columns followed by HPLC/MS/MS. Capsaicin increased the release of 2-acyl glycerols (2-AG, 2-linoleoyl glycerol, 2-oleoyl glycerol), in a concentration- and time-dependent manner, whereas levels of *N*-acyl ethanolamines (NAEs), including Anandamide, were significantly decreased. Analogous changes in 2-acyl glycerols and NAEs were measured upon ramping the temperature from 37 to 45°C. In contrast, opposite effects were measured when analyzing lipids after they were maintained at 27°C and then quickly ramped to 37°C, wherein 2-acyl glycerol levels decreased and NAEs increased. These results provide further evidence that the eCB system and TRPV1 have integrated signaling functions that are associated with the molecular response to temperature variation.

Keywords: TRPV1, lipidomics, thermoregulation, endocannabinoid, anandamide, 2-AG

INTRODUCTION

Temperature, touch, and pain are perceptions resulting from CNS processing of the transduction of stimuli by somatosensory neurons that innervate skin and organs (Lumpkin and Caterina, 2007). Among these sensations and perceptions, understanding of the transduction of temperature and heat remains elusive. It has been hypothesized that a stimulus such as heat may require more than one transduction mechanism for thermoperception to occur (Lumpkin and Caterina, 2007). Thermoregulation is a crucial, homeostatic function that promotes an organism's ability to maintain its core body temperature within a specific range (Hardy, 1961; Hensel, 1973). Changes in the environmental temperature have a more significant and immediate effect on skin temperature, compared to the body's core temperature (Schlader et al., 2011). This characteristic indicates that changes in the core body temperature cannot necessarily serve as the primary thermal signal. A straightforward example is that the human hand has evolved to perceive highly acute stimuli, with the threshold for detecting a change in temperature being $<0.5°C$, even if this is not a noxious change (Stevenson et al., 1999; Frenzel et al., 2012). Therefore, the thermoregulatory responses must be rapid, in order to prevent body core temperature challenges (Nakamura, 2011; Schlader et al., 2011), which suggests rapid modifications of the transduction apparatus by signaling molecules.

Transient receptor potential channels are ubiquitous ligand-gated ion channels, known for their role in the detection and perception of various sensations such as pain, pressure, and temperature (Montell and Rubin, 1989; Caterina et al., 1997; Patapoutian et al., 2003), and are, therefore, involved in many important physiological processes (e.g., perception of various stimuli and maintaining ion homeostasis) (Hansen et al., 2001). Eleven TRP channels have been categorized as temperature-sensitive TRPs. Six out of the eleven channels within this category have highly specific temperature thresholds and are known as thermoreceptor TRPs, or "thermoTRPs" (Montell et al., 2002; McKemy, 2005; Tominaga, 2006; Caterina, 2007). TRPV (vanilloid) cation channels 1–4 are thermoTRP channels that are able to detect thermal stimuli ranging from mild warmth to painfully hot (Clapham, 2003; Caterina, 2007; Nakamura and Morrison, 2008; Sisignano et al., 2014). TRPV1 has a temperature threshold of $\sim 42°C$, TRPV2 responds to temperatures $\geq 52°C$, and TRPV3 and TRPV4 are responsive to innocuous warmth ($\sim 26–34°C$) (Caterina et al., 1999; Smith et al., 2002; Julius, 2013). ThermoTRP channels exist as membrane proteins that enable ionic mobilization upon stimulation due to a change in temperature (Caterina et al., 1997; Story et al., 2003). If the magnitude of influx is above the threshold, the influx of these ions leads to a temporary depolarization of the cell membrane and initiates a signaling cascade to ultimately allow an organism to perceive that stimulus (Hirst et al., 2006).

The TRPV1 ion channel is the most widely studied receptor of those belonging to the TRPV family. TRPV1 was initially identified in rat dorsal root ganglion (DRG) in 1997, and coined with the name "the capsaicin receptor" (Caterina et al., 1997). These receptors are localized most notably in primary afferent nociceptive neurons in the peripheral nervous system, though they are also expressed in several non-neuronal cell types, along with higher regions of the brain, such as the striatum, hippocampus, midbrain, cerebellum, thalamus, and the hypothalamus (Caterina et al., 1997; Holzer, 2008; Starowicz et al., 2008). The TRPV1 moniker, "the capsaicin receptor," derived from the receptors' sensitivity to capsaicin, the primary pungent ingredient in hot chili peppers that elicits a "burning sensation" upon consumption (Jancso et al., 1977). The "burning pain" perception evoked upon encountering capsaicin led to the theory that, due to receptor activation by capsaicin and subsequent response mechanisms determined by the CNS, capsaicin might be eliciting the perception of pain in a manner similar to the endogenous ligands produced and released upon tissue damage (Bevan and Szolcsanyi, 1990; Caterina and Julius, 2001). This drives that hypothesis that there are endogenous ligands that regulate the function of TRPV1.

The discovery of the cannabinoid receptor 1 (CB_1) lead to the subsequent uncovering of the endocannabinoid (eCB) system. The two most studied signaling molecules within this network are Anandamide (*N*-arachidonoyl ethanolamine; AEA) and 2-arachidonoyl-*sn*-glycerol (2-AG) (Devane et al., 1992; Sugiura et al., 1995). AEA and 2-AG are both derived from membrane phospholipids that contain arachidonic acid and are hypothesized to be produced and released in response to various homeostatic disruptions (Maccarrone et al., 2000). AEA and 2-AG are recognized as lipid signaling molecules that modulate various physiological processes within the nervous system, in part by targeting the cannabinoid receptors, CB_1 and CB_2 (Di Marzo and Maccarrone, 2008). Several other members of the *N*-acyl ethanolamine (NAE) family have been identified and appear to be co-regulated by similar biosynthetic and metabolic pathways as AEA, but are conjugated to other fatty acids, such as docosahexaenoic acid, linoleic acid, oleic acid, stearic acid, or palmitic acid (Movahed et al., 2005; Dalle Carbonare et al., 2008). Although AEA was originally identified as an agonist for cannabinoid receptors, it is also capable of activating specialized, non-cannabinoid receptors, as are some of its congeners (Zygmunt et al., 1999; Smart et al., 2000; Ahern, 2003; Movahed et al., 2005). One example of these "non-cannabinoid" receptors are members of the transient receptor potential (TRP) channel superfamily (Raboune et al., 2014).

N-arachidonoyl ethanolamine (AEA) is a shared ligand between CB_1 and TRPV1, synthesized intracellularly in a PKC-mediated, calcium-dependent manner within the nervous system (Okamoto et al., 2007, 2009; Liu et al., 2008). Although not as potent as capsaicin, AEA is considered a full agonist at human TRPV1, as it displays the same maximal effect as capsaicin when administered exogenously in functional assays (Zygmunt et al., 1999; Smart et al., 2000). Additionally, it has been proposed that the activity of AEA at TRPV1 may be dependent on specific ambient temperatures, in a manner similar to capsaicin (Smart et al., 2000; Sprague et al., 2001). An earlier study suggested that 2-AG may have some effect on TRPV1 activity; but as a modulator of membrane potential, rather than as a driver of calcium mobilization (Zygmunt et al., 2013). As an agonist of CB_1, it is also possible that 2-AG may be involved in

the crosstalk between CB_1 and TRPV1, given the widespread tissue types that co-express these two receptors (Woo et al., 2008). Previously, we showed that capsaicin-stimulated TRPV1-transfected HEK cells drive an increase in production of 2-AG, providing further evidence of a molecular signaling link between the two (Leishman et al., 2017).

Here, we test the hypothesis TRPV1 activity plays an important modulatory role in the biosynthesis and metabolism of AEA, 2-AG and related lipids by analyzing their levels in TRPV1-transfected HEK cells stimulated by either capsaicin or by changes in temperature. We show that there are time and dose dependent effects with capsaicin stimulation and that many of these are mirrored with changes in temperature. Specifically, levels of 2-acyl glycerols, including 2-AG, increased and levels of NAEs, including AEA, decreased with increasing levels of capsaicin and temperature. The opposite effect in lipid production was found when TRPV1-HEK cells were cooled to 27°C in that levels of 2-acyl glycerols decreased and NAEs increased. These data provide a novel insight into the signaling mechanisms of TRPV1 activity and how this signaling system is linked to the eCB signaling system through the modulation of eCB endogenous ligands.

MATERIALS AND METHODS

Analysis of Lipids in TRPV1-HEK Cells After Stimulation by 100 nM, 1 μM, and 10 μM Capsaicin

hTRPV1-HEK cells, a kind gift from Merck (Whitehouse Station, NJ, United States), were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, United States), supplemented with 10% fetal bovine serum (Gibco, Dublin, Ireland), and 1% penicillin/streptomycin antibiotic (Thermo Fisher Scientific), as previously described (Leishman et al., 2017). Stock solutions of capsaicin were prepared in-house in 100% ethanol and stored at −80°C. Solutions of appropriate molarities were made immediately before experimentation in >99% DMSO. For each experiment, TRPV1-HEK cells at approximately 85% confluency were split into 12 individual T-25 cm^2 flasks. For each condition, the cells were gently washed twice with HEPES buffer [HEPES 0.025 mol, NaCl 0.140 mol, KCl 0.0027 mol, $CaCl_2$ 0.0018 mol, $MgCl_2$ 0.0005 mol, NaH_2PO_4 and D-glucose 0.05 mol (Sigma-Aldrich) in DI H_2O (prepared in-house)] that had been pre-warmed to 37°C in a water bath. The cells then incubated at 37°C, 5% CO_2 for 45 min in 4 mL of HEPES buffer. After the 45-min equilibration incubation, the buffer was replaced with 4 mL of the vehicle (0.1% DMSO) or capsaicin-buffer solution. The cells incubated in the capsaicin or vehicle solution for either 1 min or 5 min before lipid extraction was performed. HEPES was used to simulate our calcium-imaging protocols that validated that these cells drive calcium mobilization as previously published (Raboune et al., 2014).

After each flask's incubation period, the buffer solution was collected in a 15 mL centrifuge tube. Then, 2 mL of 100% HPLC-grade methanol (MeOH; Avantor Performance Materials, Inc., Center Valley, PA, United States) was added to the flask. The cells were harvested from the flasks using cell scrapers, and the cell solution gathered was added to the corresponding collected buffer. An additional 2 mL of MeOH was added to the flask to collect any remaining cells, which were also transferred to the 15 mL centrifuge tube. All tubes were spiked with 50 pmols deuterium-labeled *N*-arachidonoyl glycine (d_8NaGly; Cayman Chemical, Ann Arbor, MI, United States), prepared in MeOH, which served as an internal standard. After centrifugation, the supernatants were partially purified using C18 solid phase extraction columns (Agilent, Palo Alto, CA, United States) as previously described (Leishman et al., 2018).

Analysis of Lipids in TRPV1-HEK Cells After Changes in Temperature

60 mm × 15 mm glass petri dishes (Thermo Fisher Scientific) were coated with 50 μg/mL PDL prior to experimentation, to enhance cell adherence. Growth and maintenance of cells was as described above. Cells were grown to 85% confluence before experimentation.

Procedure for Temperature Ramp From 27 to 37°C

Cells in the temperature change condition were washed twice with HEPES-Tyrode buffer that had been pre-warmed to 27°C. The cells then incubated in 4 mL HEPES buffer at room temperature inside a cell culture hood for 45 min. In the hood, the cells were covered by a deep stainless-steel tray, accompanied by a beaker of hot water to provide humidity and assist in maintaining a steady temperature of 27°C, which was monitored by a digital wire probe thermometer. After the 45-min incubation period, each dish was transferred to a digital hot plate that had been pre-warmed to 27°C. The lid for the dish was removed and replaced by foil, which was fit snugly over the dish to ensure minimal heat loss and/or evaporation. A wire probe digital thermometer was positioned under the foil and submerged in the buffer solution approximately 2–3 mm from the cell layer to monitor the temperature change. The probe was situated this close in order to avoid mechanical stimulation, but close enough that the temperature being measured was as close as possible to the cell layer. The hot plate temperature was slowly raised using a manual dial while the temperature read-outs from the plate and the probe were monitored, ensuring a steady increase of 15 ± 2–4 s per degree. On average, it took 2.5 min for the buffer solution in each dish to reach 37°C. Upon reaching 37°C, the dish was removed from the hot plate, and the buffer solution was transferred to a conical tube. Subsequently, 2 mL of 100% HPLC-grade MeOH was added to the dish. The cells were then harvested from the dishes using cell scrapers and the cell solution was added to the tube containing the corresponding buffer solution. An additional 2 mL of MeOH was added to the dish to collect any remaining cells, which were also transferred to the conical tube.

Procedure for Temperature Ramp From 37 to 45°C

TRPV1-HEK cells were washed twice with HEPES buffer that had been pre-warmed to 37°C. The cells were then incubated

at 37°C, 5% CO_2 for 45 min in 4 mL of HEPES buffer, to allow for equilibration. After the 45-min incubation period, each dish was removed from the incubator and placed on a digital hot plate that was pre-warmed to 37°C. The lid for the dish was removed and replaced with a piece of aluminum foil. A wire probe digital thermometer was positioned under the foil and submerged in the buffer solution to monitor the temperature change. The hot plate temperature was slowly raised, while the temperature read-outs from the plate and the probe were monitored to ensure a steady increase of 15 ± 2–4 s per degree. On average, it took 2–2.25 min for each plate to reach 45°C. Upon reaching 45°C, the dish was removed from the hot plate and the buffer solution was transferred to a conical tube. Cells were harvested from each dish using HPLC-grade MeOH, as described above.

The cells in the control condition for each temperature ramp group were washed twice with HEPES-Tyrode buffer, which was pre-warmed to 37°C, and were then incubated under standard cell culture incubator conditions in 4 mL HEPES buffer for 45 min. The temperature of the solution in each dish was taken after each 45-min incubation period, to ensure the temperature had been maintained at 37°C, ±1°C. The buffer solution was then transferred to a 15 mL conical centrifuge tube. Cells were harvested from each dish using HPLC-grade MeOH, as described above for the temperature-change condition.

To test the hypothesis that the effects of the change in temperature were dependent on TRPV1 activation, cells were treated with 500 nM of the TRPV1 antagonist, iRTX (iodo-resiniferatoxin) prior to the 45-min incubation period and then the identical procedure as outlined above was performed.

All cell solutions from each condition were spiked with 50 pmols d_8 N-arachidonoyl glycine (NAGly), which served as an internal standard. The spiked samples were

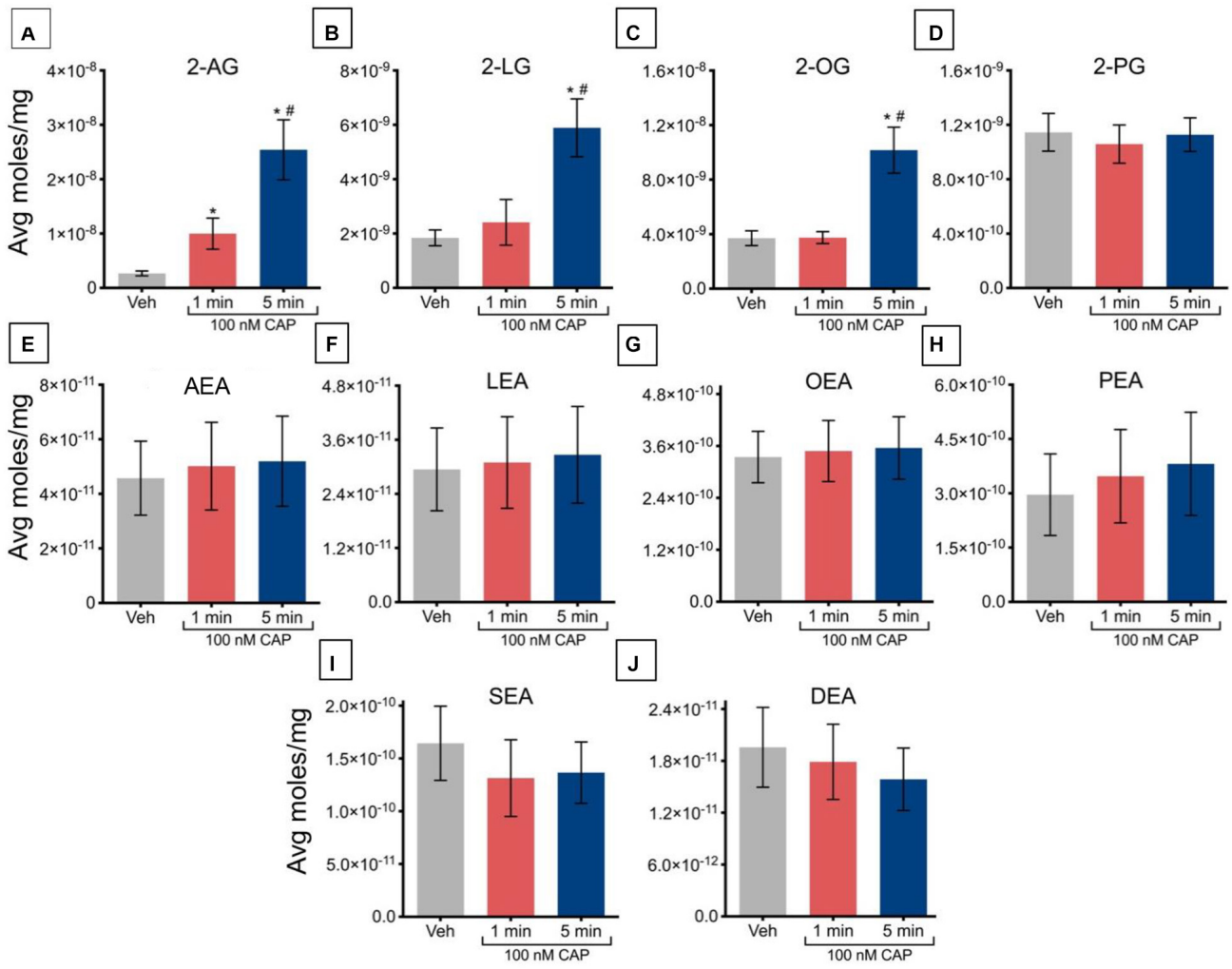

FIGURE 1 | Changes in lipid levels of 2-acyl glycerols **(A–D)** and *N*-acyl ethanolamines **(E–J)** after TRPV1-HEK293 cells incubated in 100 nM capsaicin (CAP) for 1 min or 5 min. Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min or 5 min to vehicle is indicated by * ($p \leq 0.05$). Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min of incubation vs. 5 min of incubation is indicated by # ($p \leq 0.05$). Data were averaged over 12 total data points per condition ($n = 3$ individual experiments) and presented as average moles/mg (y-axis).

centrifuged at 3,000 rpm for 15 min at 24°C. After centrifugation, the supernatants were partially purified using C18 solid phase extraction columns as previously described (Leishman et al., 2018).

HPLC/MS/MS Quantification and Analysis

Analysis of the samples was carried out using an Applied Biosystems API 3000 triple quadrupole mass spectrometer with electrospray ionization (Applied Biosystems Sciex, Foster City, CA, United States) in MRM mode as previously described (Leishman et al., 2017, 2018). Analyst 1.4.2 software (Applied Biosystems) was used to quantify the HPLC/MS/MS data. The software generated chromatograms by determining the retention times of each analyte with a programmed parent ion and a fragmentation ion mass. Quantification of the analytes were calculated by coupling the calibration curves of the synthetic standards and the recovery adjustments, determined by the d_8NAGly standard.

Concentrations of each analyte were reported as moles per gram, after measuring the weights of each dried cell pellet. Recoveries were calculated by measuring the amount of d_8NaGly remaining in each sample after lipid extraction and comparing those values to the recovery standard that represented 100% recovery. IBM SPSS Statistics 23 (IBM, Armonk, NY, United States) was used to determine statistical differences between conditions, implementing one-way ANOVAs. Data comparisons from the capsaicin incubations were made across all three conditions (vehicle, 1-min incubations, and 5 min incubations). A 95% confidence interval was calculated for the means, and statistical significance was defined as $p \leq 0.05$. For temperature experiments, comparisons were made between the vehicle conditions and the heat-challenged conditions using SPSS one-way ANOVAs. A 95% confidence interval was calculated for the means, and statistical significance was defined as $p \leq 0.05$.

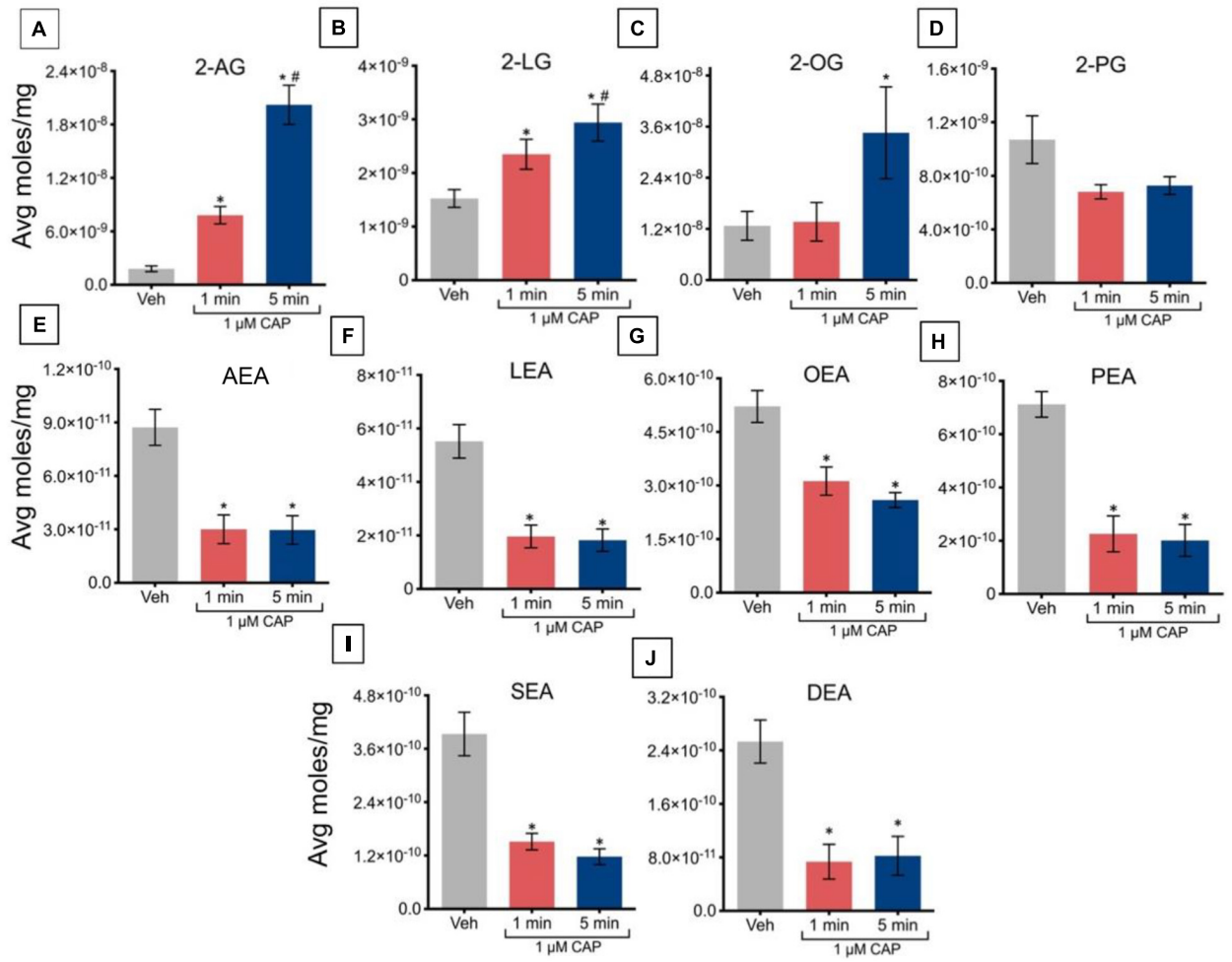

FIGURE 2 | Changes in lipid levels of 2-acyl glycerols **(A–D)** and *N*-acyl ethanolamines **(E–J)** after TRPV1-HEK293 cells incubated in 1 μM capsaicin (CAP) for 1 min or 5 min. Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min or 5 min to vehicle is indicated by * ($p \leq 0.05$). Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min of incubation vs. 5 min of incubation is indicated by # ($p \leq 0.05$). Data were averaged over 8 total data points per condition ($n = 2$ individual experiments) and presented as average moles/mg (*y*-axis).

GraphPad Prism 7.0 (GraphPad, San Diego, CA, United States) was used to generate graphs.

RESULTS

Lipidomics Analysis of TRPV1-HEK Cells After Capsaicin Stimulation

The following lipids were detected and analyzed in all samples: 2-arachidonoyl glycerol (2-AG), 2-linoleoyl glycerol (2-LG), 2-oleoyl glycerol (2-OG), 2-palmitoyl glycerol (2-PG), AEA, *N*-linoleoyl ethanolamine (LEA), *N*-oleoyl ethanolamine (OEA), *N*-palmitoyl ethanolamine (PEA), *N*-stearoyl ethanolamine (SEA), and *N*-docosahexaenoyl ethanolamine (DEA). Data are analyzed by time; however, they are presented independently by concentration of capsaicin and not as a direct comparison of concentrations. This is because these are data from independent experiments and not on the same cells, which precludes the ability for reliable direct comparisons of absolute lipid concentrations with capsaicin concentrations.

Capsaicin-Induced Changes in Lipids Levels After TRPV1-HEK Cells Incubated in 100 nM Capsaicin for 1 min or 5 min

Incubation of TRPV1-HEK293 cells with 100 nM capsaicin for 1 min caused a significant increase in 2-AG concentrations but had no significant effect on the other 2-acyl-glycerols measured. Incubation with 100 nM capsaicin for 5 min drove significant increases in 2-AG, 2-LG, and 2-OG, relative to vehicle (**Figures 1A–C**). Levels of 2-AG, 2-LG, and 2-OG were significantly higher after 5 min than after 1 min, indicating that regulation of these lipids may be time-dependent at this

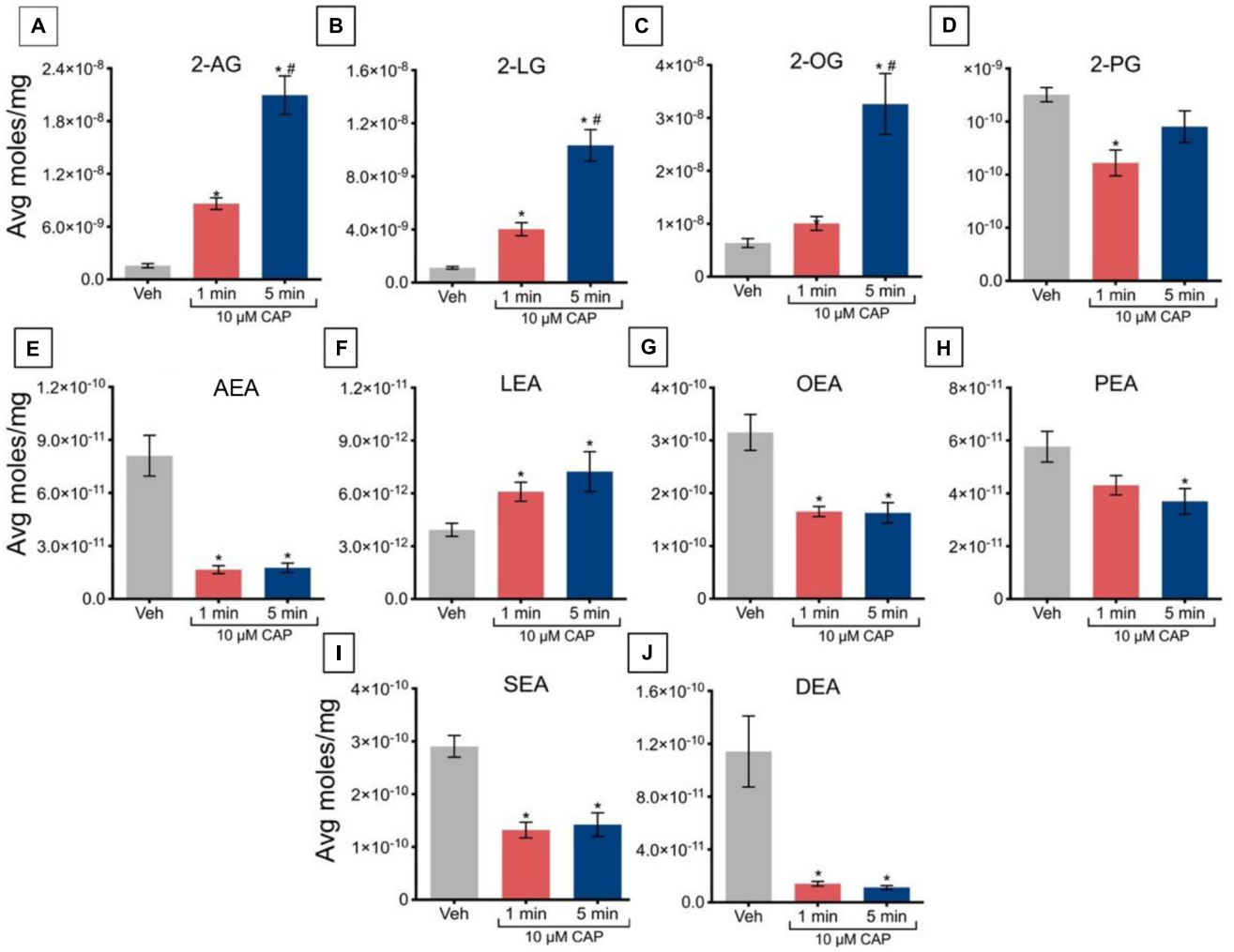

FIGURE 3 | Changes in lipid levels of 2-acyl glycerols **(A–D)** *N*-acyl ethanolamines **(E–J)** after TRPV1-HEK293 cells incubated in 10 μM capsaicin (CAP) for either 1 min or 5 min. Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min or 5 min to vehicle is indicated by * ($p \leq 0.05$). Significant changes in lipid levels when comparing capsaicin-induced changes after 1 min of incubation vs. 5 min of incubation is indicated by # ($p \leq 0.05$). Data depicted in each graph were averaged over 16 total data points per condition ($n = 4$ individual experiments) and presented as average moles/mg (*y*-axis).

concentration of capsaicin (**Figures 1A–C**). There were no significant changes seen in levels of 2-PG at both 1 min and 5 min (**Figure 1D**). Likewise, no changes were measured in any of the NAEs at either time point (**Figures 1E–J**).

Capsaicin-Induced Changes in Lipids Levels After TRPV1-HEK Cells Incubated in 1 μM Capsaicin for 1 min or 5 min

Incubation of TRPV1-HEK293 cells with 1 μM capsaicin for 1 min caused a significant increase in levels of 2-AG and 2-LG, relative to vehicle, with no changes in levels of 2-OG or 2-PG (**Figures 2A–D**). Capsaicin incubation for 5 min significantly increased levels of 2-AG, 2-LG, and 2-OG, but not 2-PG, relative to vehicle. The increased levels of 2-AG and 2-LG after 5 min of incubation was significantly higher than levels at 1 min, indicating that regulation of these lipids is time-dependent at this concentration of capsaicin. Incubation of TRPV1-HEK293 cells with 1 μM capsaicin for 1 min and 5 min significantly decreased levels of all NAEs, including the eCB anandamide. In contrast to effects on 2-AG and 2-LG, which were time-dependent, the effects on NAEs were independent of time, showing similar decreases at both time points (**Figures 2E–J**).

Capsaicin-Induced Changes in Lipids Levels After TRPV1-HEK Cells Incubated in 10 μM Capsaicin for 1 min or 5 min

Incubation of TRPV1-HEK293 cells with 10 μM capsaicin for 1 min significantly increased levels of 2-AG, 2-LG, and 2-OG, relative to vehicle (**Figures 3A–C**); however, this incubation significantly decreased levels of 2-PG (**Figure 3D**). At 5 min, levels of 2-AG, 2-LG, and 2-OG remained elevated relative to vehicle, but no significant changes were observed in the levels of 2-PG. Effects on 2-acyl glycerols were time-dependent, with significantly higher levels of 2-AG, 2-LG, and 2-OG after 5 min of incubation versus 1 min. Incubation of TRPV1-HEK293 cells with 10 μM capsaicin for 1 min and 5 min significantly reduced levels of AEA, OEA, SEA, and DEA, relative to vehicle (**Figures 3E,G,I,J**), whereas LEA levels increased relative to vehicle (**Figure 3F**), while levels of PEA remained unchanged (**Figure 3H**). No significant effects of time were detected for

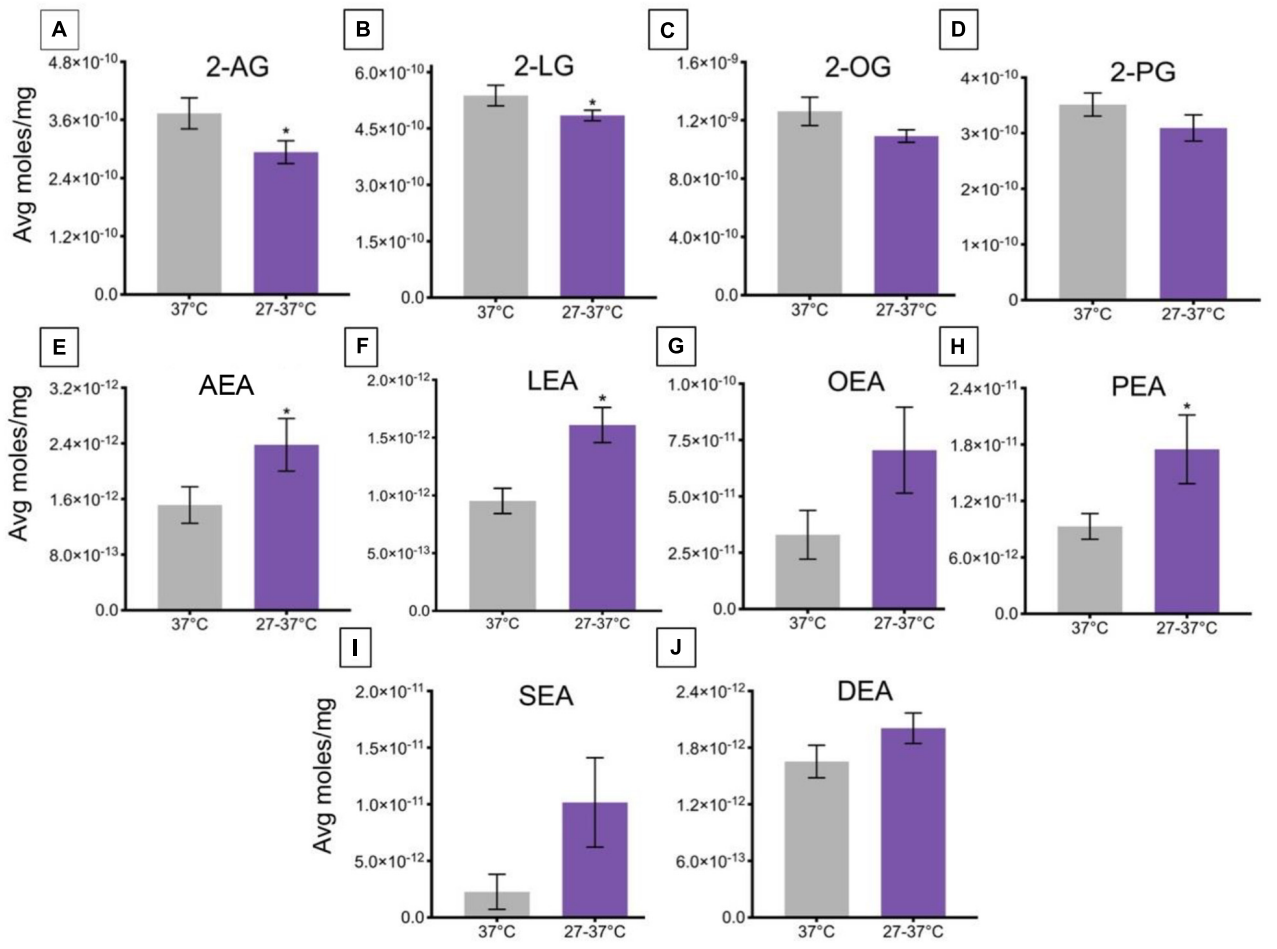

FIGURE 4 | Changes in lipid levels of 2-acyl glycerols **(A–D)** and *N*-acyl ethanolamines **(E–J)** upon an increase in temperature from 27 to 37°C. Significant temperature-induced changes after TRPV1-HEK293 cells were exposed to a change in ambient temperature from 27 to 37°C is indicated by * ($p \leq 0.05$). Data depicted in each graph were averaged over 15 total data points per condition ($n = 3$ individual experiments) and are presented as average moles/mg (*y*-axis).

NAEs in these experiments, in that levels of NAEs did not further change at 5 min versus 1 min (**Figures 3E–J**).

Lipidomics Analysis of TRPV1-HEK Cells After Changes in Temperature

Temperature-Induced Changes in Lipid Levels After Change From 27 to 37°C in TRPV1-HEK Cells

A change in ambient temperature to 27°C for 45 min and then a ramp to 37°C over ∼2 min caused a significant decrease the levels of 2-AG and 2-LG, relative to controls maintained at a temperature of 37°C (**Figures 4A,B**). Levels of 2-OG and 2-PG were unaffected by this change in temperature (**Figures 4C,D**). This change in temperature drove a significant increase in levels of AEA, LEA, and PEA, relative to a maintained temperature of 37°C (**Figures 4E,F,H**), whereas no significant changes occurred in levels of OEA, SEA, and DEA (**Figures 4G,I,J**).

Temperature-Induced Changes in Lipid Levels After Change From 37 to 45°C in TRPV1-HEK Cells

A change in ambient temperature from 37 to 45°C over ∼2.5 min caused a significant increase in levels of 2-AG, 2-LG, and 2-OG (**Figures 5A–C**), whereas there was no significant change in levels of 2-PG (**Figure 5D**). This change in temperature significantly decreased levels of AEA, LEA, and DEA (**Figures 5E,F,J**), whereas the change in temperature caused a significant increase in lipid levels of SEA (**Figure 5I**). No significant changes were measured in levels of OEA or PEA (**Figures 5G,H**).

Temperature-Induced Changes in Lipid Levels After Change From 37°C–45°C in iRTX-Treated TRPV1-HEK Cells

To determine the extent to which TRPV1 activation during heat ramp stimulation drives changes in these lipids the same testing protocol was used on HEK TRPV1 cells with a pre-treatment of 500 nM iRTX, a potent TRPV1 antagonist. **Table 1** shows that there is only one significant change in any of the lipids measured. 2-LG was still significantly increased in the presence of iRTX; however, the magnitude of effect is much reduced. These data support the involvement of TRPV1 in the modulation of these lipids.

DISCUSSION

The detection of ambient temperature is vital for organisms that seek temperatures that are optimal for life, and to avoid any potentially damaging environmental conditions. The activity

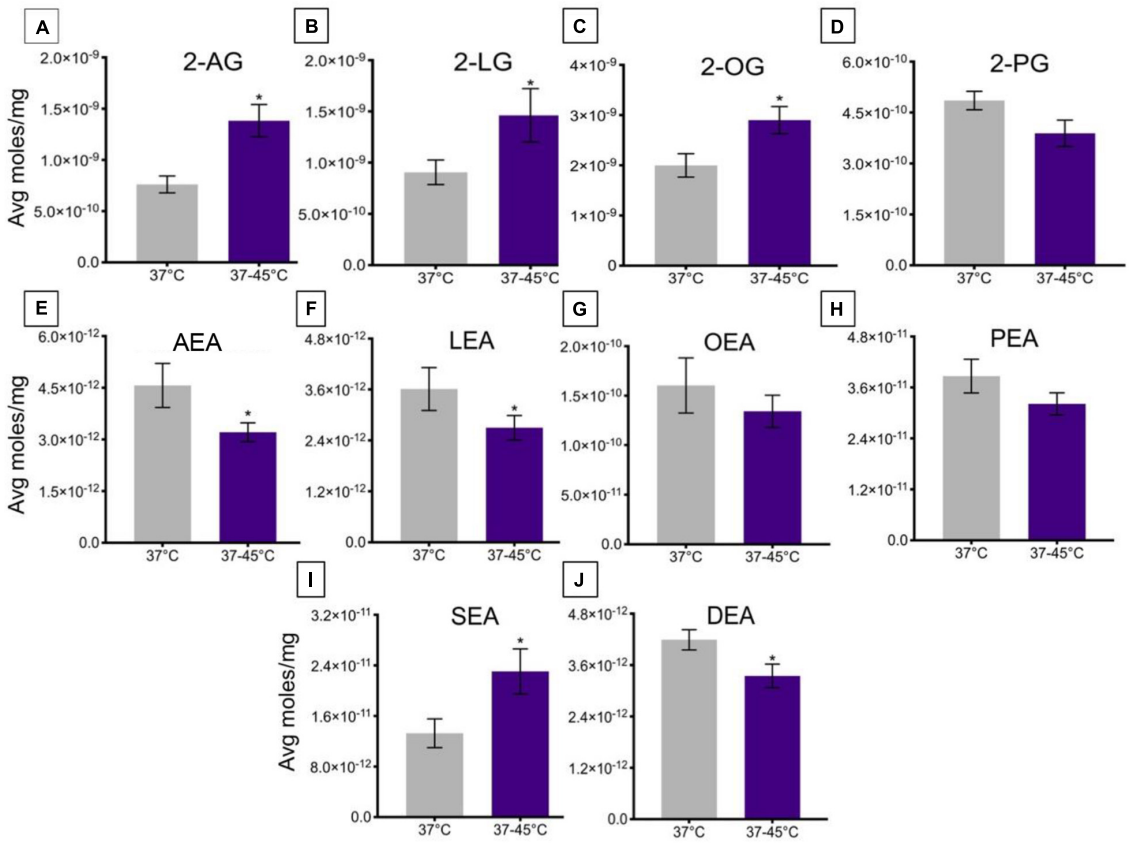

FIGURE 5 | Changes in lipid levels of 2-acyl glycerols **(A–D)** and *N*-acyl ethanolamines **(E–J)** upon an increase in temperature from 37 to 45°C. Significant temperature-induced changes after TRPV1-HEK293 cells were exposed to a change in ambient temperature from 37 to 45°C, is indicated by * ($p \leq 0.05$). Data depicted in each graph were averaged over 15 total data points per condition ($n = 3$ individual experiments) and are presented as average moles/mg (*y*-axis).

TABLE 1 | Levels of lipids in HEK-TRPV1 cells after heat ramp from 37–45°C in the presence of the TRPV1 antagonist, iRTX.

	500 nM iRTX	Mean	SEM
2-AG	37°C	3.07E-10	2.25E-11
	37–45°C	3.39E-10	3.40E-11
2-LG	37°C	3.43E-10	2.34E-11
	37–45°C	**4.44E-10**	3.00E-11
2-OG	37°C	5.88E-09	2.45E-10
	37–45°C	6.90E-09	5.22E-10
2-PG	37°C	4.56E-10	3.63E-11
	37–45°C	4.69E-10	3.71E-11
AEA	37°C	7.56E-11	6.31E-12
	37–45°C	6.18E-11	5.26E-12
LEA	37°C	6.53E-10	3.87E-11
	37–45°C	7.40E-10	3.32E-11
OEA	37°C	5.16E-11	4.01E-12
	37–45°C	6.01E-11	3.02E-12
PEA	37°C	2.73E-10	1.87E-11
	37–45°C	3.21E-10	2.04E-11
SEA	37°C	4.04E-10	2.77E-11
	37–45°C	3.56E-10	2.99E-11
DEA	37°C	1.83E-10	1.22E-11
	37–45°C	2.27E-10	2.05E-11

All lipids levels are expressed as mols/gram dried cell pellet weight. Bold indicates $p < 0.05$. See text for abbreviations.

of thermoTRPs facilitates the ability of organisms to adjust other physiological functions and behaviors, upon detecting an environmental cue such as temperature (Castillo et al., 2018). This type of regulation can ensure that prolonged periods of core body temperature elevation or depression are minimized, preserving a hospitable environment for necessary enzymatic reactions to occur (Romanovsky, 2007). As a thermoTRP, a notable characteristic of TRPV1 channels is that they are responsive to exogenous and endogenous chemical stimuli (capsaicin) and physical stimuli, such as heat (Caterina et al., 1997; Clapham, 2003). This characteristic ability to respond to such a range of stimuli categorizes TRPV1 as a polymodal receptor. As a polymodal molecular integrator, TRPV1 has evolved an ability to have several mechanisms/pathways by which it can transduce information gathered from a diverse set of ligands (Julius and Basbaum, 2001; Raboune et al., 2014). Our current data suggests that TRPV1 activation drives changes in eCB and related lipid mediators by both chemical and thermal stimuli. These data presented in this study provide a novel insight into the dynamic molecular signaling systems involved in the thermoregulatory system at the level of TRPV1 activation.

The surprising finding that increases in levels of 2-AG corresponded with decreases in levels of AEA with both capsaicin and heat in a time range of only 1–5 min suggests a potential molecular link in their biosynthesis and/or metabolism. It is a typical assumption when measuring an increase in a signaling molecule to infer that the metabolism has been inhibited. Likewise, there is often the inference of an upregulation in metabolism when the levels of a molecule decrease. The other side of the equation rests with the regulation of the biosynthesis wherein increases in a molecule can denote an upregulation of this biosynthesis process and a downregulation with decreases. Both processes may also be at play in the rapid modulation to a cellular event, especially when two divergent pathways for biosynthesis share a common precursor.

While AEA and 2-AG both contain arachidonic acid (AA), their biosynthetic pathways are not through conjugation of free AA; instead, they are both produced through enzymatic modulation of AA-containing membrane phospholipids (Liu et al., 2008; Okamoto et al., 2009; Leishman et al., 2016; Mock et al., 2020). Three separate synthesis pathways have been hypothesized to generate AEA (Ueda et al., 2013). Each of these pathways require availability of *N*-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is the product of the membrane phospholipid, phosphatidylethanolamine (PE) through an acyl transfer from phosphatidylcholine (PC) via an *N*-acyl transferase (NAT) enzyme in a calcium-dependent manner (Di Marzo et al., 1994). More recently, the Cravatt group isolated the serine hydrolase PLA2G4E as a specific NAT in CNS tissue that produces the NAPE species that are precursors to NAEs including AEA (Ogura et al., 2016). NAPE-PLD cleaves the NAPE glycerophosphate group via hydrolysis, yielding NAEs (Di Marzo et al., 1994; Leishman et al., 2016). The second pathway first requires conversion of NAPE into lyso-NAPE, which occurs when the two NAPE-specific phospholipases (A1/A2) cleave the molecule into lyso-NAPE and glycerophosphoanandamide. From here, a specific PLD can hydrolyze the intermediate molecule to yield NAEs (Simon and Cravatt, 2006). The third pathway requires the use of a NAPE-specific PLC to cleave NAPE and acquire phospho-NAE. Dephosphorylation of this intermediate using a lipid phosphatate yields NAEs (Liu et al., 2008).

Biosynthesis of 2-AG is like AEA in that both are derived from membrane phospholipids. Diacylglycerols (DAGs) are analogous to NAPEs in that they are likewise generated by multiple pathways (Stella et al., 1997; Sugiura et al., 2006). One of the pathways to generate DAGs is through the hydrolysis of phosphatidic acid (PA), which can be generated from PC enzymatically via phospholipase D (PLD) or phospholipase C (PLC), and a third is through the direct hydrolysis of triacylglycerols (Stella et al., 1997; Sugiura et al., 2006). DAGs are then hydrolyzed via diacylglycerol lipase (DAGL) into monoacylglycerols (MAGs) such as the 2-acyl glycerols including the eCB, 2-AG (Sugiura et al., 2006).

It is important to note that PC is a precursor to both AEA and 2-AG. We hypothesize that the regulation of PC metabolism may be a factor in shunting toward one pathway over the other. The evidence that lower temperatures drive the opposite effect with increases and NEAs and decreases in 2-acyl glycerols further supports this hypothesis. **Figure 6** outlines the specific pathways that involve PC in both NAE and 2-acyl glycerol biosynthesis. We hypothesize that TRPV1 activity is driving a shift in the amount of PC being shunted in the PLD pathway toward DAGs, which is creating a precursor deficit for NAPE and then NAE production. As with all hypotheses, this one comes with caveats.

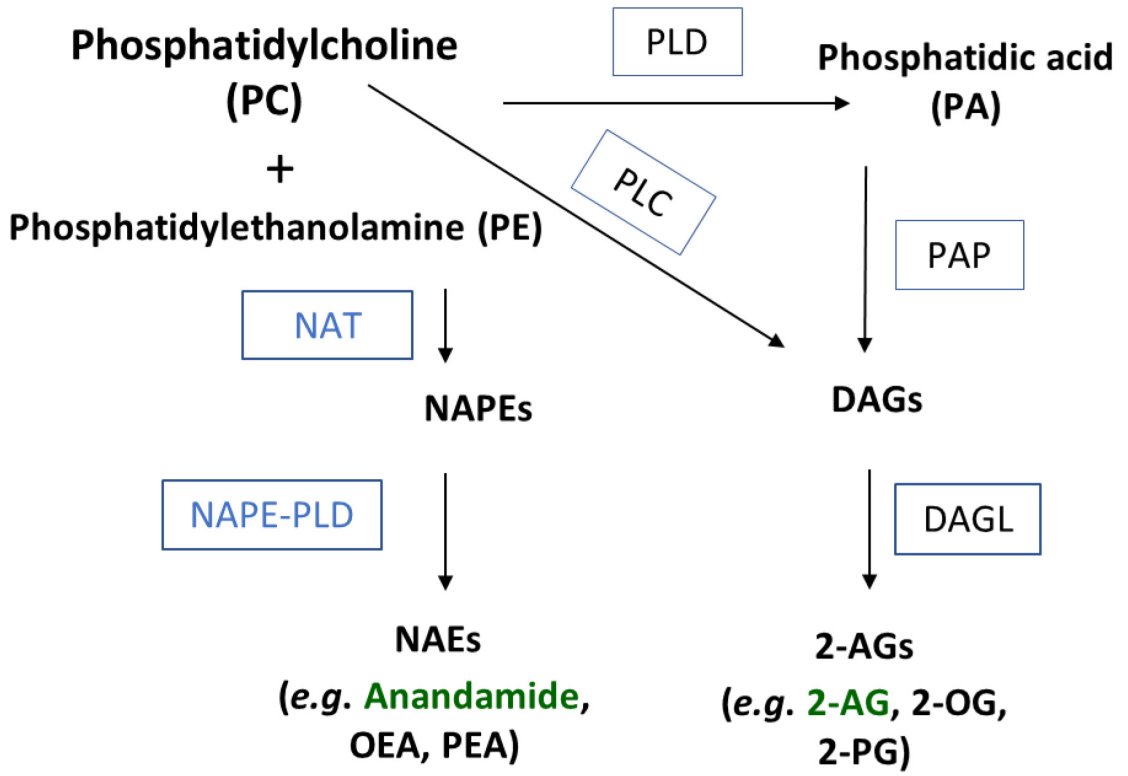

FIGURE 6 | Schematic of NAE and 2-acyl glycerol biosynthesis through a phosphatidylcholine (PC) precursor. Substrates and products in bold black. Enzymes abbreviated in boxes. NAT, N-acyl transferase; PLD, phospholipase D; PLC, phospholipase C; PA, phosphatidic acid; NAPE, N-acyl phosphatidylethanolamine; PAP, phosphatidic acid phosphohydrolase; DAG, diacylglycerol; DAGL, diacylglycerol lipase.

While AEA and 2-AG as well as OEA and 2-OG have a very predictable inverse relationship with TRPV1 activity at 1 and 10 μM capsaicin and heat, the linoleoyl derivatives (2-LG and LEA) only show this tight relationship with 1 μM capsaicin and heat. Whereas PEA and 2-PG do not show this same relationship, wherein 2-PG and PEA are less effected by capsaicin and heat overall, but both decrease with 10 μM capsaicin. The standards for 2-stearoyl glycerol and 2-docosahexaenoyl glycerol were not available for analysis during these experiments, so we were unable to ascertain the relationships between the species. However, it is important to note that SEA increased with heat instead of decreased like the rest of the NAEs; therefore, it is likely that there are multiple ways that these signaling molecules are regulated with temperature. What the purpose of these lipid signaling molecules is another important aspect of study for future experiments.

CONCLUSION

Here, we demonstrate that activity of TRPV1 via capsaicin and temperature drives increases in levels of 2-acyl glycerols and decreases in NAEs in a time and dose dependent manner. These novel findings provide a unique insight in the signaling systems involving TPRV1 activity and in lipid signaling molecule regulation by temperature. The data further illustrate that the modulation of intracellular calcium is only one of the many signaling cascades that are relevant to TRPV1 signaling.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MM, KS, and AA performed the TRPV1 HEK cell experiments (MM and KS with capsaicin, MM and AA with temperature). EL performed the mass spectrometric analysis. MM and KS performed the statistical analyses. MM, EL, and HB shared contribution to the manuscript. MM and HB conceived of the idea and developed methods for experimental implementation. All authors contributed to the article and approved the submitted version.

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