INVOLVEMENTS OF TRP CHANNELS, OXIDATIVE STRESS AND APOPTOSIS IN NEURODEGENERATIVE DISEASES

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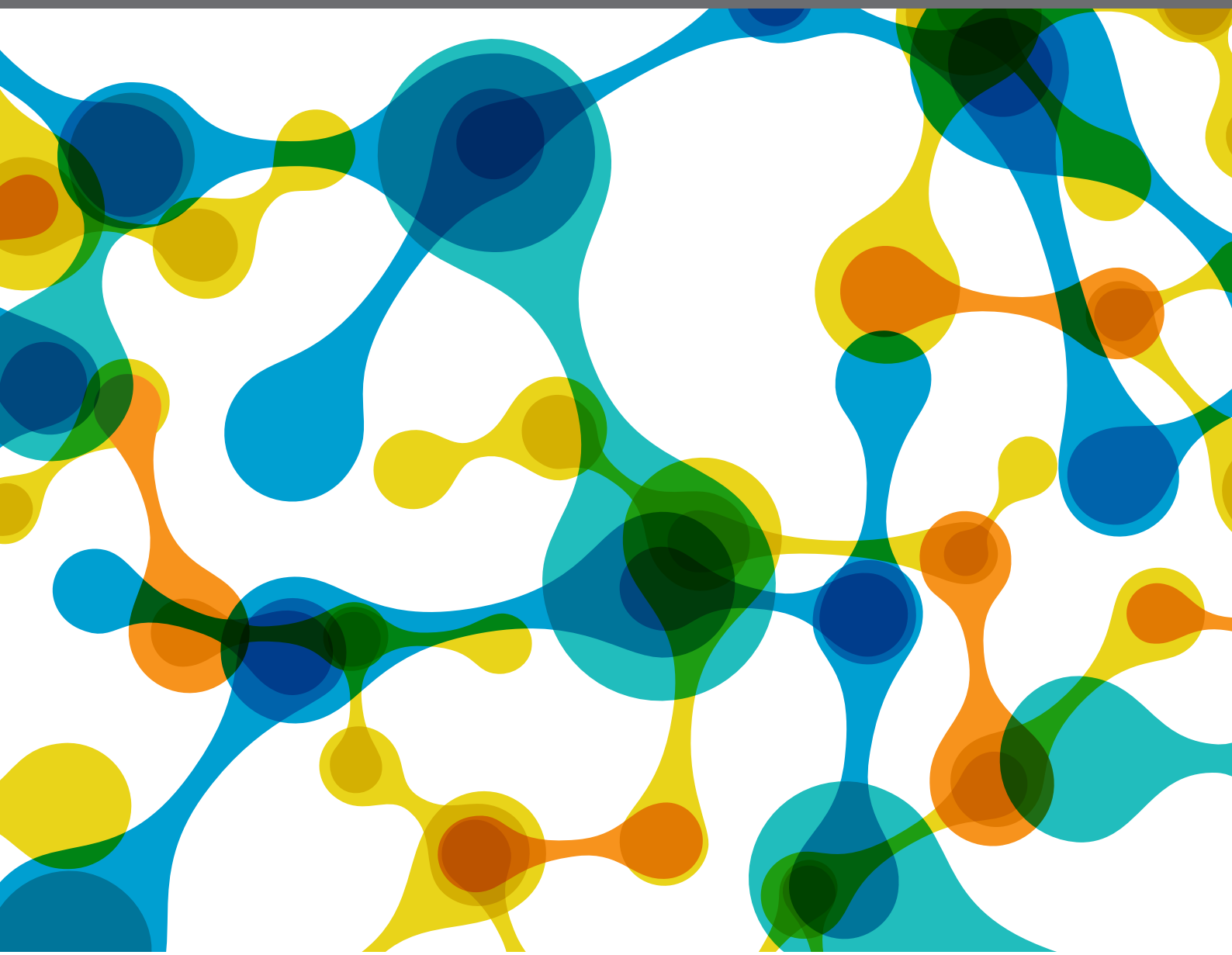

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INVOLVEMENTS OF TRP CHANNELS, OXIDATIVE STRESS AND APOPTOSIS IN NEURODEGENERATIVE DISEASES

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EDITORIAL
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Editorial: Involvements of TRP Channels, Oxidative Stress and Apoptosis in Neurodegenerative Diseases

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Editorial on the Research Topic

Involvements of TRP Channels, Oxidative Stress and Apoptosis in Neurodegenerative Diseases

The oxygen free radicals generated during metabolism can cause cumulative oxidative damage to nucleic acids, lipids, and protein, resulting in structural degeneration, apoptosis, functional decline, and age-related degenerative diseases involving the cardiovascular, endocrine, neurologic, immune, respiratory, gastrointestinal, and reproductive systems. The transient receptor potential (TRP) protein superfamily is composed of several cation-permeable channels that are widely distributed in mammalian cells. TRP channels can be divided into six subfamilies that are dependent on their sequence identity. These channels play a crucial role in the regulation of oxidative stress and should be considered as likely targets for the treatment of age-related neurodegenerative diseases associated with chronic oxidative stress, decreases in metabolic regulation, and cell viability.

At least three subfamilies of TRP channels are associated with oxidative stress. These include the TRPV subfamily (characterized by chemical, mechanical, and physical stimuli); the TRPC subfamily (characterized by receptor operated calcium entry channels); and the TRPM subfamily (with roles in cell proliferation and death).

The articles in this Research Topic review current thinking with regards to the role of TRP channels in oxidative stress, aging, and neurodegenerative diseases, and highlight the involvement of these channels in the pathobiology of selected neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. Therapeutic strategies that modulate the activation of specific TRP channels may be beneficial for attenuating cellular damage due to oxidative stress in neurological disorders.

The first article by Duitama et al. provides a broad perspective of the role of TRP-dependent mechanism(s), which can mediate pain sensation in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. It discusses the therapeutic approaches available to palliate pain and neurodegenerative symptoms throughout the regulation of these channels. TRP channels are postulated to be involved in the pathobiology of neurodegenerative diseases and pain nociception through modulation of intracellular Ca2+ signaling, oxidative stress, and the production and release of inflammatory mediators.

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The second article by Hong et al. provides an overview of the role of TRP channels as potential targets for neurodegenerative diseases. The review provides an up-to-date summary of the involvement of TRP channels in neurological disorders and discusses recent work on the development of therapeutic candidates for neurodegenerative disorders targeting TRP channels. As the structures of TRP channels are beginning to be elucidated using cryo-electron microscopy, TRP channel antagonists are beginning to be developed to mitigate symptoms in neurodegenerative disorders.

Some TRP channels can be activated downstream of NMDA receptor activation and contribute to reduced synaptic transmission, excitotoxicity, and cell death. The original research article by Doğan et al. examined the interaction between the purinergic receptors (PzX7Rs) and the NMDA receptor in a rat model for absence epilepsy. The findings of this study demonstrate that P2X7Rs play an independent role in the formation of absence seizures. More specifically, treatment with a P2X7Rs agonist lowered the antioxidant activity of the NMDA receptor antagonist memantine whereas the agonist of P2X7Rs lowered the anticonvulsant action of memantine, suggesting a partial interaction between P2X7 and NMDA receptors with potential implications for TRP channels in absence epilepsy. Ru et al. showed that tea polyphenols can attenuate methamphetamine-induced neuronal cell loss by protecting against apoptosis and DNA damage in PC12 cells. Furthermore, ubiquitination appears to be regulated to some extent by TRP channels. The systematic review by Momtaz et al. highlights the involvement of the ubiquitin-proteosome pathway in neurodegenerative diseases and how natural products can interfere with this complex regulatory system at various stages of the disease.

It is well-established that TRPV1 is involved in oxidative stress-induced pain and neuronal injury, associated with neuropathy reported in neurodegenerative diseases and glaucomatous optic neuropathy. An original research article by McGrady et al. provides renewed insight into the role of TRPV1 on optic nerve axon excitability in an animal model for glaucoma. The study found that in the absence of TRPV1, energy demand following intraocular pressure-related stress is increased, leading to alterations to axon transport and maintain optimal voltage-dependent axon signaling. Therefore, in glaucoma, TRPV1 may modulate the expression of voltage-gated sodium channels in neurons exposed to stress to maintain axonal excitability and preserve energy resources.

The TRPV1 channel has also been proposed to act as a steroid receptor to protect tissues against oxidant stress. Ramirez-Barrantes et al. demonstrated that TRPV1 is necessary for 17β-estradiol to improve metabolic function in vulnerable cells. As well, 17β-estradiol but not 17α-estradiol increases the effect of TRPV1 single channel activity leading to increased open probability. The protective effects of 17β-estradiol were found to be independent of estrogen receptor pathway activation, membrane started and stereospecific. These findings suggest that TRPV1 is a 17β-estradiol-activated ionotropic membrane receptor coupling that can influence mitochondrial function and cell viability.

The TRPCs serve as a redox-sensitive ion channel that can play a prominent role in neurodegeneration. Maria-Ferreira et al. review the role of TRPCs in the pathogenesis of Parkinson's disease. The review discusses the role of TRPCs in the various biochemical and molecular processes associated with the pathobiology of Parkinson's disease that consequently led to increased oxidative stress, impaired dopaminergic signaling, and apoptosis.

The TRPM family member TRPM2 has several physiologic isoforms that are present in a variety of cell types and respond to oxidant stress, pro-inflammatory mediators such as TNFα, and β-amyloid peptide. The perspective article by Wang et al. discusses the important role of TRPM2 in Alzheimer's diseases, citing recent studies using divergent cell systems and techniques including overexpression, channel depletion or inhibition, and calcium chelation. The review also provides a causative relationship between exposure to particular matters, TRPM2 channel activation, Alzheimer's disease risk, and age-related brain damage. Therapeutic strategies targeting the TRPM2 channel represent a potential strategy for lowering the risk of exposure to particular matters in Alzheimer's disease.

The mini review article by Santoni et al. provides an overview of the role of endosome/lysosome Ca^{2+} permeable channels known as mucolipins (TRPML) in the regulation of calcium signaling associated with oxidative stress induced oxidative stress. TRPMLs represent a key candidate for the treatment of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, ALS, mucolipidosis type IV, and Neimann-Pick disease.

TRPM7 has been strongly implicated in the regulation of intracellular Ca^{2+} influx and anoxic neuronal cell death. In the last article, Sun et al. demonstrated in a neuroblastoma cell line that treatment with the β-adrenergic receptor (β-AR) agonist isoproterenol could enhance Mg^{2+} influx and cell survival in the presence of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and potentiate TRPM7 channel activation that leads to an increase in intracellular Mg^{2+} levels. The effect of isoproterenol was reversed following the addition of 2APB, a known TRPM7 channel antagonist. Moreover, TRPM7 expression and function neurotoxins were inhibited following exposure to neurotoxins. These findings suggest a positive role for β-AR in activating TRPM7 channels, modulating Mg^{2+} homeostasis, and promoting the survival of SH-SY5Y cells following exposure to potent neurotoxins.

The sum of the articles adds to our recent work in the area of TRP channel signaling and neurodegenerative diseases. The articles in this special issue provide a summary of the multiple roles of TRP channels in the pathogenesis of neurodegenerative disorders and provide emerging evidence for TRP channels as a target for the development of therapeutic agents to improve neurological dysfunction.

AUTHOR CONTRIBUTIONS

NB wrote the manuscript. TS and MN edited the manuscript. All authors reviewed and approved the final manuscript.

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ORIGINAL RESEARCH
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Tea Polyphenols Attenuate Methamphetamine-Induced Neuronal Damage in PC12 Cells by Alleviating Oxidative Stress and Promoting DNA Repair

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DNA integrity plays a crucial role in cell survival. Methamphetamine (METH) is an illegal psychoactive substance that is abused worldwide, and repeated exposure to METH could form mass free radicals and induce neuronal apoptosis. It has been reported that free radicals generated by METH treatment can oxidize DNA and hence produce strand breaks, but whether oxidative DNA damage is involved in the neurotoxicity caused by METH remains unclear. Tea polyphenols exert bioactivities through antioxidant-related mechanisms. However, the potential neuroprotective effect of tea polyphenols on METH-induced nerve cell damage and the underlying mechanism remain to be clarified. In this study, oxidative stress, DNA damage, and cell apoptosis were increased after METH exposure, and the expressions of DNA repair-associated proteins, including the phosphorylation of ataxia telangiectasia mutant (p-ATM) and checkpoint kinase 2 (p-Chk2), significantly declined in PC12 cells after high-dose or long-time METH treatment. Additionally, tea polyphenols could protect PC12 cells against METH-induced cell viability loss, reactive oxide species and nitric oxide production, and mitochondrial dysfunction and suppress METH-induced apoptosis. Furthermore, tea polyphenols could increase the antioxidant capacities and expressions of p-ATM and p-Chk2 and then attenuate DNA damage *via* activating the DNA repair signaling pathway. These findings indicate that METH is likely to induce neurotoxicity by inducing DNA damage, which can be reversed by tea polyphenols. Supplementation with tea polyphenols could be an effective nutritional prevention strategy for METH-induced neurotoxicity and neurodegenerative disease.

Keywords: tea polyphenols, methamphetamine, apoptosis, DNA damage, oxidative stress

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INTRODUCTION

The synthetic central stimulant methamphetamine (METH) is widely abused in the world. Clinical toxicology surveys have shown that METH can induce neuronal damage in abusers (Gold et al., 2009; O'Dell and Marshall, 2014; Ren et al., 2016). In line with these clinical reports, numerous animal studies have revealed that METH can induce long-term damage to

dopaminergic neurons and cause cell apoptosis (Li et al., 2017; Dang et al., 2018). The neurotoxicity of METH is mainly believed to be dependent on its structural similarity to dopamine (DA), which makes it easier for it to enter dopaminergic neurons *via* the DA transporter (DAT) and causes DA to be over-released into the cytoplasm, where DA can undergo auto-oxidation rapidly to form a large number of toxic materials such as superoxide radicals, resulting in oxidative stress, decreased mitochondrial membrane potential ($\Delta\Psi m$), and neuronal apoptosis (Krasnova and Cadet, 2009). METH treatment may also lead to a decline in superoxide dismutase (SOD) and glutathione peroxidase activities, with increased lipid peroxidation and levels of reactive oxygen species (ROS) (Qie et al., 2017). Pretreatment with antioxidants such as N-acetylcysteine has been shown to exert neuroprotection against the nerve damage caused by METH (Nakagawa et al., 2018). However, little is known concerning how METH impairs adaptation to cellular stresses such as oxidant injury and can thus cause cellular dysfunction leading to disease.

Genome integrity is important for cell survival. DNA damage is closely related to the growth status and function of cells, so nerve damage caused by METH may be related to DNA damage. Based on the generally accepted theory, highly conserved DNA repair system including ataxia telangiectasia mutant (ATM) and checkpoint kinase 2 (Chk2) can deal with both exogenous and endogenous DNA damage under normal conditions, resulting in damage at low homeostasis levels compatible with normal cellular function (Terabayashi and Hanada, 2018). However, endogenous damage cannot be repaired in a timely manner under the condition of DNA repair deficiency and keeps accumulating over time, leading to unscheduled alterations in the genome or instability, which can induce cell damage or apoptosis (Mirza-Aghazadeh-Attari et al., 2018). The neurotoxicity induced by the accumulation of DNA damage has been widely reported in neurodegenerative disease (Fernandez-Bertolez et al., 2018; Wu et al., 2018). For instance, alcohol abuse may significantly increase the level of ROS, which leads to DNA damage and may trigger apoptosis *via* activation of the mitochondrial pathway (Fowler et al., 2012; Kotova et al., 2013). Repeated exposure to METH could form large amounts of free radicals and causes DNA oxidation and strand breaks (Johnson et al., 2015). Therefore, we speculated that DNA damage may be an important cause of neurotoxicity induced by METH and that free radicals may be involved in DNA damage and apoptosis, while reducing the levels of free radicals could partially inhibit METH-induced neuronal DNA damage and apoptosis.

Tea polyphenols are natural compounds extracted from tea leaves and show good antioxidant capacities both *in vitro* and *in vivo* (Mao et al., 2017; Qi et al., 2017a, 2018). However, there have been few reports regarding whether tea polyphenols have a protective effect on METH-induced neuronal damage. Therefore, the purpose of the current research was to study whether tea polyphenols could alleviate apoptosis induced by METH through the inhibition of oxidative stress and DNA damage in dopaminergic neurons. For this purpose, we determined cell survival rates, apoptotic rates, $\Delta\Psi m$, ROS production, oxidative enzyme activities, nitric oxide (NO) production, and expressions of DNA damage and repair-related proteins in rat adrenal pheochromocytoma cells (PC12). PC12 cells were selected because they can synthesize and store DA, and they have many biochemical mechanisms related to dopaminergic cells (Greene and Tischler, 1976; Li et al., 2017). The results of this study demonstrate that METH exposure can increase oxidative stress and DNA damage and that tea polyphenols may be considered an effective protective substance to mitigate the DNA damage and apoptosis caused by METH in future clinical practice.

MATERIALS AND METHODS

Chemicals and Drug Preparations

Methamphetamine (METH) was provided by the Hubei Provincial Public Security Department. Tea polyphenols were purchased from Beijing Yihua Tongbiao Technology Co. Ltd. (tea polyphenol purity >98%, including catechin content >70%, epigallocatechin gallate content >40%; Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich, Inc. (St Louis, USA). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Thermo Fisher Scientific (Carlsbad, USA). The Muse MitoPotential Kit, Muse Multi-Color DNA Damage kit, and Muse Annexin V & Dead Cell Kit were procured from Millipore Corporation (Darmstadt, Germany). The lactate dehydrogenase (LDH) cytotoxicity assay kit, reduced glutathione (GSH) assay kit, total superoxide dismutase (T-SOD) assay kit, and malondialdehyde (MDA) assay kit were procured from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The NO and $\Delta\Psi m$ detection kit were obtained from Beyotime Biotechnology (Haimen, China). The RIPA lysis buffer, phosphatase inhibitors, and protease inhibitor cocktail were purchased from Boster Biological Technology Co. Ltd. (Wuhan, China). Antibodies against cleaved caspase-3, phospho-ATM (p-ATM), phospho-Histone H2AX (γ-H2AX), and phospho-Chk2 (p-Chk2) were purchased from Cell Signaling Technology, Inc. (Beverly, USA). All other chemicals were of analytical grade.

Cell Culture

Rat adrenal pheochromocytoma cells (PC12, high differentiation) were provided by the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% FBS (complete medium). Cells were passaged every 3 days to maintain exponential growth.

Cell Proliferation Experiment

Cell proliferation was examined by using MTT assay. Cells were seeded in 96-well plates and incubated overnight. MTT solution (final concentration of 0.5 mg/ml) was added after treatment with different substances, and incubation was continued for 4 h. The culture medium was then discarded, and DMSO was added into each well. Subsequently, a microplate reader (Thermo Scientific, USA) was used to measure the absorbance at 570 nm.

Lactate Dehydrogenase Release Assay

The cytotoxicity of cells exposed to different treatments was determined by LDH activity in culture medium. After being treated with different substances, the cultural supernatant of each well was transferred as the measured group. According to the instructions of the manufacturer, the blank group, control group, and standard group were also prepared. Finally, optical density (OD) was measured at 450 nm using the microplate reader. The activity of LDH was calculated according to the following formula:

$$\text{LDH activity}\left(\frac{\text{U}}{\text{L}}\right)=\frac{\text{OD measured}-\text{OD control}}{\text{OD standard}-\text{OD blank}}\times 0.2\ \text{mmol/L}\times 1{,}000$$

Cell Apoptosis Detection

Cell apoptosis was detected by flow cytometry. After treatment, cells were harvested and resuspended in a mixture of complete medium and Annexin V and Dead cell reagent. The mixture was incubated with gentle oscillation at 25°C for 20 min. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V has a high affinity for PS and binds to cells with exposed PS. In Annexin V and Dead cell reagent, Annexin V is conjugated to fluorescein (FITC). This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. In addition, the reagent includes 7-Amino-Actinomycin (7-AAD), which can bind tightly to the nucleic acids in cells and is impermeant to live cells and early apoptotic cells but stains dead cells and late apoptotic cells. The percentage of apoptotic cells was quantified with flow cytometry (Muse Cell Analyzer, Germany).

Analysis of Mitochondrial Membrane Potential

The changes in $\Delta\Psi$m were detected by using JC-1 in PC-12 cells treated with METH with or without tea polyphenols. JC-1 is a cationic dye that accumulates in energized mitochondria. JC-1 is predominantly a monomer that yields green fluorescence with an emission of 530 ± 15 nm at low $\Delta\Psi$m and aggregates, yielding a red-colored emission (590 ± 17.5 nm), at high $\Delta\Psi$m. After being treated, cells were stained with JC-1 working solution, rinsed twice with ice-cold staining buffer, resuspended in complete medium, and immediately examined with a fluorescent microscope (IX71, Olympus, Japan). The excitation wavelength of JC-1 monomers was 488 nm, and the emission wavelength was 535 nm. The excitation wavelength of JC-1 aggregates was 525 nm, and the emission wavelength was 595 nm.

The proportion of cells in which $\Delta\Psi$m had declined was measured by using a Muse MitoPotential Kit. After being treated, cells were harvested and suspended in assay buffer. Changes in $\Delta\Psi$m were then evaluated according to the instructions of the manufacturer.

DNA Damage Detection

Alkaline Comet Assay was applied to detect DNA strand breaks. Cells were suspended in low-melting agarose, plated on pre-coated microscope slides, and lysed in pre-chilled lysis solution for 1 h at 4°C. After incubation in alkaline buffer for 20 min, cells were electrophoresed for 15 min at 25 V, soaked in neutralization buffer for 5 min, and dyed with ethidium bromide in the dark. Images were analyzed using a fluorescent microscope 20 min later, and comets were analyzed with the Comet Assay Software Project. The percentage of DNA in the tail was used to reflect the extent of DNA damage.

A Muse Multicolor DNA Damage Kit was also used to investigate the DNA damage. After being treated, cells were harvested, washed with cold PBS, and fixed for 10 min on ice. The percentage of DNA-damaged cells was quantified after being permeabilized according to the manufacturer's instructions.

Measurement of Nitric Oxide Release

Quantitative determination of nitrite ions was applied as an indirect method for determining the level of NO. In simple terms, cells were seeded and incubated for 24 h. After exposed to different substances, cell culture mediums were collected to analyze the release of NO, following the manufacturer's instructions.

Detection of Reactive Oxygen Species

DCFH-DA can be oxidized by ROS into 2′,7′-dichlorofluorescin (DCF) after entering cells, so the intensity of DCF can represent the level of intracellular ROS. After different treatments, cells were suspended in DCFH-DA solution, and the cell suspensions were incubated at 37°C for 20 min. PBS was then used to resuspend the cells, and the intensity of DCF was measured with a fluorescent microplate reader (excitation 485 nm, emission 500 nm).

Determination of Antioxidant System and Lipid Peroxidation

The oxidative stress induced by METH and the protection from tea polyphenols were assessed by using the oxidative enzyme system (SOD, GSH, and MDA) to examine the oxygen reactivity of PC12 cells. After treatment with different substances, cells were harvested, resuspended, sonicated, and centrifuged for 15 min at 4,000 rpm. Subsequently, supernatants were individually used to measure the activities of the antioxidases SOD and GSH using corresponding diagnostic kits. The concentration of MDA in supernatants, which could express the degree of lipid peroxidation, was also determined according to the instructions of the manufacturer.

Western Blotting

RIPA buffer supplemented with phosphatase inhibitors and cocktail was used to lyse PC12 cells, and lysed cells were centrifuged for 15 min at 12,000 rpm to gather the supernatant. Stain-Free Gels (Bio-Rad) were used to separate proteins, and isolated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking, the PVDF membrane was incubated with primary antibodies (anti-cleaved caspase-3, anti-γ-H2AX anti-p-ATM, and anti-p-Chk2) overnight at 4°C, followed by a horseradish-peroxidase-conjugated secondary antibody. Finally, the PVDF membrane was incubated

with ECL substrate (Thermo Fisher Scientific Inc.) and scanned with a ChemiDoc Touch Imager (Bio-Rad). The results of Western Blotting were analyzed with Image J. All gels were imaged after electrophoresis. It has been reported in previous studies that normalization of samples to total protein density values is more reliable than normalization to individual protein levels (Vegh et al., 2014).

Statistical Analysis

All data are shown as means ± SEM. Differences among groups were calculated by one-way ANOVA using SPSS 20.0 software, and Tukey's HSD was applied as a post-hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Methamphetamine on Cell Viabilities in PC12 Cells

Both MTT assay and LDH cytotoxicity assay were used to measure the effect of METH on the viabilities of PC12 cells. **Figure 1A** shows that METH could significantly inhibit the proliferation of PC12 cells in MTT assay and that exposure to 3 mmol/L METH caused a 40.5 ± 5.3% reduction in the number of viable PC12 cells ($p < 0.01$). Meanwhile, the activities of LDH progressively increased by 23.6, 29.2, and 43.1%, respectively, after incubation with 3, 5, and 7 mmol/L METH for 24 h compared with the control group ($p < 0.05$, **Figure 1B**). Collectively, these results suggested that METH exposure could induce significant neurotoxic effects in PC12 cells *in vitro*.

Effect of Methamphetamine on Nitric Oxide and Reactive Oxygen Species Levels in PC12 Cells

To investigate whether oxidative stress is involved in the cytotoxic effect of METH, the production of NO and ROS was examined (**Figure 2**). The results showed that levels of NO and ROS were remarkably increased after METH exposure compared with the control group. For instance, exposure to 1.0 mmol/L METH for 24 h increased NO- and ROS-production by 4.96-fold and 2.05-fold, respectively ($p < 0.05$), and these increases reached 12.00-fold and 2.63-fold, respectively, when the dose of METH was 3.0 mmol/L ($p < 0.01$). The levels of NO and ROS production were 2.83-fold and 1.60-fold higher respectively after METH (3.0 mmol/L) treatment for 3 h ($p < 0.05$), and these effects increased gradually over time. These results indicated that METH induced significant oxidative stress in PC12 cells.

Effect of Methamphetamine on the ΔΨm in PC12 Cells

The loss of ΔΨm, an indication of mitochondrial function, is one of the important indicators of apoptosis. Compared with the control group, METH induced a significant increase in total mitochondrial depolarization (**Figures 3A,B**, $p < 0.05$). For instance, compared to the control group, the proportion of depolarized cells in 3 mmol/L and 6 mmol/L METH-treated cells were increased significantly by 2.35-fold and 2.85-fold, respectively, after 24-h exposure ($p < 0.05$).

Effect of Methamphetamine on Cell Apoptosis in PC12 Cells

The flow cytometry results showed that METH treatment could remarkably increase cell apoptosis, and the apoptosis rate increased with an increase in the concentration and incubation time (**Figures 3C,D**). For example, after exposure to METH (1 mmol/L) for 24 h, the rate of apoptotic cells increased 2.2-fold ($p < 0.01$), and this increase reached 3.4-fold when the dose of METH was 6 mmol/L ($p < 0.01$). In addition, the rate of apoptotic cells was increased to 2.1-fold higher after 3 mmol/L METH treatment for 6 h ($p < 0.01$), and this effect increased gradually over time. As shown in **Figure 4**, the protein levels of cleaved caspase-3 in METH-treated groups were also significantly higher than that of the control group ($p < 0.05$).

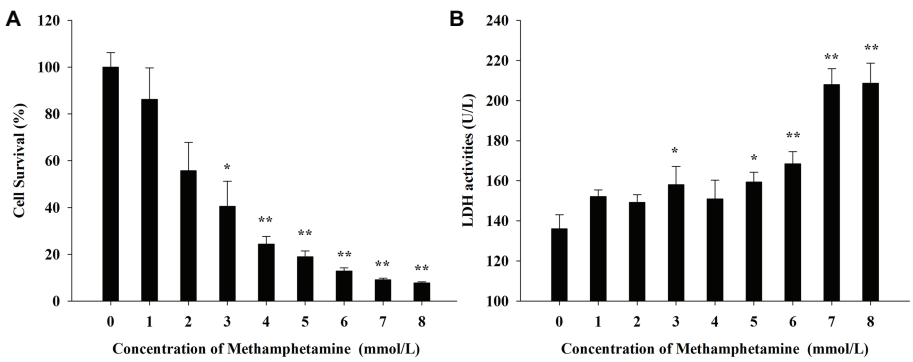

FIGURE 1 | Effect of methamphetamine on the viability in PC12 cells. **(A)** After PC12 cells were treated with different concentrations of methamphetamine for 24 h, the cell viability was measured by MTT assay. **(B)** After PC12 cells were treated with different concentrations of methamphetamine for 24 h, the activities of LDH in the supernatant of culture medium were detected. Data are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group.

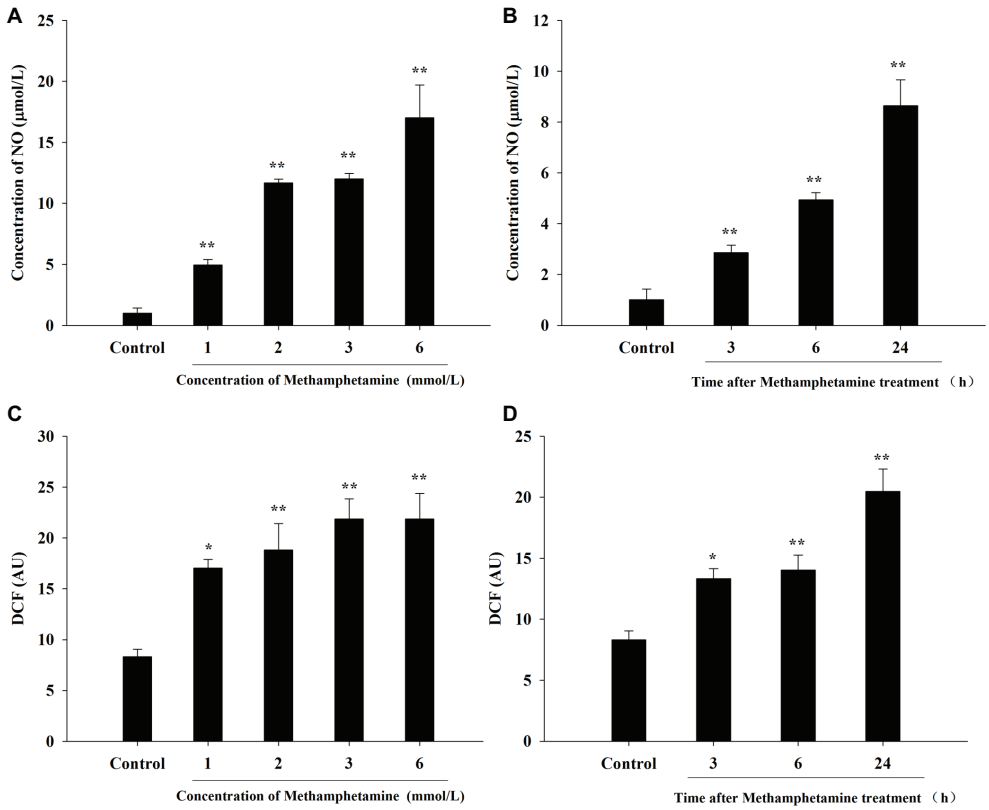

FIGURE 2 | Effect of methamphetamine on NO and ROS production in PC12 cells. PC12 cells were treated with 1–6 mmol/L METH for 24 h **(A,C)** or with 3.0 mmol/L METH for 3–24 h, as indicated **(B,D)**. The supernatant from each group was collected to determine the production of NO **(A,B)**. DCFH-DA was incubated with cells for 1 h in a CO_2 incubator, and then the fluorescent signal was obtained to evaluate the intracellular ROS level in different groups **(C,D)**. Data are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group.

Effect of Methamphetamine on DNA Damage in PC12 Cells

The expressions of DNA damage and repair-related protein markers in PC12 cells after METH treatment were determined to evaluate the influence of METH on DNA damage. As showed in **Figure 4**, METH exposure greatly increased the expression of γ-H2AX. For instance, exposure to METH (2.0 mmol/L) for 24 h increased the expression of γ-H2AX 2.45-fold ($p < 0.05$), and this increase reached 5.11-fold when the dose of METH was 6.0 mmol/L ($p < 0.01$). In addition, the expression of γ-H2AX increased gradually with an increase in meth concentration ($p < 0.05$). These findings indicated that METH could induce DNA damage in PC12 cells.

The results in **Figure 4** also show that low-dose or short-time METH treatment could significantly increase the expressions of p-ATM and p-Chk2, while high-dose or long-time METH treatment could reduce their expression levels (**Figure 4**). For instance, exposure to 1.0 mmol/L METH for 24 h and exposure to 3.0 mmol/L METH for 3 h could significantly increase the protein levels of p-ATM and p-Chk2 ($p < 0.05$), while their expressions were decreased substantially compared with the control group by 24-h exposure to 3.0 mmol/L METH ($p < 0.05$). These results showed that METH could induce significant DNA damage and inhibit the activation of the DNA repair system in PC12 cells, and the cell apoptosis caused by METH may be related to the oxidative stress and DNA damage that it induces.

Effect of Tea Polyphenols on the Cytotoxicity of PC12 Cells Caused by Methamphetamine

As shown in **Figure 5A**, the cell survival rate of METH-treated group was significantly lower than that of the control group ($p < 0.01$), and the survival rates of groups treated with different doses of tea polyphenols (5, 10, 20, and 40 μmol/L; $p < 0.05$) were increased significantly compared with the METH group. For instance, the cell survival rate of the 40-μmol/L tea polyphenols group was 99.93 ± 0.91%, while that of the METH group was 60.23 ± 0.97% ($p < 0.01$).

Effect of Tea Polyphenols on Nitric Oxide and Reactive Oxygen Species Production After Methamphetamine Exposure in PC 12 Cells

As shown in **Figure 5B**, NO production was increased significantly in the METH group compared with the control group ($p < 0.01$). When the concentration of tea polyphenols increased to 20 μmol/L, the contents of NO were decreased significantly

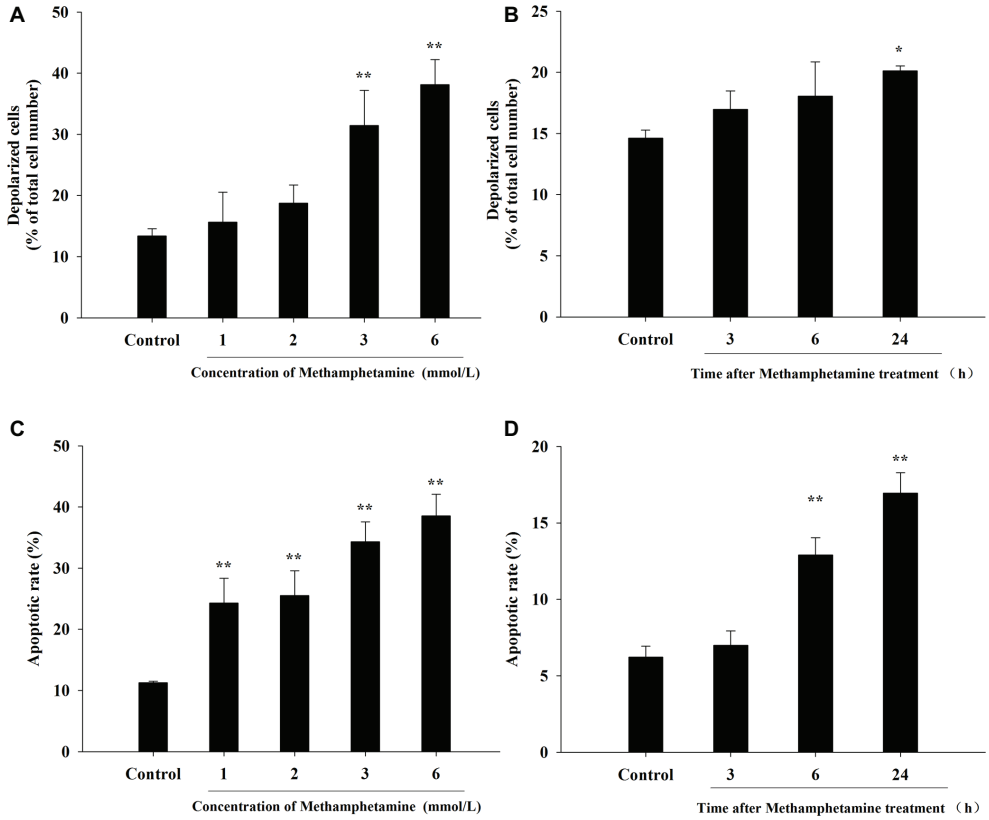

FIGURE 3 | Effect of methamphetamine on mitochondrial membrane potential and apoptosis in PC12 cells. PC12 cells were treated with 1–6 mmol/L METH for 24 h **(A,C)** or with 3.0 mmol/L METH for 3–24, as indicated **(B,D)**. Mitochondrial membrane potentials in different groups were analyzed by Muse MitoPotential assay **(A,B)**. Cell apoptosis was analyzed by Muse Annexin V & Dead Cell Assay **(C,D)**. Data are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group.

compared with the METH group ($p < 0.05$). As shown in **Figure 5C**, METH also significantly promoted the generation of ROS ($p < 0.01$). Additionally, with incremental increases in the dose of tea polyphenols, the levels of ROS gradually decreased, and 40 μmol/L tea polyphenols significantly decreased the ROS production in comparison with the METH group ($p < 0.05$).

Effect of Tea Polyphenols on the Antioxidant System and Lipid Peroxidation After METH Exposure in PC12 Cells

Figure 6 presents the data from the SOD, GSH, and MDA detection experiments. Compared with the control group, SOD activities in the METH group were significantly reduced in PC12 cells (71.21 ± 1.52 vs. 33.51 ± 1.59, $p < 0.01$), and 10, 20, and 40 μmol/L of tea polyphenols significantly increased SOD activities compared with the METH group (**Figure 6A**, $p < 0.05$). Additionally, METH significantly decreased GSH levels compared with the control group (1.83 ± 0.13 vs. 5.64 ± 0.21, $p < 0.01$), and 10, 20, and 40 μmol/L of tea polyphenols significantly increased GSH levels (**Figure 6B**, $p < 0.05$). Finally, the MDA contents were greatly increased after METH treatment (5.57 ± 0.42 vs. 11.36 ± 0.65, $p < 0.01$) compared with the control group, and all doses of tea polyphenols decreased the MDA contents significantly (8.00 ± 0.68, 7.23 ± 0.29, 7.82 ± 0.21, 6.02 ± 0.10 vs. 11.36 ± 0.65, **Figure 6C**, $p < 0.05$).

Effect of Tea Polyphenols on ΔΨM and Cell Apoptosis After Methamphetamine Exposure in PC 12 Cells

JC-1 assay and flow cytometry were both used to measure mitochondrial depolarization, which occurs in the early phase of apoptosis. In the JC-1 assay, decreased red fluorescence and increased green fluorescence represented decreased ΔΨm in mitochondria. As shown in **Figure 7A**, the increased number of green-stained cells indicated that METH had a strong pro-apoptotic effect on PC 12 cells, and different concentrations of tea polyphenols could effectively inhibit the METH-induced decrease in ΔΨm. Muse MitoPotential assay was applied to further detect the proportion of cells with decreased membrane potential (**Figure 7B**). **Figure 7C** shows that METH could significantly increase the rate of mitochondrial depolarized cells in comparison with the control group ($p < 0.01$), indicating that a remarkable dissipation of ΔΨm was induced by METH. In addition, 20 and 40 μmol/L tea polyphenols could greatly reduce the total depolarization of mitochondria compared with the METH group ($p < 0.01$).

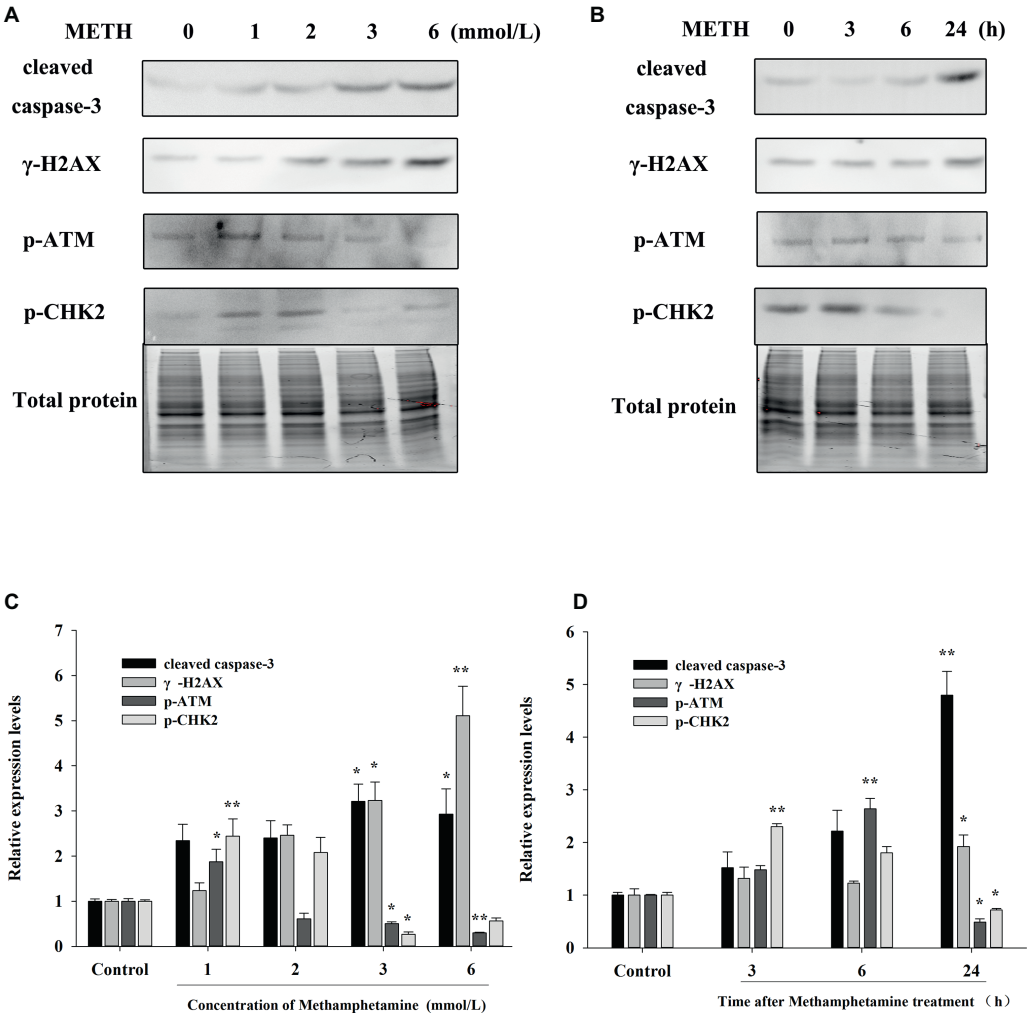

FIGURE 4 | Effect of methamphetamine on apoptosis and DNA damage in PC12 cells. **(A)** PC12 cells were treated with 1–6 mmol/L METH for 24 h, and the expressions of cleaved caspase-3, γ-H2AX, p-ATM, and p-CHK2 were detected using Western Blot; total protein was used as a loading control. Densitometric analysis of the blots is shown in **(B)**. **(C)** PC12 cells were treated with 3.0 mmol/L METH for 3–24 h, and the expressions of cleaved caspase-3, γ-H2AX, p-ATM, and p-CHK2 were detected using Western Blot; total protein was used as a loading control. Densitometric analysis of the blots is shown in **(D)**. Data are presented as mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group.

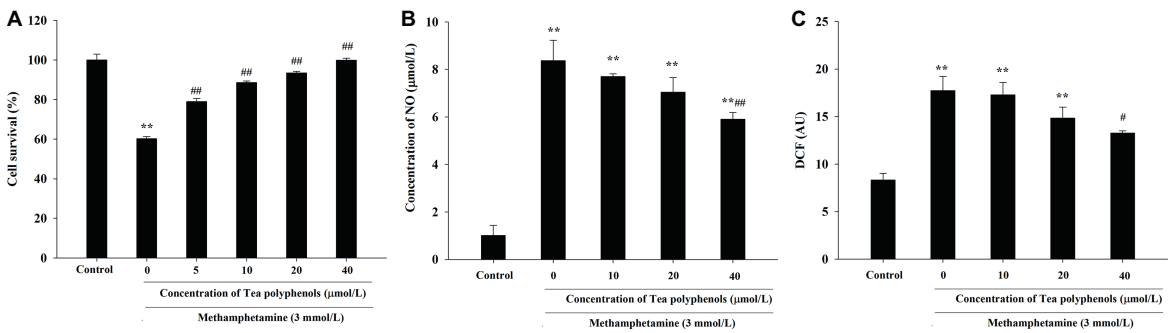

FIGURE 5 | Intervention effects of tea polyphenols on viability and oxidative stress in PC12 neuronal cells. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (5, 10, 20, and 40 μmol/L) for 24 h. **(A)** Cell viability was measured by MTT assay. **(B)** The supernatant from each group was collected to determine the production of NO. **(C)** DCFH-DA was incubated with cells for 1 h in a CO_2 incubator, and then the fluorescent signal was obtained to evaluate the intracellular ROS level in different groups. Data are presented as mean ± SEM; $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the METH group.

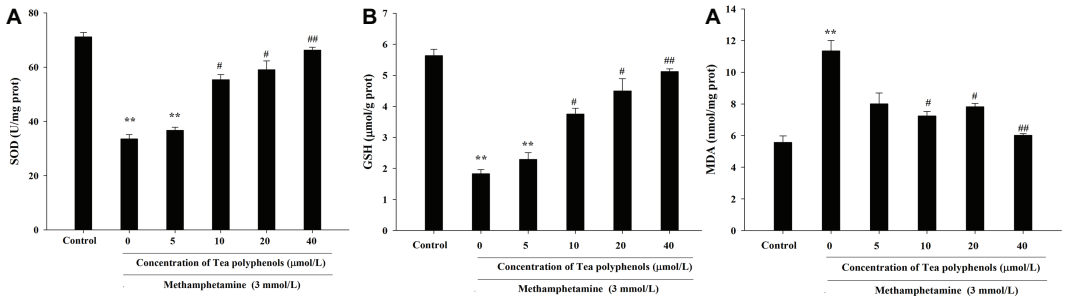

FIGURE 6 | Intervention effects of tea polyphenols on the oxidative enzyme system in PC12 neuronal cells. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (5, 10, 20, and 40 μmol/L) for 24 h. Cell samples were collected for measurements of the levels of SOD **(A)**, GSH **(B)**, and MDA **(C)** using corresponding commercial detection kits. Data are presented as mean ± SEM; $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the METH group.

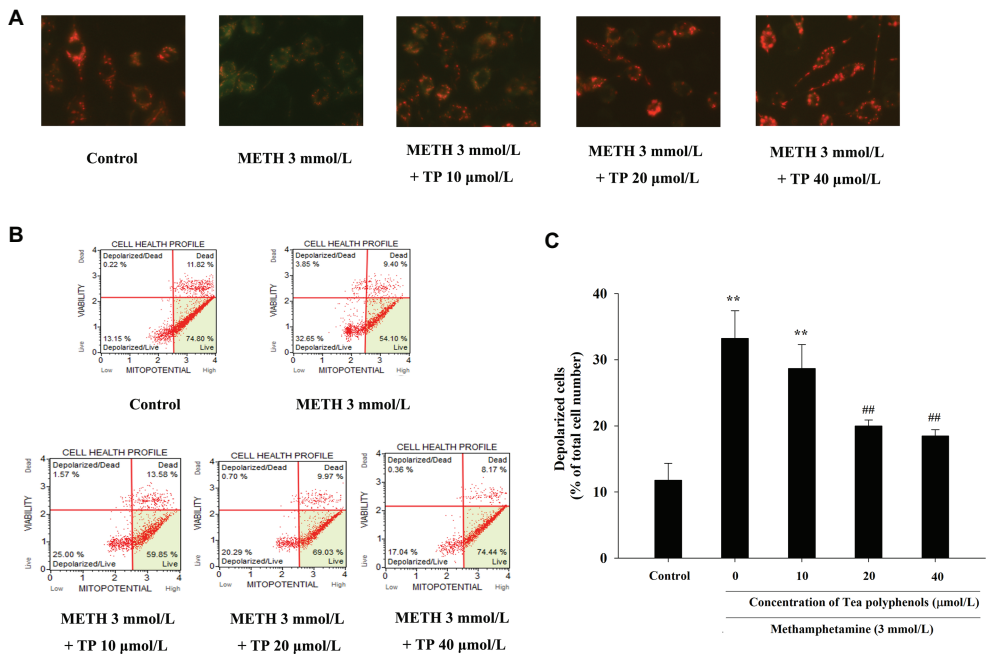

FIGURE 7 | Effect of tea polyphenols on mitochondrial membrane potential in PC 12 cells after METH exposure. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (5, 10, 20, and 40 μmol/L) for 24 h. **(A)** The mitochondrial membrane potential was examined by JC-1 staining (×200). The mitochondrial membrane potential in PC 12 cells was also analyzed by Muse MitoPotential assay. Representative dot plots in the live, depolarized/live, depolarized/dead, and dead phases are shown in the left panel **(B)**, and the mean percentage of depolarized cells is expressed in a histogram in the right panel **(C)**. Data are presented as mean ± SEM; $^{**}p < 0.01$ versus the control group; $^{\#\#}p < 0.01$ versus the METH group.

METH caused a remarkable increase in the apoptosis rate compared with the control group ($p < 0.01$). Treatments of 20 and 40 μmol/L tea polyphenols greatly decreased the apoptotic rates compared with the METH group (**Figure 8**, $p < 0.05$).

Effect of Tea Polyphenols on DNA Damage After METH Exposure in PC12 Cells

Immunostaining combined with flow cytometry, comet assay, and Western Blot were performed after exposure of METH with or without tea polyphenols to investigate the protection provided by tea polyphenols against DNA damage in PC12 cells. After METH exposure, the percentage of DNA-damaged cells increased compared with the control group (**Figure 9**). All concentrations of tea polyphenols could remarkably reduce the percentage of DNA-damaged cells ($p < 0.01$). The results of comet assay showed that the METH exposure group had a higher percentage of tail DNA than the control group (**Figures 10A,B**, $p < 0.01$). The METH-induced DNA damage was remarkably decreased in the tea polyphenols pretreatment groups ($p < 0.05$). Furthermore, the protein expression of γ-H2AX in the METH group was substantially higher than that in the control group ($p < 0.01$).

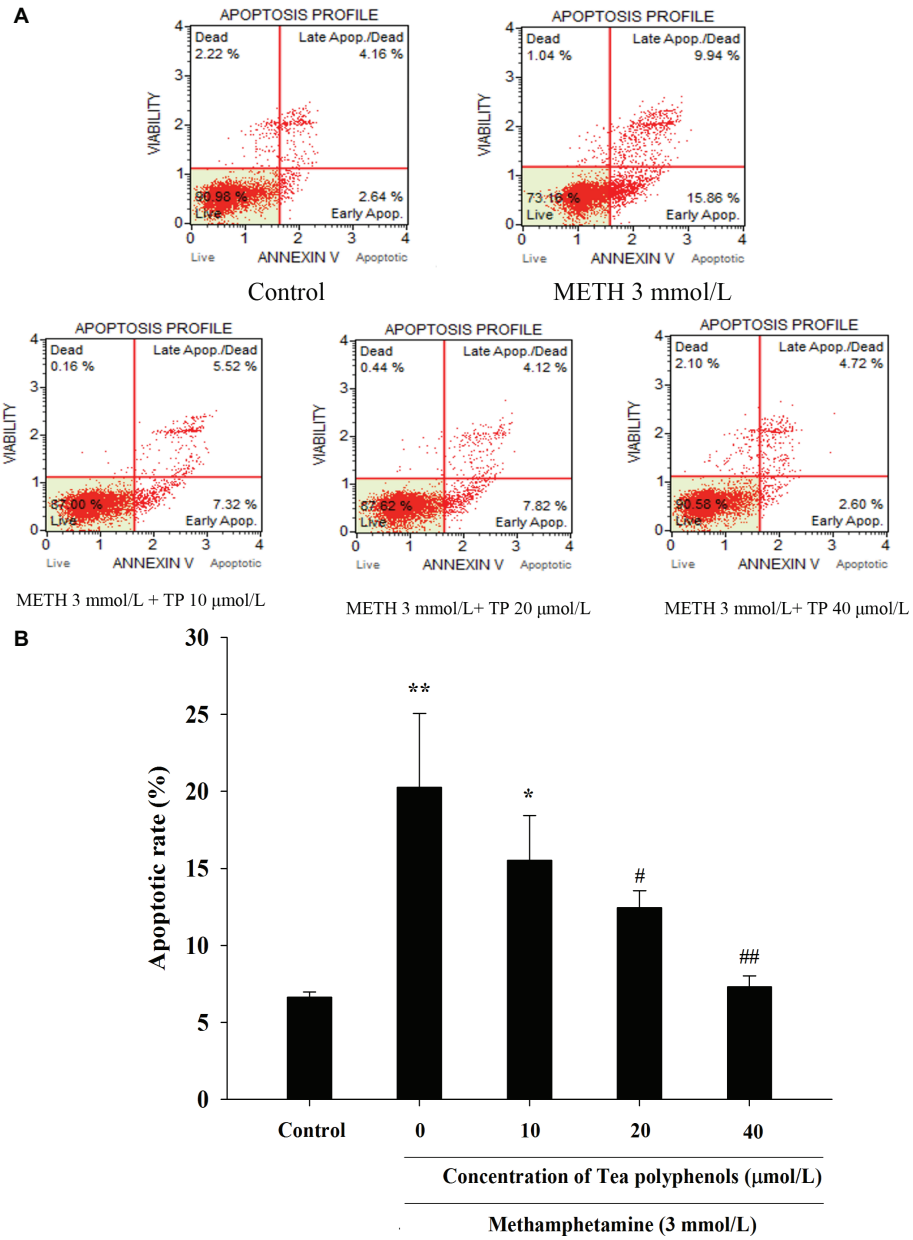

FIGURE 8 | Effect of tea polyphenols on apoptosis in PC12 cells after METH exposure. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (10, 20, and 40 μmol/L) for 24 h. Cell apoptosis was analyzed by Muse Annexin V & Dead Cell Assay. Representative dot plots in the live, dead, late apoptotic/dead, and early apoptotic phases are shown in the upper panel **(A)**, and the mean percentage of cell apoptosis is expressed in a histogram in the lower panel **(B)**. Data are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the METH group.

In contrast, 40 μmol/L tea polyphenols significantly decreased protein expression of γ-H2AX ($p < 0.05$).

Additionally, the protein expressions of p-Chk2 and p-ATM were significantly decreased after 3 mmol/L METH treatment ($p < 0.05$). In contrast, 20 μmol/L tea polyphenols significantly increased the protein levels of p-ATM and p-Chk2 (**Figures 10C,D**, $p < 0.05$). Therefore, the above findings indicated that tea polyphenols could reduce METH-induced DNA damage by increasing the expressions of DNA repair-related proteins in PC12 cells.

DISCUSSION

METH is a widely abused central neurostimulant that has been shown to produce complex neurotoxicity (Du et al., 2018; Li et al., 2018). The exact mechanism of the toxic effects of METH has not been fully elucidated, despite increasing evidence regarding the nerve cell damage induced by METH. Moreover, there is still a lack of effective treatment strategies for the neurotoxicity caused by METH, and more effective candidates need to be developed. In the present study, levels of NO and

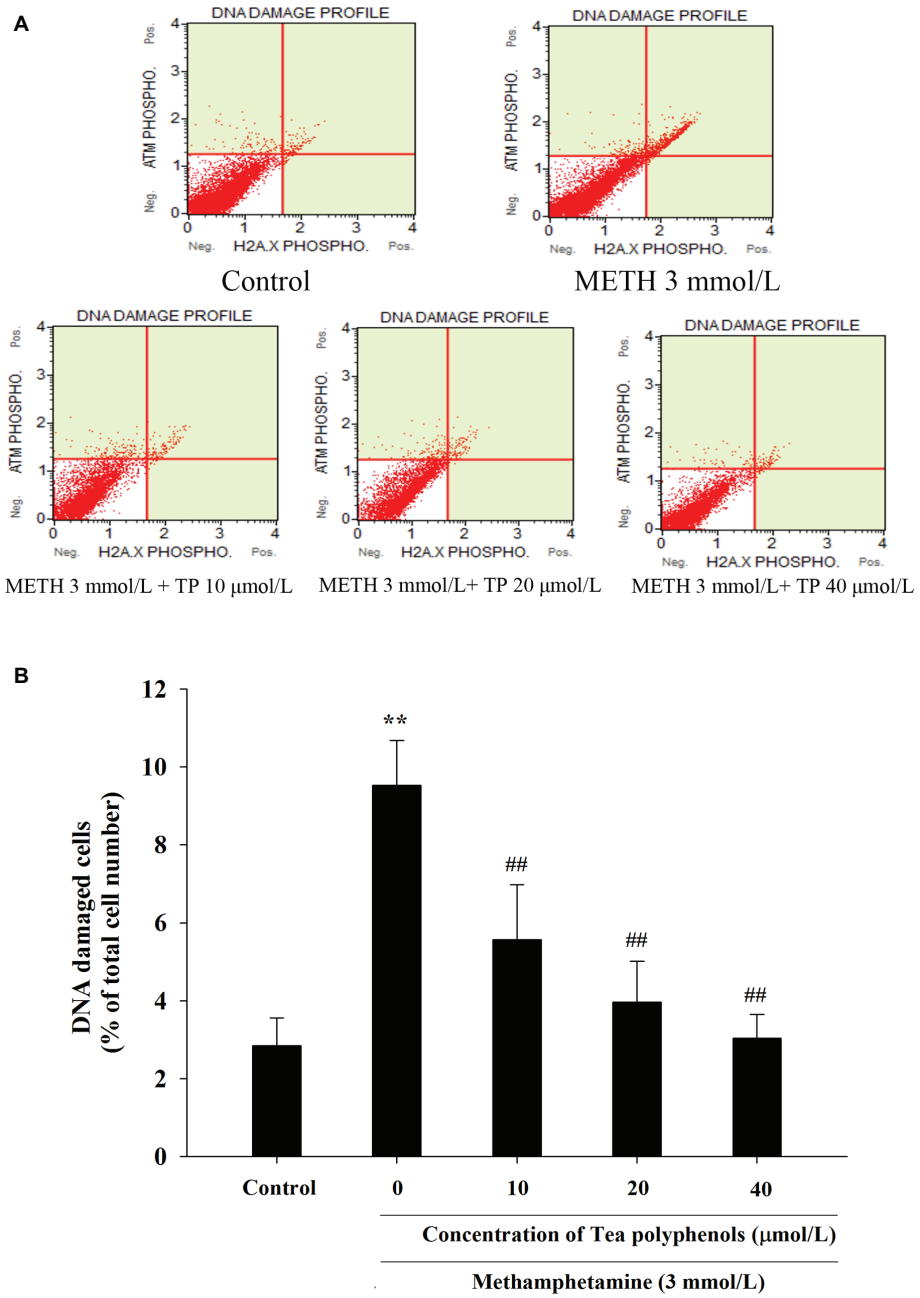

FIGURE 9 | Effect of tea polyphenols on DNA damage in PC12 cells after METH exposure. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (10, 20, and 40 μmol/L) for 24 h. Cell DNA damage was analyzed by Muse Multi-Color DNA damage assay. Representative dot plots are shown in the upper panel **(A)**, and the mean percentage of DNA damaged cells is expressed in a histogram in the lower panel **(B)**. Data are presented as mean ± SEM; $^{**}p < 0.01$ versus the control group; $^{\#\#}p < 0.01$ versus the METH group.

ROS were significantly increased after METH treatment *in vitro*. We also found that DNA damage and apoptosis were triggered by METH in PC12 cells, while tea polyphenols could alleviate METH-induced DNA damage and apoptosis *via* increasing antioxidant capacity and the expressions of DNA damage repair-associated proteins. These findings indicated that METH can cause a significant increase in free radicals and induce DNA damage and cell apoptosis and that this can be reversed by tea polyphenols.

Mounting evidence suggests that the mass formation of free radicals and oxidative stress may be involved in the neurotoxicity induced by METH (Huang et al., 2017; Yang et al., 2018), although the exact underlying mechanism is not yet clear. Oxidative stress induced by METH can cause damage to proteins, lipids, and DNA, altering cellular signal transduction (Krasnova and Cadet, 2009; Shokrzadeh et al., 2015). In line with these findings, we found that METH could significantly increase levels

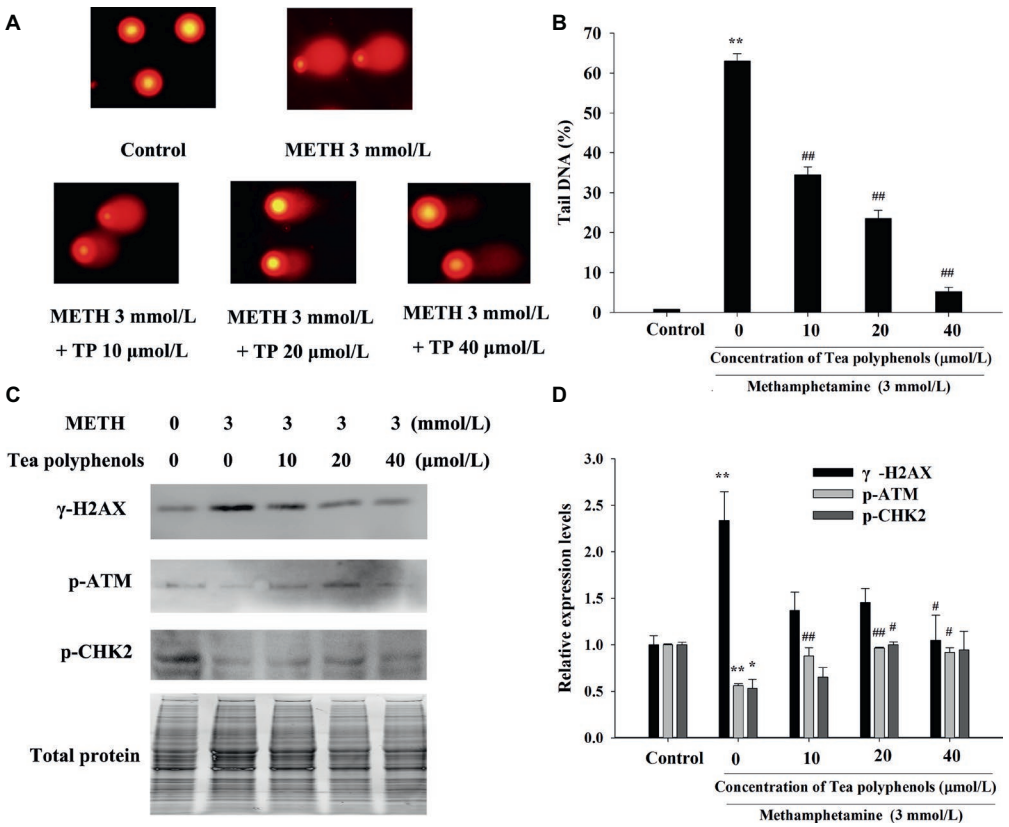

FIGURE 10 | Effect of tea polyphenols on DNA damage and related protein expression in PC12 cells after METH exposure. PC12 cells were treated with METH (3.0 mmol/L) with or without treatment with tea polyphenols (10, 20, and 40 μmol/L) for 24 h. Cell DNA damage was analyzed by comet assay **(A)**, and the statistical result is expressed by a histogram in the right panel **(B)**. The expressions of γ-H2AX, p-ATM, and p-CHK2 were detected using Western Blot **(C)**; total protein was used as a loading control. Densitometric analysis of the blots is shown in **(D)**. Data are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the METH group.

of NO and that the increase was positively correlated with METH exposure time and concentration. The induced free radicals may be the triggering factors to induce DNA strand breaks and mitochondrial-mediated apoptosis (Chen et al., 2017). We found that the level of γ-H2AX, an indicator of DNA strand breaks, was remarkably elevated after METH treatment, and levels of γ-H2AX were also positively correlated with METH exposure time and concentration. Previous reports have shown that METH could significantly increase the apoptosis rate and elevate the protein levels of cleaved PARP, cleaved caspase-3, and Bax (Li et al., 2018; Sharikova et al., 2018; Zhao et al., 2018). In agreement with these findings, the level of cleaved caspase-3 was increased significantly after METH treatment, and METH remarkably increased depolarization of the mitochondria and the cell apoptotic rate compared with the control group. Our data, combined with previous reports, indicate that highly active free radicals and oxidative damage may be partially involved in the apoptosis caused by METH.

Unless an effective repair mechanism corrects the damage to the double helix, DNA damage may cause persistent abnormalities after mitosis and in irreplaceable cells such as neurons (Milanese et al., 2018). Fortunately, cells have evolved DNA damage repair (DDR) mechanisms to alleviate a variety of damages (Henssen et al., 2017). Once DNA damage is triggered by exogenous and endogenous factors such as free radicals, the DDR can be activated to alter expressions of the damage sensor γ-H2AX and subsequent signal transduction pathways such as ATM/Chk2 pathway-related proteins (Ronco et al., 2017; He et al., 2018). If the damage is mild, it can be repaired through DDR; otherwise, it will result in gene mutation or apoptosis (Jackson and Bartek, 2009). However, whether DNA damage and repair-related proteins are involved in the neurotoxicity induced by METH remains unclear. In the present study, the expressions of p-ATM and p-Chk2 were significantly increased at 3 h after METH treatment and were reduced at 24 h. Consequently, we speculated that PC12 cells excited the expressions of DDR-associated proteins as a stress defense mechanism to prevent cytotoxicity at the early phase after METH treatment. However, if DNA damage is not repaired, the cellular protective effect may not overcome the toxicity induced by METH, and cells are likely to undergo programmed cell death such as mitochondria-mediated apoptosis.

Previous studies have found that pretreatment with antioxidants such as N-acetylcysteine and ascorbic acid can prevent METH-induced cell damage, and these reports further confirm the

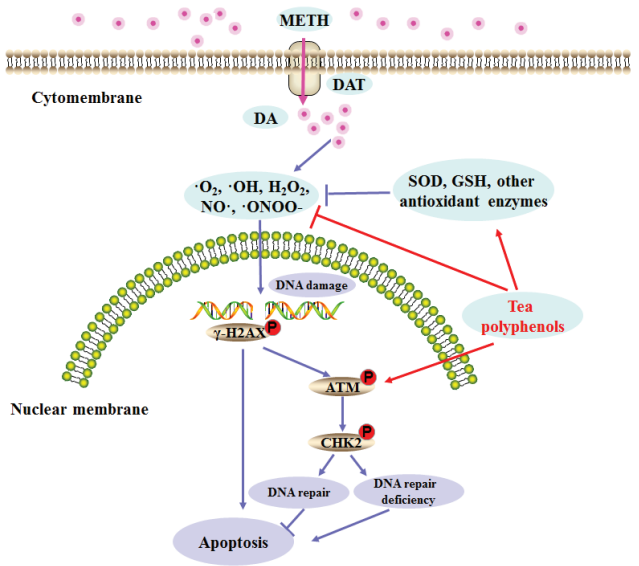

FIGURE 11 | Schematic representation depicting the role of DNA damage and repair signaling pathway in METH-induced neurotoxicity.

potential role of oxidation mechanisms in METH neurotoxicity (Chandramani Shivalingappa et al., 2012; Huang et al., 2017; Zeng et al., 2018). Tea polyphenols are bioactive catechins that have been shown to exert protection against neuronal cell damage (Ding et al., 2017; Chen et al., 2018). For example, tea polyphenols can suppress the ROS release and reduction of SOD activities and apoptosis induced by glutamate in primary cortical neurons (Cong et al., 2016). Furthermore, tea polyphenols also possess neuroprotective activities *via* the activation of the Keap1/Nrf2 pathway *in vitro* and *in vivo* (Qi et al., 2017b). In this study, we found that tea polyphenols have protective effects against METH-induced toxicity. Similar to previous studies, tea polyphenols were able to reverse the decline of SOD and GSH significantly and inhibit the increase in MDA contents as well as the production of NO and ROS that is induced by METH exposure. We further verified that tea polyphenols are highly likely to reduce the apoptosis in PC12 cells induced by METH through the mitochondria-mediated pathway. Additionally, our results indicated that tea polyphenols increased levels of DDR related proteins (p-ATM and p-Chk2) and decreased METH-induced DNA damage marker γ-H2AX expression. Based on these results, a series of events might occur in the procedure of the apoptotic pathway, and we speculated that tea polyphenols may attenuate ROS and NO production, promote the expressions of the oxidative enzyme system and DDR-related proteins, protect against DNA damage, and prevent apoptosis during treatment with METH. A schematic representation presenting the relationship among oxidative stress, DNA damage, and apoptosis after METH treatment and the underlying mechanism of action of tea polyphenols on the apoptosis induced by METH is shown in **Figure 11**.

In summary, oxidative stress, DNA damage, and apoptosis are interrelated in the pathology of many nerve system diseases. In the current study, we found that tea polyphenols protected against the neurotoxicity induced by METH in PC12 cells. Furthermore, we have demonstrated that the protective effect of tea polyphenols was mediated through attenuated oxidative stress, DNA damage, and mitochondrial apoptosis. Therefore, our research supports the hypothesis that supplementation with tea polyphenols might effectively prevent METH-induced neurotoxicity and neurodegenerative disease, and it is necessary to carry out further investigation in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

QR and CL designed the study. QR, QX, and XT processed the MTT assay, LDH assay, apoptosis detection, oxidative stress, and DNA damage tests. LC and MZ collected and analyzed data. YL interpreted the data. QR wrote and edited the manuscript. All authors critically reviewed the content and approved the final version for publication.

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PERSPECTIVE
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Predisposition to Alzheimer's and Age-Related Brain Pathologies by PM2.5 Exposure: Perspective on the Roles of Oxidative Stress and TRPM2 Channel

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Accumulating epidemiological evidence supports that chronic exposure to ambient fine particular matters of <2.5 μm (PM2.5) predisposes both children and adults to Alzheimer's disease (AD) and age-related brain damage leading to dementia. There is also experimental evidence to show that PM2.5 exposure results in early onset of AD-related pathologies in transgenic AD mice and development of AD-related and age-related brain pathologies in healthy rodents. Studies have also documented that PM2.5 exposure causes AD-linked molecular and cellular alterations, such as mitochondrial dysfunction, synaptic deficits, impaired neurite growth, neuronal cell death, glial cell activation, neuroinflammation, and neurovascular dysfunction, in addition to elevated levels of amyloid β (Aβ) and tau phosphorylation. Oxidative stress and the oxidative stress-sensitive TRPM2 channel play important roles in mediating multiple molecular and cellular alterations that underpin AD-related cognitive dysfunction. Documented evidence suggests critical engagement of oxidative stress and TRPM2 channel activation in various PM2.5-induced cellular effects. Here we discuss recent studies that favor causative relationships of PM2.5 exposure to increased AD prevalence and AD- and age-related pathologies, and raise the perspective on the roles of oxidative stress and the TRPM2 channel in mediating PM2.5-induced predisposition to AD and age-related brain damage.

Keywords: Alzheimer's disease, age-related brain pathologies, PM2.5, oxidative stress, TRPM2 channel

INTRODUCTION

Air pollution has increasingly become an environmental risk to public health worldwide, particularly to people living in large cities. This has been supported by compelling evidence for strong association of chronic exposure to ambient air pollution with increased morbidity and mortality of respiratory and cardiovascular diseases (Liu et al., 2019). There is growing evidence

to show that exposure to polluted ambient air is also injurious to the brain (Brockmeyer and D'Angiulli, 2016; Clifford et al., 2016; Power et al., 2016; Babadjouni et al., 2017; Cohen et al., 2017; Sripada, 2017; Underwood, 2017; Bencsik et al., 2018). Among other air pollutants, the fine particulate matters (PM) with an aerodynamic diameter of <2.5 μm (PM2.5), which include ultrafine PM with a size of <200 nm (PM0.2) and nanometer-sized PM (nPM) or nanoparticles (NPs), has attracted particular attentions for their potential damage to the brain because they more readily enter the brain; they can penetrate the olfactory epithelium and, alternatively and/or additionally, travel deep into the airways and lungs, infiltrate into the blood circulation, and finally cross the blood–brain barrier (BBB) (Heusinkveld et al., 2016; Maher et al., 2016; Underwood, 2017; Bencsik et al., 2018). Such tiny particles in ambient air can be mainly derived from diesel exhaust (DE) and traffic/combustion-related air pollution, and also increasingly result from manufacturing, application, and subsequent release of nanomaterials (Bencsik et al., 2018). In general, the smaller their size, the greater their capacity of inducing oxidative stress and thus the more severe the resulting cytotoxicity is (Underwood, 2017). It has been shown that exposure to traffic-related air pollution and PM2.5 during early life can damage brain and cognitive development and increase the prevalence of autism spectrum disorders (Raz et al., 2015; Saenen et al., 2015; Sunyer et al., 2015; Sram et al., 2017; Sripada, 2017; Fu et al., 2019; Jo et al., 2019; Pagalan et al., 2019a,b). Accumulating evidence also supports predisposition by PM2.5 exposure of both children and adults to various brain pathologies including Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, and stroke and depressive disorders (Mushtaq et al., 2015; Kioumourtzoglou et al., 2017; Seelen et al., 2017; Sram et al., 2017; Bencsik et al., 2018; Bazyar et al., 2019; Fu et al., 2019; Shou et al., 2019). Air pollution has increasingly become a major environmental risk factor inducing dementia (Underwood, 2017). AD represents the most common cause of age-related brain damage and dementia. In this article, we discuss the studies showing predisposition by PM2.5 exposure to AD and age-related brain damage, and hypothesize the roles of oxidative stress and the oxidative stress-sensitive transient receptor potential melastatin 2 (TRPM2) channel in PM2.5-induced AD and age-related brain pathologies.

ALZHEIMER'S PATHOLOGIES AND OXIDATIVE STRESS

Alzheimer's disease is an age-related neurodegenerative condition manifested by progressive decline and loss of cognitive function. AD patients in the later disease stage suffer brain structural alterations, including shrinking of hippocampus and cerebral cortex (Drew, 2018). Prior to such structural atrophy, the AD brain is more often than not characterized at the microscopic level by widespread aggregation and deposition of extracellular amyloid β (Aβ) peptides in amyloid plaques and intra-neuronal hyper-phosphorylated tau proteins into neurofibrillary tangles (NFT). Genetically, AD can be familial and sporadic. Familiar AD (FAD), identified in a very small number of cases, arises predominantly from mutations in amyloid precursor protein (APP), presenilin 1 (PS1), and PS2 that lead to excessive Aβ generation and neurotoxic Aβ fibrillary formation, a process often referred to as amyloidogenesis. Sporadic form, accounting for a vast majority of cases, results from aging, genetic [e.g. carrying apolipoprotein E (APOE) ε4 allele which is associated with a reduced capacity of clearing and degrading Aβ], and environmental risk factors that aggravate amyloidogenesis (Blennow et al., 2010; Buxbaum, 2019; Licher et al., 2019).

The amyloid cascade hypothesis of AD posits that Aβ directly or indirectly causes synaptic deficits and neuronal loss, leading to cognitive dysfunction (Blennow et al., 2010; Selkoe and Hardy, 2016). The direct toxicity of Aβ to synapses and neurons is well attested by *in vitro* studies exposing cultured neurons to Aβ alongside *in vivo* studies using various strains of transgenic AD mice that express AD-linked human mutant genes leading to elevated Aβ levels (e.g. APP/PS1, 5xFAD, or APOE ε4 mice) or wild-type animals, predominantly mice and rats, with intra-hippocampal administration of neurotoxic Aβ (Blennow et al., 2010; Buxbaum, 2019). Recent studies have disclosed AD-related alterations in the genetic and functional profile of microglia, the immune-competent cells in the brain, and association of mutations in microglia-specific genes (e.g. TREM2) with AD, which triggers an escalating interest in microglia, particularly microglia-mediated neuroinflammation, and recognition of its importance in AD pathogenesis and progression (McQuade and Blurton-Jones, 2019; Wang and Colonna, 2019). The brain is highly vulnerable to oxidative damage, due to its high oxygen consumption, high content of fatty acids, and weak antioxidant capacity. Aβ can promote ROS generation and in return ROS can enhance Aβ generation and aggregation. ROS are potent in modifying functionally important molecules (e.g. DNA and proteins) and damaging intracellular organelles (e.g. lysosomes and mitochondria) (Jiang et al., 2016; Butterfield, 2018). Aβ and ROS synergistically can damage synapses and neurons, induce microglial activation and neuroinflammation, and impair neurovascular and BBB function (Sweeney et al., 2018). Oxidative damage is a prominent and common feature of many neurodegenerative diseases and accepted as an important neurodegeneration mechanism (Jiang et al., 2016; Butterfield, 2018; Trist et al., 2019).

CAUSATIVE RELATIONSHIPS OF PM2.5 EXPOSURE WITH AD AND AGE-RELATED PATHOLOGIES

PM2.5 Exposure Induces Predisposition to Dementia, AD, and Age-Related Brain Damage

The interest in PM2.5-induced brain damage and cognitive dysfunction was in fact triggered by a histochemical study revealing widespread pathological modifications (e.g. degenerating cortical neurons, apoptotic white matter glial cells, NFT, and BBB impairment) in the brains of demented dogs living in a highly air polluted urban region of Mexico

City (Calderon-Garciduenas et al., 2002). Subsequent studies by the same group have documented numerous early pathological indicators of neurodegenerative diseases, including accumulation of Aβ42, oxidative stress, neuroinflammation, and neurovascular damage in the brains of children and young people in Mexico City experiencing chronic exposure to high levels of air pollution and PM2.5 (Calderon-Garciduenas et al., 2004, 2008, 2012, 2016, 2018; Gonzalez-Maciel et al., 2017). Consistently, epidemiological studies support significant association of chronic exposure to PM2.5 or traffic-related air pollution with increased risk to dementia and AD (Kioumourtzoglou et al., 2016; Chen et al., 2017; Fu et al., 2019) and age-related cerebral atrophy (Wilker et al., 2015). Chronic PM2.5 exposure has also been associated with accelerated loss of gray and white matters or increased risk of cognitive impairment in older women (Casanova et al., 2016; Cacciottolo et al., 2017). Collectively, these studies support predisposition to AD and age-related brain damage by chronic PM2.5 exposure. Further supporting evidence comes from studies examining cognitive function and neuro-behaviors in rodents after exposure to ambient PM2.5 with various doses and durations. An early study using 4-week-old male mice reported that PM2.5 exposure (94.4 $\mu g/m^3$, 6 h per day, and 5 days per week) for 10 months impaired learning and memory and also resulted in depressive-like responses (Fonken et al., 2011). A recent study shows that PM2.5 exposure (3 mg/kg every other day) for 4 weeks also damaged learning and memory in young mice (4 weeks). However, such PM2.5 exposure-induced detrimental effects were not observed in adult (4 months) and middle-aged (10 months) mice (Ning et al., 2018), suggesting an age-ceiling effect. Another recent study using 2-month-old male rats reports that intra-tracheal injection of PM2.5 (20 mg/kg every 7 days) for 3–12 months damaged sensory functions as well as learning and memory (Zhang et al., 2018). As discussed in detail below, studies provide further evidence to suggest that PM2.5 exposure predisposes humans to AD and development of AD-related pathologies in rodents via exacerbating the pathological pathways that are known to be implicated in AD, namely, directly causing synaptic deficits and neuronal cell death, or indirectly inducing microglia-mediated neuroinflammation and disrupting neurovascular function (**Figure 1**).

PM2.5 Exposure Impairs Neurite Growth, Expression of Synapse Proteins and Receptors, and Neuronal Cell Viability

Studies have investigated the effects of PM2.5 exposure on neurons, drawing particular attention to neurite growth, synaptic structure and function, and neuronal cell viability. In the above-mentioned study, exposure of 4-week-old mice to PM2.5 for 10 months reduced dendritic spine density of hippocampal neurons in the CA1 region and also dendritic length and branching in the CA3 region (Fonken et al., 2011). Another study using 8-week-old male mice showed that exposure to PM2.5 (65.7 ± 34.2 $\mu g/m^3$, 6 h per day, and 5 days per week) for 9 months induced synaptic alterations by increasing the expression of postsynaptic density protein 95 (PSD95) without effect on the expression of presynaptic protein synaptophysin (Bhatt et al., 2015). A recent study using 3-month-old mice shows that exposure to ambient nPM (5 h per day and 3 days per week) for 10 weeks caused white matter damage in the CA1 and DG regions of the hippocampus, and suppressed neurite outgrowth in the CA1 region (Woodward et al., 2017). The same study examined the receptors for glutamate, the key excitatory neurotransmitter in the hippocampus. The expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) class receptor GluR1 subunit was reduced, whereas the expression of GluR2 subunit, or *N*-methyl-D-aspartate (NMDA) class receptor NR2A or NR2B subunit remained unchanged. Such nPM-induced effects on white matter and GluR1 expression in 3-month-old mice were similar to these in 18-month-old mice due to aging. Moreover, in the old mice, nPM exposure induced no further detrimental effect, again indicating an age-ceiling effect (Woodward et al., 2017). Another recent study shows that exposure of female mice to nPM for 10 weeks selectively reduced neurite density in the CA1 region and attenuated the GluR1 expression without effect on the expression of GluR2, NR2A, NR2B, synaptophysin, and PSD95 (Cacciottolo et al., 2017). The study also shows that nPM exposure selectively decreased neurite density and the GluR1 expression in hippocampus of $5xFAD^{+/-}$ mice carrying the human APOE ε4 gene as observed in wild-type mice. In addition, PM2.5-induced AD-related pathologies are associated with neuronal death. For example, a recent study demonstrates that intra-tracheal injection of PM2.5 in 2-month-old male rats induced necrosis and apoptosis of cortical neurons (Zhang et al., 2018). A more recent study using human neuroblastoma SH-SY5Y cells, a widely used cell model in the study of neurodegeneration mechanism (Xicoy et al., 2017), also shows that exposure to PM2.5 (25–250 mg/mL) for 24 h reduced cell viability (Wang et al., 2019). Collectively, these studies suggest that PM2.5 exposure can lead to neurodegeneration by compromising neurite growth, expression of synapse proteins and receptors, and neuronal cell viability (**Figure 1**).

PM2.5 Exposure Induces Microglial Cell Activation and Generation of Proinflammatory Cytokines

As has been introduced above, microglia-mediated neuroinflammation has attracted increasing attention for its role in AD. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 are the major neurotoxic pro-inflammatory cytokines in AD-related neuroinflammation. Studies, both *in vivo* using rodents and *in vitro* using cultured cells, have provided evidence to suggest that PM2.5 exposure can induce deleterious effects on the brain via neuroinflammation, mainly through excessive generation of these proinflammatory cytokines by microglia (Brockmeyer and D'Angiulli, 2016; Jayaraj et al., 2017). An early study showed that exposure of 4-week-old male mice to PM2.5 for 10 months upregulated the expression of IL-1β and TNF-α in the brain (Fonken et al., 2011). Another early study using 12-to-14-week-old male rats reported that exposure to DE (0.5 and 2.0 mg/m^3, 4 h per day, and 5 days per week) for 1 month resulted in concentration-dependent

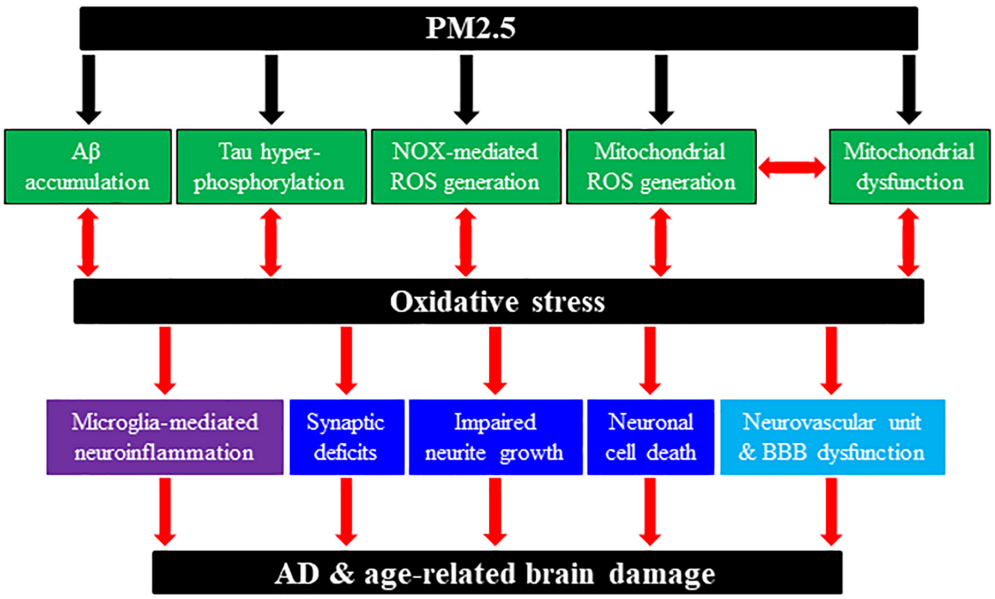

FIGURE 1 | Schematic summary of potential molecular and cellular pathways involved in PM2.5-induced predisposition to AD and age-related brain damage. Chronic exposure to PM2.5 in ambient polluted air can predispose humans to AD or rodents to AD-related brain damage through generation or activation of multiple pathological factors and pathways. PM2.5 exposure can induce or enhance amyloid β (Aβ) accumulation; tau hyper-phosphorylation; NADPH oxidase (NOX)-mediated reactive oxygen species (ROS) generation; mitochondrial dysfunction; and mitochondrial ROS generation. In addition, PM2.5 exposure-induced AD-related brain pathologies engage multiple cellular pathways, including synaptic deficits, impaired neurite growth and cell death in neurons; microglia (and astrocytes) activation and generation of neurotoxic proinflammatory meditators [e.g., interleukin (IL)-1β, tumor necrosis factor-α, IL-6, ROS]; neurovascular unit and blood–brain barrier (BBB) dysfunction. We have hypothesized the roles of oxidative stress and subsequent activation of the TRPM2 channel (not depicted here) in coupling PM2.5 exposure to predisposition to AD and age-related brain damage leading to cognitive dysfunction.

increases in the expression of ionized calcium-binding adaptor molecule 1 (Iba-1), a microglial cell marker, and IL-6 in the whole brain (Levesque et al., 2011). More specifically, such exposure elevated the levels of Iba-1, IL-1β, IL-6, and TNF-α in the cortex and midbrain regions. The same study also showed that intra-tracheal administration of single high dose of DE-derived particles (20 mg/kg) increased the TNF-α level in the serum and whole brain and that exposure to DE-derived nPM (50 μg/mL) enhanced TNF-α generation from rat immortalized microglial cells prior primed by lipopolysaccharide (LPS) (Levesque et al., 2011). Similarly, a recent study using 3-month-old mice shows that exposure to ambient nPM for 10 weeks increased the Iba-1 expression in the CA1 and DG regions of the hippocampus and the TNF-α expression level in the whole brain (Woodward et al., 2017). Another recent study using 3-month-old mice also reports that exposure to DE containing 250–300 μg/m^3 PM2.5 for 6 h induced morphological changes of microglial cells and elevated Iba-1 expression in the hippocampus, similarly in male and female mice (Cole et al., 2016). Furthermore, nPM exposure massively elevated the levels of IL-1β, IL-6, TNF-α, and IL-3 and the level of malondialdehyde (MDA), a biomarker of oxidative stress, in the hippocampus, and such brain inflammation and oxidative stress were noticeably higher in male mice than in female mice, suggesting sex difference (Cole et al., 2016). A separate study using cultured rat microglial and astrocytes demonstrated that exposure to traffic-derived PM0.2 (6–12 μg/ml) induced the TNF-α expression (Cheng et al., 2016). The study also showed that the neurite length of rat cortical neurons, when cultured in media conditioned by PM0.2-exposed microglia, astrocytes, or mixed cell cultures, was significantly shorter. PM0.2-induced reduction in neurite growth was prevented by siRNA-mediated knockdown of the TNF-α expression, indicating that PM0.2-induced TNF-α generation by glial cells mediates such neurotoxicity (Cheng et al., 2016). Another recent study using 15/16-week-old mice shows that exposure to traffic-derived nPM (330 μg/m^3, 5 h per day, and 3 days per week) for 10 weeks induced microglial cell activation and increased the deposition of complement C5/C5α proteins and C5a receptor 1 in the corpus callosum (Babadjouni et al., 2018). Taken together, these studies support that PM2.5 exposure causes AD- and age-related brain pathologies via inducing neuroinflammation (**Figure 1**).

Both *in vivo* studies using APP/PS1 mice and *in vitro* studies using cultured microglial cells have revealed an important role for Aβ-induced activation of the multi-protein complex NLRP3 inflammasome and caspase-1 and ensuring generation of IL-1β in AD pathologies (Heneka et al., 2015; Wes et al., 2016; White et al., 2017). Consistently, a recent study using LPS-primed cultured mouse microglial cells shows that exposure to PM2.5 (50 μg/ml) for 4 h enhanced Aβ-induced NLRP3 inflammasome activation and IL-1β generation (Wang et al., 2018). Such PM2.5-induced effects were dependent upon both NADPH oxidase (NOX)- and mitochondria-dependent generation of ROS. Furthermore,

PM2.5 exposure enhanced the capacity of Aβ-treated microglia to induce neuronal cell death in cortical neuron/microglia co-cultures, where microglia and neurons were separately seeded in the upper and lower chambers, respectively, in trans-well plates. Such PM2.5-induced microglia-mediated neuronal cell death was prevented by pharmacological inhibition of NOX-mediated and mitochondrial ROS generation or caspase-1 activation (Wang et al., 2018). These results therefore suggest that PM2.5 exposure can induce neuroinflammation via sequential induction of oxidative stress, NLRP3 inflammasome activation, and IL-1β generation.

PM2.5 Exposure Induces Neurovascular Unit and BBB Dysfunction

Impairment in the neurovascular unit and BBB function can render enhanced infiltration of peripheral immune cells and proinflammatory mediators to intensify brain inflammation as well as the entry of PM2.5 into the brain. Evidence exists to indicate that Aβ can induce neurovascular unit and BBB dysfunction and thereby increase susceptibility to AD (Park et al., 2014; Sweeney et al., 2018). As mentioned above, histochemical studies of the brain of young people who lived in Mexico City suggest that chronic exposure to PM2.5 or combustion-derived nNPs can compromise the neurovascular unit and BBB function (Calderon-Garciduenas et al., 2016; Gonzalez-Maciel et al., 2017) and thereby aggravate AD-related pathologies (**Figure 1**).

INDUCTION OF AD-ASSOCIATED MOLECULAR ALTERATIONS BY PM2.5 EXPOSURE

PM2.5 Exposure Enhances Aβ Accumulation and Tau Hyper-Phosphorylation

There is evidence to indicate that PM2.5 exposure enhances the levels of Aβ and tau hyper-phosphorylation. As shown in an aforementioned recent study using 8-week-old male mice, exposure to ambient PM2.5 (65.7 ± 34.2 μg/m^3, 6 h per day, and 5 days per week) for 9 months reduced the level of APP protein and increased the levels of beta-site APP cleaving enzyme (BACE) protein and Aβ40 in the cerebral temporal cortex (Bhatt et al., 2015). A recent study using 2-month-old female 5xFAD$^{+/-}$/APOE ε4 mice reports that nPM exposure for 15 weeks accelerated amyloid deposition and plaque formation and elevated the level of Aβ oligomers, which may contribute to nPM-induced selective reduction in neurite density in the CA1 region (Cacciottolo et al., 2017). The same study also shows that exposure of mouse neuroblastoma N2a cells expressing Swedish mutant APP to 10 μg/ml nPM for 24 h enhanced Aβ42 generation. Another recent study using 10-week-old female 5xFAD mice reports that exposure to DE (0.95 mg/m^3, 6 h per day, and 5 days per week) for 3 weeks elevated the levels of cortical Aβ plaque load and whole brain Aβ42 (Hullmann et al., 2017). However, prolonged exposure for 13 weeks resulted in no effect on the levels of Aβ plaque load and whole brain Aβ42, which were already high due to aging and AD progression (Hullmann et al., 2017). Such an observation further indicates an age-related ceiling effect as previously reported in wild-type mice (Woodward et al., 2017). Another recent study using 10-month-old mice has found that exposure to ambient PM2.5 (3 mg/kg) for 4 weeks increased the level of tau hyper-phosphorylation as well as altering neuronal mitochondria, inducing ROS generation and reducing the cellular ATP content (Gao et al., 2017). Therefore, PM2.5 exposure can induce AD-related pathologies via stimulating Aβ generation and tau hyper-phosphorylation (**Figure 1**).

PM2.5 Exposure Stimulates ROS Generation and Oxidative Stress

As discussed above, excessive ROS generation and ensuing oxidative damage play an important role in AD. There is increasing evidence to indicate that PM2.5-induced AD-related pathologies are associated with increased ROS generation and oxidative stress. For example, a recent study using 2-month-old male rats shows that intra-tracheal injection of PM2.5 significantly reduced the activities of superoxide dismutase (SOD), a superoxide radical scavenger, and glutathione (GSH) peroxidase, an important antioxidant enzyme catalyzing the reduction of hydrogen peroxide (H_2O_2) by GSH, and increased the level of MDA (Zhang et al., 2018). There were also substantial mitochondrial dysfunction and loss of cristae within mitochondria in cortical neurons of PM2.5-exposed rats (Zhang et al., 2018). A more recent study using SH-SY5Y cells shows that exposure to PM2.5 (25, 100, and 250 mg/mL) concentration-dependently increased the levels of intracellular Ca^{2+} and ROS, and reduced the cellular ATP content and GSH/GSSG ratio (Wang et al., 2019). PM2.5 exposure also induced mitochondrial fragmentation and increased the level of optic atrophy 1 (OPA1) protein, which is critical for mitochondrial fusion, without change in the level of dynamin-related protein 1 (Drp1) protein, which is important for mitochondrial fission. Furthermore, PM2.5 exposure triggered the opening of mitochondrial permeability transition pore, decreased the mitochondrial membrane potential and mitochondrial SOD activity, and elevated the mitochondrial content of MDA (Wang et al., 2019). Therefore, accumulating evidence suggests that ambient PM2.5 exposure induces oxidative stress and mitochondrial dysfunction, leading to neuronal cell death (**Figure 1**).

PERSPECTIVE ON ROLES OF OXIDATIVE STRESS AND TRPM2 CHANNEL IN PM2.5-INDUCED DISPOSITION TO AD AND AGE-RELATED BRAIN DAMAGE

TRPM2 Channel Is Critical in Mediating AD- and Age-Related Cognitive Dysfunction

The TRPM2 channel, member of the mammalian TRP channel superfamily, is a Ca^{2+}-permeable cationic channel gated

by intracellular ADP-ribose (ADPR) and related compounds (Perraud et al., 2001; Yu et al., 2017, 2019). The TRPM2 channel is highly sensitive to ROS due to the potent capacity of ROS to promote ADPR generation (Jiang et al., 2010). Studies using transgenic TRPM2-knockout (TRPM2-KO) mice and/or derived cell cultures provide compelling evidence to show the TRPM2 channel expression in neurons, microglia, astrocytes, neuro-endothelial cells, and pericytes in the brain and demonstrate its crucial role in mediating brain damage induced by various pathological conditions (Li et al., 2015; Jiang et al., 2018; Malko et al., 2019; Mai et al., 2020). For example, a recent study introducing TRPM2-KO in the APP/PS1 mice has disclosed a key role of the TRPM2 channel in Aβ-induced synaptic deficits, microglial cell activation, and age-related impairment in learning and memory (Ostapchenko et al., 2015). Studies using cultured hippocampal neurons have revealed TRPM2 channel activation to be essential in a positive feedback loop that couples ROS/Aβ-induced NOX-mediated and mitochondrial ROS generation, lysosomal and mitochondrial dysfunction to neuronal cell death (Li et al., 2017; Li and Jiang, 2018, 2019). Studies using cultured microglial cells support a critical role for the TRPM2 channel in microglial cell activation, ROS generation, and production of proinflammatory cytokines induced by exposure to ROS, Aβ, and other pathological stimuli (Aminzadeh et al., 2018; Syed Mortadza et al., 2018). In addition, there is evidence to indicate a significant role for the TRPM2 channel in endothelial cells in mediating Aβ-induced oxidative damage to the neurovascular unit and BBB function (Park et al., 2014). As illustrated in **Figure 2**, accumulating evidence supports the roles of oxidative stress and the TRPM2 channel in AD-related pathologies via mediating Aβ-induced synaptic deficits, neuronal cell death, microglia-mediated neuroinflammation, and neurovascular and BBB dysfunction (Jiang et al., 2018).

A recent study has examined the effect of TRPM2-KO on age-related loss of cognitive function in mice (Kakae et al., 2019). There was noticeable decline in working and cognitive memory in middle-age WT mice at the age of 12–16 months and significant impairment in spatial memory in aged WT mice reaching 20–24 months compared with young WT mice of 2–3 months. Such age-related cognitive dysfunction in WT mice was not observed in age-matched TRPM2-KO mice. This study also has documented substantial white matter damage, loss of neuronal cells in hippocampus, and an increase in the number of Iba1-positive microglial/macrophage cells and CD3-positive T cells and a greater level of TNF-α in the corpus callosum and hippocampus in aged WT mice. Interestingly, all of these age-related detrimental effects were almost completely prevented by TRPM2-KO. These results provide clear evidence to suggest an important role for the TRPM2 channel in mediating age-related loss of cognitive function, at least in part via neuroinflammation.

Oxidative Stress and TRPM2 Channel Activation Are Important in NPs-Induced Damaging Effects

There is increasing evidence to show important roles for ROS generation and TRPM2 channel activation in mediating multiple cellular effects induced by ultrafine PM, particularly various types of NPs. For example, our study showed that in human embryonic kidney 293 (HEK293) cells expressing a low level of the TRPM2 channel, exposure to silica NPs for 3–6 h initially induced TRPM2-independent generation of ROS, which sufficiently activated the TRPM2 channel and up-regulated the NOX2 expression to further provoke oxidative stress and subsequent cell death (Yu et al., 2015). Intriguingly, silica NPs-induced cell death in HEK293 cells was attenuated by elevating the TRPM2 expression. This was due to selective and TRPM2-dependent down-regulation of the NOX4 expression and ROS generation. There was similar TRPM2 expression-dependence of silica NPs-induced cell death in bone marrow-derived macrophages. Collectively, this study suggests a dual role of the TRPM2 channel in NPs-induced effect on cell viability. A recent study also supports a significant role of the TRPM2 channel in mediating the cytotoxicity of mesoporous silica NPs to HEK293 cells (Mohammadpour et al., 2019). A separate study showed that exposure to lanthanide-based nanoparticles (LNs) induced NLRP3 inflammasome activation and IL-1β generation from LPS-primed mouse bone marrow-derived macrophages, human THP-1, and mouse peritoneal macrophages *in vitro* and also from mice intraperitoneally injected with LNs *in vivo* (Yao et al., 2016). LNs-induced NLRP3 inflammasome activation and IL-1β generation were reduced by inhibiting mitochondrial ROS

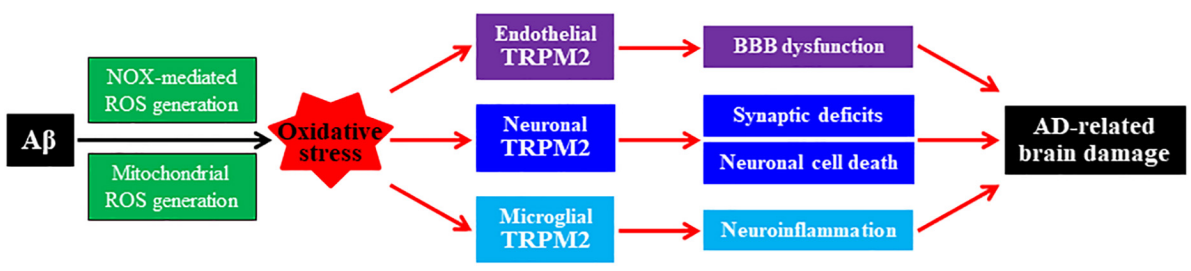

FIGURE 2 | Roles of oxidative stress and TRPM2 channel in mediating Aβ-induced AD-related brain damage. Chronic exposure to elevated levels of amyloid β peptide (Aβ) induces NADPH oxidase (NOX)-mediated and mitochondrial generation of reactive oxygen species (ROS), giving rise to oxidative stress in neuronal, microglial, and endothelial cells in the brain. Activation of the TRPM2 channels in these cells by ROS or under oxidative stress, respectively, mediates synaptic deficits, neuronal cell death, microglial cell activation and generation of neurotoxic proinflammatory mediators, neurovascular unit, and blood–brain barrier (BBB) dysfunction. These changes contribute to Alzheimer's disease (AD)-related brain damage leading to cognitive dysfunction.

generation and strongly suppressed by inhibiting NOX, and also by pharmacological inhibition of the TRPM2 channel or genetic depletion of the TRPM2 expression. These results support critical roles of ROS generation and subsequent TRPM2 channel activation in NPs-induced NLRP3 inflammasome activation and IL-1β generation (Yao et al., 2016). Our recent study shows that zinc oxide NPs (ZnO-NPs) induced brain pericyte cell death, which was prevented by siRNA-mediated knockdown of the TRPM2 expression in cultured pericytes and in mice by TRPM2-KO (Jiang et al., 2017). ZnO-NPs induced pericyte cell death was also suppressed by inhibiting nitric oxide synthase and scavenging peroxynitrite. Moreover, our study provides evidence to show that ZnO-NPs-induced TRPM2 protein nitration acts as a molecular inducer of autophagy that mediates pericyte cell death (Jiang et al., 2017). Collectively, accumulating evidence shows important roles of oxidative stress and TRPM2 channel in NPs-induced cellular effects.

CONCLUDING REMARKS

It is clear from the above discussion that epidemiological studies support association of PM2.5 exposure with increased risk to AD and age-related brain damage. Experimental studies consistently support causative relationships of PM2.5 exposure to AD and age-related pathologies and, in addition, identify engagement of multiple pathological factors such as oxidative stress and multiple pathways. Nonetheless, it is noticeable that the current understanding is largely piecemeal and remains poor with respect to the underlying molecular and signaling mechanisms. Emerging evidence also suggests important roles of oxidative stress and the TRPM2 channel in mediating various NPs-induced cellular effects, prompting an attractive hypothesis that oxidative stress and the TRPM2 channel play similar roles in mediating PM2.5 exposure-induced AD predisposition and age-related brain damage. Further investigations are required to support or refute this hypothesis. Our hypothesis, if proves true, raises an interesting perspective on targeting the TRPM2 channel as a preventative and therapeutic strategy to limit the risk of PM2.5 exposure to AD and age-related brain damage in humans.

AUTHOR CONTRIBUTIONS

L-HJ proposed and elaborated the concept and drafted the manuscript. LuW and YY elaborated the concept and revised the manuscript. All authors participated in the discussion, and revised and approved the manuscript.

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MINI REVIEW
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Pathophysiological Role of Transient Receptor Potential Mucolipin Channel 1 in Calcium-Mediated Stress-Induced Neurodegenerative Diseases

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Mucolipins (TRPML) are endosome/lysosome Ca^{2+} permeable channels belonging to the family of transient receptor potential channels. In mammals, there are three TRPML proteins, TRPML1, 2, and 3, encoded by *MCOLN1-3* genes. Among these channels, TRPML1 is a reactive oxygen species sensor localized on the lysosomal membrane that is able to control intracellular oxidative stress due to the activation of the autophagic process. Moreover, genetic or pharmacological inhibition of the TRPML1 channel stimulates oxidative stress signaling pathways. Experimental data suggest that elevated levels of reactive species play a role in several neurological disorders. There is a need to gain better understanding of the molecular mechanisms behind these neurodegenerative diseases, considering that the main sources of free radicals are mitochondria, that mitochondria/endoplasmic reticulum and lysosomes are coupled, and that growing evidence links neurodegenerative diseases to the gain or loss of function of proteins related to lysosome homeostasis. This review examines the significant roles played by the TRPML1 channel in the alterations of calcium signaling responsible for stress-mediated neurodegenerative disorders and its potential as a new therapeutic target for ameliorating neurodegeneration in our ever-aging population.

Keywords: neurodegenerative disease, TRPML1, lysosomal storage disease, oxidative stress, mitochondria, autophagy, Ca^{2+} signaling

INTRODUCTION

Neurodegenerative diseases entail progressive destruction and loss of neural cells and impairment of both motor and cognitive functions. They include Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic lateral sclerosis (ALS), as well as pathologies caused by lysosomal accumulation, such as Mucolipidosis type IV (MLIV) and Niemann-Pick disease (NPD).

All neurodegenerative disorders are marked by the accumulation of abnormally aggregated proteins and mitochondrial dysfunction. Some genes involved in PD or ALS are related to mitochondria, the main source of reactive oxygen species (ROS) in aging cells (Indo et al., 2015). In

addition, aggregated misfolded proteins can inhibit mitochondrial functions and induce oxidative stress (Abramov et al., 2017).

Several studies demonstrated the importance of maintaining the balance between oxidative stress and the antioxidant system (Li et al., 2013). Under physiological conditions, low levels of ROS are required in processes such as inflammation, synaptic plasticity, learning and memory. On the other hand, high ROS levels are dangerous for the cells themselves, due to their high reactivity against biological structures (Kishida and Klann, 2007). In this regard, the central nervous system is particularly susceptible to oxidative stress and its related damage, because of high oxygen consumption and poor counteracting antioxidant defenses.

These antioxidant defenses are generally classified as enzymatic or non-enzymatic. Among the former are superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, while the latter include glutathione, selenium and vitamins A, E and C (Rego and Oliveira, 2003).

Evidence indicates that transient receptor potential (TRP) channels play a central role in the modulation of oxidative stress and lysosome functions, in particular by regulating calcium ion influx and efflux (Sterea et al., 2018). In the TRP family, the mucolipin (TRPML) subfamily is of particular interest because it localizes to the endo-lysosomal compartment. The best-characterized member is TRPML1, encoded by the *MCOLN1* gene. TRPML1 is permeable to Ca^{2+}, Na^{+}, Fe^{2+}, Mg^{2+}, and K^{+} (Xu et al., 2007; Dong et al., 2008, 2009). It has an intraluminal loop that can be protonated activating the channel (Xu et al., 2007; Dong et al., 2008). It is activated by phosphatidylinositol-3,5-biphosphate (PtdIns(3,5)P2), voltage, low pH, and the synthetic compounds MK6-83 and ML-SA1 (Raychowdhury et al., 2004; Dong et al., 2010; Grimm et al., 2010; Shen et al., 2012; Zhang et al., 2012; Chen et al., 2014). It is inhibited by phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P2), sphingomyelins, and lysosomal adenosine (Shen et al., 2012; Zhang et al., 2012). Some studies indicate that TRPML1 is also involved in lysosomal storage, transportation and acidic homeostasis and in this way it promotes the cation efflux into the cytosol (Morgan et al., 2011). TRPML1 is also classified as an important regulator of autophagy, given that TRPML1 mutations affect lysosomal storage and lysosomal impairment is responsible for autophagy defects. TRPML1 can also be negatively regulated through the phosphorylation of Ser572 and Ser576 residues by the target of rapamycin (TOR) with a consequent autophagy decrease (Onyenwoke et al., 2015). Autophagy can target oxidized and damaged molecules for lysosomal degradation. ROS are able to induce autophagy and their major sources are mitochondria, localized in proximity of lysosomes (Elbaz-Alon et al., 2014; Li et al., 2015). Zhang et al. demonstrated that endogenous ROS are able to regulate lysosomal activities through the TRPML1 channel, which functions as a "ROS sensor" (Zhang et al., 2016). In this way, lysosomal Ca^{2+} release induces nuclear translocation of transcription factor EB (TFEB) (Medina et al., 2015), followed by autophagosome and lysosome biogenesis, induction of autophagic flux and re-establishment of redox homeostasis. Hence, we are interested in the interplay between TRPML1, calcium flux and neurodegenerative diseases.

There are two other members in the TRPML subfamily, TRPML2 and TRPML3, encoded by *MCOLN2* and *MCOLN3* genes. Like TRPML1, they are active in late endosomes/lysosomes; in addition, TRPML2 and TRPML3 are active in early endosomes, and TRPML2 also in recycling (Chen et al., 2017; Plesch et al., 2018). They have not been correlated with neurodegeneration in humans so far.

The aim of this review is to highlight the role of TRPML1 in neurodegenerative diseases, reporting the current data available in the literature. The following sections describe some of the most important neurodegenerative diseases, with attention to the role of TRPML1 functions.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by marked cognitive disabilities, ranging from memory loss to synapse disappearance. Pathologic changes occur in the brain such as pyramidal neuron damage, extracellular accumulation of β-amyloid aggregates and neurofibrillary tangles containing hyperphosphorylated Tau protein (Selkoe, 2001). A central hallmark of AD pathogenesis is Ca^{2+} dyshomeostasis. Mutations in the β-amyloid precursor protein (APP) or in presenilin (PS) 1/2, characteristics of familial AD, are associated with aberrant Ca^{2+} concentrations responsible for apoptosis and excitotoxicity in neurons (Yamamoto et al., 2007). In particular, models of AD show an atypical efflux of lysosomal Ca^{2+}, which leads to impaired autophagy, a process in which lysosomes degrade proteins or cytoplasmic organelles (Komatsu et al., 2006). Autophagy also contributes to β-amyloid secretion and metabolism, and its dysfunction is associated with the induction of neuronal lesions (Nixon, 2017). Related to autophagy, anomalies of the endosomal-lysosomal network are characteristic of AD. Studies performed in PS1 mutated neurons demonstrated that the loss of PS1 disrupts lysosome acidification and thus impairs autophagy.

In APP/PS1 transgenic mice, neuronal TRPML1 is downregulated, the mTOR pathway is inhibited and beclin and LC3 protein upregulated. Conversely, TRPML1 overexpression triggers autophagy by activating the mTOR pathway (Zhang et al., 2017) thus diminishing neuronal apoptosis. When primary neurons, isolated from hippocampus of APP/PS1 transgenic mice, were treated with β-amyloid peptides, cell viability was impaired and lysosomal Ca^{2+} concentration was reduced. The upregulation of TRPML1 expression is able to strongly attenuate these effects, and thus it is possible that the channel plays an important role in the maintenance of lysosomal homeostasis (Zhang et al., 2017).

Lee and coworkers demonstrated that PS1 knock-out (KO) cells, used as model of early AD, display elevated lysosomal pH due to vATP-ase deficiency. This alkaline lysosomal pH inhibits the function of the two-pore channel 2 (TPC2) and stimulates an abnormal TRPML1-mediated depletion of lysosomal Ca^{2+} (Lee et al., 2015). Their results indicated that the endogenous TRPML1

is present in a hyperactive state in PS1 KO cells and contributes to Ca^{2+} efflux from lysosomes thus leading to autophagy impairment. In addition, the observation that treatment of PS1 KO cells with an inhibitor of NAADP-dependent channels resets Ca^{2+} homeostasis suggests that there is a complex interplay between TRPML and NAADP signaling. However, normalization of Ca^{2+} levels is not able to reverse proteolytic and autophagic defects in PS1 KO cells. Rather, the associated changes in lysosomal pH appear to be more functionally significant. No data are reported in this study about changes in β-amyloid peptide ratio, production and clearance; thus, the involvement, in β-amyloid alterations, of the lysosomal Ca^{2+} efflux evoked by TRPML1 has not been clarified so far in PS1 KO cells.

However, in a triple transgenic gp120/APP/PS1 mouse model, a role of TRPML1 in the regulation of β-amyloid peptide clearance has been suggested. In fact, there is evidence in the brain of HIV-infected patients that β-amyloid peptides accumulate causing cognitive deficits that overlap with those of the AD. It has been demonstrated that the viral protein gp120 promotes the accumulation of β-amyloid peptides, sphingomyelin and Ca^{2+} inside lysosomes and autophagic compartments. The activation of TRPML1, by its agonists, induces Ca^{2+} efflux from lysosomes with consequent pH acidification that promotes the clearance of intraneuronal β-amyloid/sphingomyelin deposits (Bae et al., 2014). So, these findings showed that the induction of lysosomal acidification by activating the TRPML1-induced Ca^{2+} efflux reduces the deposition of β-amyloid peptides in the HIV-infected brain.

Among the potential factors implicated in AD, an impairment of the blood brain barrier (BBB), responsible for the increase in LDL flux from the peripheral circulation into the brain, has been described. Moreover, high plasma levels of cholesterol are found to be able to compromise the BBB. Once inside the brain, LDL can enter into endolysosomes and deacidify them, thus blocking their function. This mechanism is responsible for the LDL-induced increases in β-amyloid peptides generation in neurons. It has been demonstrated that the TRPML1 agonist ML-SA1 is able to prevent LDL-induced increases in β-amyloid peptides, while TRPML1 silencing potentiates LDL-induced effects (Hui et al., 2019).

PARKINSON'S DISEASE

Parkinson's disease (PD) is characterized by the progressive degeneration of the dopaminergic neurons located in the substantia nigra pars compacta (SNc) (Lima et al., 2009). The main hallmarks of PD are progressive neuronal loss and intracellular inclusions known as Lewy bodies and neurites, predominantly composed of misfolded and aggregated forms of α-synuclein (Lotharius and Brundin, 2002). The causes involved are mitochondrial dysfunction and oxidative stress supported by *PTEN-induced kinase 1*, *Parkin*, *Protein deglycase-1*, and *Leucine-rich repeat kinase 2 (LRRK2)* genes that regulate mitochondrial and ROS homeostasis (Kilpatrick, 2016).

Recent studies have reported that in Parkinson's disease, the mitochondrial Ca^{2+} dynamics are altered when impaired formation of membrane connections between mitochondria and the endoplasmic reticulum (ER) or other components of Ca^{2+} signaling cause neurodegeneration in SNc neurons, which are already vulnerable due to excessive Ca^{2+} influx. Indeed, SNc neurons are subjected to an excessive influx of Ca^{2+} through voltage-gated calcium (Cav1.3) channels (Guzman et al., 2009). This Ca^{2+} exposure comes at an energetic cost to mitochondria. As a result, neurons experience oxidative stress, which might make them less tolerant to stressors (Guzman et al., 2010).

Since mitochondria, ER and lysosomes communicate through Ca^{2+} signals, and since TRPML1, like other endo-lysosomal Ca^{2+} channels, crosstalks with ER Ca^{2+} channels, it may be that alterations in TRPML1 activity contribute to PD (Kilpatrick, 2016).

Lysosomes are also involved in endocytic, autophagic and secretory pathways. Since lysosomes degrade α-synuclein through chaperone-mediated autophagy (CMA) (Cuervo et al., 2004), the accumulation of α-synuclein implicates lysosomal dysfunction in PD. Lysosomal Ca^{2+} content is impaired in a beta-glucocerebrosidase GBA1-mutated PD model and is related to altered endo-lysosomal morphology. In addition, the LRRK2-mutated PD model shows deregulated lysosomal Ca^{2+} signaling and altered morphology. It has been suggested that excessive activation of TRPML channels, caused by changes in lysosomal pH, depletes lysosomal Ca^{2+} (Lee et al., 2015). If this is the case, then the increased NAADP-evoked Ca^{2+} signals measured in LRRK2-mediated PD (Hockey et al., 2015) probably drain the lysosomes of Ca^{2+}.

Moreover, in a PARK9-mutant PD model, the loss of lysosomal type 5 P-type ATPase function leads to α-synuclein accumulation. Indeed, PARK9 regulates lysosomal exocytosis, a pathway that could be potentiated to reduce α-synuclein accumulation. Tsunemi demonstrated that TRPML1 agonists are able to increase lysosomal exocytosis, thus impairing α-synuclein intracellular levels (Tsunemi et al., 2019).

Since neuroinflammation seems to be essential for PD pathogenesis (Whitton, 2007; Ransohoff, 2016), Gao et al. (2003) conceived a PD mouse model based on treatment with the inflammogen lipopolysaccharide (LPS) plus the neurotoxin 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP). In this model, NADPH-oxidase-dependent ROS generation has a central role (Gao et al., 2003). Through metabolomics analysis, Huang et al. exploited the LPS-MPTP model to identify adenosine and adenosine deaminase (ADA) as the most promising therapeutic targets for PD (Huang et al., 2019). Previously it had already been demonstrated that the neuromodulator adenosine is able to weaken oxidative stress and excitotoxicity (Fredholm, 2007; Min et al., 2008). However, its use is limited by several adverse side effects, rapid metabolism and difficulty in penetrating the blood brain barrier (Phillis and Wu, 1981). Therefore, increasing its local release through the inhibition of ADA could be a promising approach. Indeed, compared to controls, mice exposed to MPTP have impaired adenosine concentration and increased ADA activity. Treatment with the ADA inhibitor deoxycoformycin (DCF) is able to reverse the effects of MPTP (Huang et al., 2019). However, in lysosomes of ADA mutant B-lymphocytes, adenosine accumulation impairs TRPML1 activity and triggers

lysosome enlargement and dysfunction (Zhong et al., 2017). These data suggest that the lack of TRPML1 activity could lead to an increased susceptibility to oxidative stress and cell death. Therefore, rigorous experiments should be conducted to further explore the possible role of TRPML1 as a therapeutic target in PD.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that leads to progressive loss of motor neurons in the anterior horn of the spinal cord, with muscle weakness, wasting, and spasticity (Kiernan et al., 2011). It is classified as either sporadic or familial: for familial ALS mutations in superoxide dismutase 1 (SOD1) enzyme, TAR DNA binding protein 43 and proteins involved in autophagic pathway and lysosome function are present (Chen et al., 2013). The latter two are regulated by intracellular Ca^{2+} flux inside the cell. In particular, lysosomal Ca^{2+} can be released by intracellular signals, such as NAADP (Kauppila et al., 2017) and PI(3,5)P2 (López-Otín et al., 2013). TRPML1 could play an important role in restoring autophagy and lysosome function in ALS, given that Ca^{2+} release is crucial for lysosome function, that TRPML1 is implicated in lysosomal Ca^{2+} release, and that PI(3,5)P2 levels are impaired in ALS. In the ALS mouse model, chronic exposure to the neurotoxin L-BMAA impairs autophagy in primary motor neurons, leading to ER stress and cell death (Tedeschi et al., 2019a; **Figure 1**). In these neurons, TRPML1 protein levels are downregulated; however, early channel activation induced by the ML-SA1 agonist is able to counteract TRPML1 impairment and reduce ER stress proteins and Caspase-9 upregulation, thus rescuing motor neurons from death. Under normal conditions in motor neuronal cells, the lysosomal TRPML1 colocalizes with the ER Ca^{2+} sensor STIM1, which suggests that there is cross-talk between ER and lysosomes, in which lysosomal Ca^{2+} efflux through TRPML1 plays a pivotal role (Tedeschi et al., 2019b). The depletion of ER Ca^{2+} stores affects the lysosomal Ca^{2+} release that takes place through the action of TRPML1. These data suggest that ER is a key source of lysosomal Ca^{2+} in motor neurons, as demonstrated also in HEK 293 cells that stably express GCaMP3-TRPML1 (Garrity et al., 2016); altered Ca^{2+} homeostasis in one of these organelles has dramatic implications on the other stores (Tedeschi et al., 2019a).

Furthermore, ER dysfunction is common to different forms of ALS, from sporadic ALS, which is characterized by misfolding of wild-type SOD1, to the L-BMAA-induced ALS mouse model. Tedeschi et al. demonstrated that the agonist ML-SA1 is able to prevent the increase of ER stress markers. Thus it can be assumed that the proximity to ER store and lysosomes means that lysosomal Ca^{2+} release through TRPML1 may contribute to ER Ca^{2+} concentration and stress prevention by continuous refilling of Ca^{2+} (Tedeschi et al., 2019b).

MUCOLIPIDOSIS TYPE IV

Mucolipidosis type IV (MLIV) is an autosomal recessive lysosomal storage disorder due to *MCOLN1* gene mutations (Bargal et al., 2000; Sun et al., 2000). Neurodegeneration with spasticity, hypotonia, and the inability to walk independently are common hallmarks (Altarescu et al., 2002).

Some publications have connected TRPML1 mutations with the lower lysosomal pH registered in MLIV patients compared with normal control (Raychowdhury et al., 2004; Soyombo et al., 2006), although these results are different from data reported by Bach (Bach et al., 1999). In particular, Soyombo et al. has demonstrated that TRPML1 is able to prevent lysosomal overacidification because it is permeable to H^+ and thus it dissipates high H^+ concentration to maintain lysosomal homeostasis under normal condition (Soyombo et al., 2006). In the absence of TRPML1 regulation of pH, the acidic conditions result in the dysfunction of lysosomal hydrolase and thus substrates accumulation.

Given its permeability to Ca^{2+}, TRPML1 activation is required to allow the attachment of vesicles to motor proteins along the microtubules and the fusion with plasma membrane in normal cells. In MLIV, the loss of TRPML1 function is related to defects in lysosomal biogenesis and exocitosis (LaPlante et al., 2006).

Fibroblasts of MLIV patients contain soluble protein and lipid aggregates (Bach, 2001; Altarescu et al., 2002; Smith et al., 2002) due to abnormal sorting and/or transport of these macromolecules along the late endocytic pathway (Bargal and Bach, 1997; Chen et al., 1998). Typical aspects of MLIV are mitochondrial fragmentation and decreased mitochondrial Ca^{2+} buffering efficiency (Jennings et al., 2006; Kiselyov et al., 2007b). Since lysosomes are significant players in the autophagic recycling of mitochondria, defects in their function may affect recycling and thus lead to the storage of fragmented mitochondria and the failure to buffer cytoplasmic Ca^{2+}. The reduced buffering capacity could make cells more sensitive to pro-apoptotic signals (Kiselyov et al., 2007a; Venugopal et al., 2009; Demers-Lamarche et al., 2016).

As described above, TRPML1 induces TFEB transcriptional activity, and TRPML1 is itself the target of TFEB (Medina et al., 2015). This creates a feedback loop that activates autophagy. In addition, a new TFEB-independent pathway has been demonstrated (Scotto Rosato et al., 2019). Acute activation of TRPML1 is able to increase phagophore formation, thus activating calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) and AMP-activated protein kinase (AMPK), and also inducing the formation of the Beclin1/VPS34 autophagic complex and the production of phosphatidylinositol 3-phosphate (PI3P). PI3P-enriched ER subdomains act as platforms for phagophore formation. These results are of considerable importance because in the cells of MLIV patients, defective production of PIP3 impairs recruitment of PI3P-binding proteins (WIPI2 and DFCP1) to the phagophore during autophagy induction.

Moreover, TRPML1 could also have a role in the preservation membrane potential useful for the efficient transport of chaperone-mediated autophagy (CMA) substrate proteins for degradation. The intraluminal loop of TRPML1 seems to interact directly with heat shock cognate protein of 70 kDa and heat shock proteins of 40 kDa, members of a molecular chaperone complex required for protein transport into the lysosome during CMA

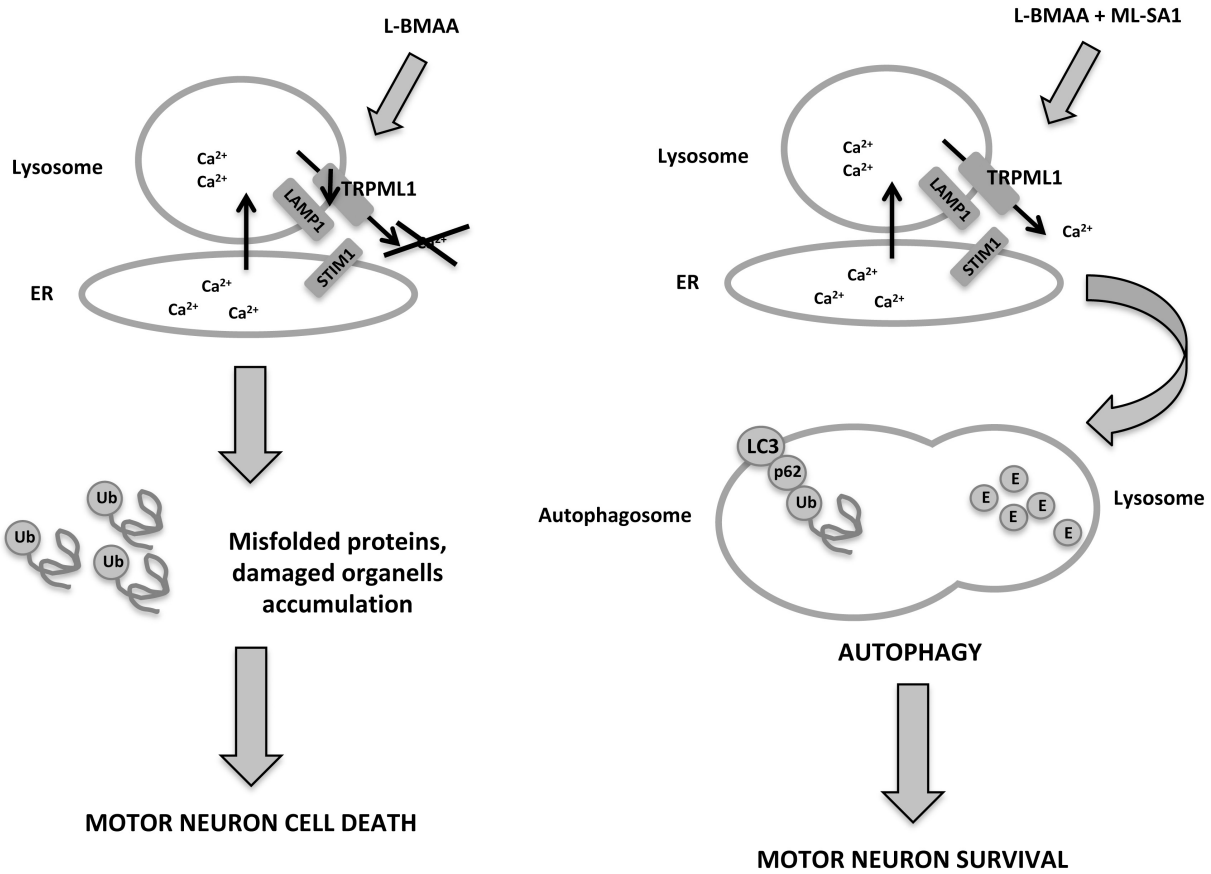

FIGURE 1 | In a neurotoxin L-BMAA-induced ALS mouse model, TRPML1 is downregulated, autophagy is impaired and motor neurons die from accumulation of misfolded proteins. However, administration of the TRPML1 agonist, ML-SA1, activates the channel and leads to lysosomal Ca^{2+} release, autophagic flux and motor neuron survival.

(Venugopal et al., 2009). Of note, lysosomes from MLIV patients exhibit a reduction in CMA. Also, in MLIV lysosomes there is a reduced amount of lysosomal-associated membrane protein type 2A essential for the chaperones complex bound to the lysosome membrane. Related to the impairment of CMA, MLIV fibroblasts increase the oxidized protein levels that sensitize neurons to apoptosis, thus leading to neuronal degeneration (Venugopal et al., 2009).

Neurodegenerative effects have been also correlated to zinc accumulation in lysosomes in MLIV fibroblasts or in *TRPML1*-knockdown HEK-293 cells (Eichelsdoerfer et al., 2010). This accumulation is not reverted by treatment with the TRPML1 agonist MK6-83; in contrast, treatment with MK6-83 significantly reduces zinc accumulation in F408del TRPML1 mutant-expressing fibroblasts (Chen et al., 2014).

Several patients show *MCOLN1* gene mutations that introduce premature stop signals and result in an absent TRPML1 protein, or a protein lacking the ion-conducting pore between TMD5 and TMD6. Some have single point mutations that maintain an open reading frame (Altarescu et al., 2002; Manzoni et al., 2004), some have mislocalized proteins, some have TRPML1 correctly localized but incapable of responding to endogenous ligands. In the latter situation, there may be promise in therapy based on the use of an agonist of TRPML1 to enhance its activity. Indeed, *in vitro* results demonstrated that small-molecule ligands are able to recover endogenous channel activity and also endo-lysosomal trafficking defects and accumulation of zinc (Chen et al., 2014).

NIEMANN-PICK DISEASE

Niemann-Pick diseases (NPD) are lipid storage pathologies associated with central nervous system impairment due to lipid accumulation (Patterson and Walkley, 2017; Torres et al., 2017). Three types of NPD have been identified. Types A and B are characterized by deficient activity of acid sphingomyelinase, which degrades lysosomal sphingomyelin; type C shows defective function in cholesterol efflux from lysosomes (Patterson and Walkley, 2017; Schuchman and Desnick, 2017; Torres et al., 2017) as a consequence of mutation in *NPD type C1 (NPC1)* or *NPD type C2 (NPC2)* genes, responsible for cholesterol transport. This causes an increase in concentration of cholesterol with accumulation of unesterified cholesterol in late endosomes/lysosomes. Accumulation of sphingomyelin and

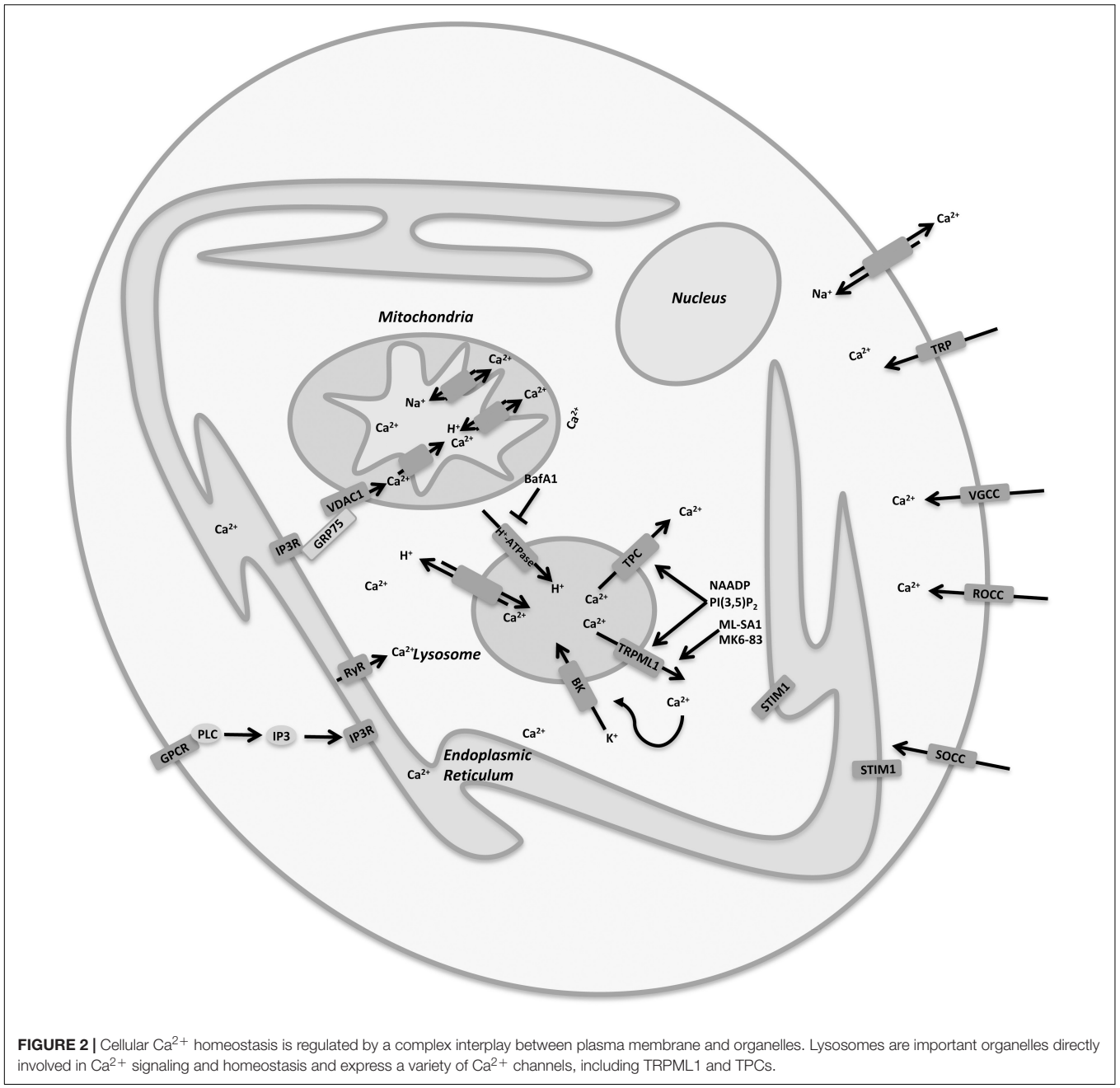

FIGURE 2 | Cellular Ca^{2+} homeostasis is regulated by a complex interplay between plasma membrane and organelles. Lysosomes are important organelles directly involved in Ca^{2+} signaling and homeostasis and express a variety of Ca^{2+} channels, including TRPML1 and TPCs.

cholesterol affects lysosomal Ca^{2+} release and blocks endocytosis and fusion between late endosomes and lysosomes, resulting in endocytosis and autophagy dysfunction (Samie and Xu, 2014; Höglinger et al., 2019; Tancini et al., 2019).

Sphingomyelin is able to inhibit Ca^{2+} efflux through the TRPML1 channel. Therefore, by inhibiting TRPML1 activity, the accumulation of sphingomyelin could influence both lysosomal pH and Ca^{2+} signaling through ER and mitochondria (Lloyd-Evans and Platt, 2010; Wheeler et al., 2019). Moreover, TRPML1 forms a complex with the large conductance Ca^{2+}-activated K^+ channels (BK) in lysosomes. The BK channels are activated by TRPML1-mediated Ca^{2+} release to maintain the negative membrane potential needed for sustained lysosomal Ca^{2+} release (Cao et al., 2015). Either TRPML1 or BK deficiency results in lysosomal Ca^{2+} accumulation, defective lysosomal membrane trafficking, and lysosome storage. Furthermore, upregulation of TRPML1 or BK reverses the impaired lysosome Ca^{2+} release and membrane trafficking in NPC1 fibroblasts. Moreover, in NPC1 or NPC2 KO HeLa cells, cholesterol accumulates in late endosomes, and the treatment with 2-hydroxypropyl-ß-cyclodextrin reduces cholesterol content (Vacca et al., 2019). Here TRPML1 silencing abrogates this effect: this may suggest that TRPML1 is directly implicated in the regulation of endo-lysosome secretion.

DISCUSSION

Neurodegenerative diseases are serious health problems. Numerous efforts have been made to identify neuropathological, biochemical and genetic biomarkers for them. Mitochondrial function and resistance to oxidative stress are compromised during the aging phase, and this is a starting point for the onset of neurodegenerative diseases (Cenini et al., 2019). Other factors that promote oxidative stress are excitotoxicity and aberrant protein processing, which lead to outcomes such as impairment of lysosome integrity. Given that lysosomes are the major contributors to autophagic recycling of mitochondria, to misfolded protein and to damaged organelles, it may be that defects in lysosome function affect mitochondrial recycling, cause accumulation of fragmented mitochondria, and block the ability to buffer cytoplasmic Ca^{2+}, and that these processes in turn sensitize cells to pro-apoptotic signals. In this regard, several reports suggest that lysosomal Ca^{2+} impairment is involved in the pathogenesis of neurodegenerative diseases. For this reason, the calcium channels expressed on lysosomes have been attracting a lot of attention lately, especially as potential new targets for fighting neurodegeneration. It is now well known that TPCs and TRPMLs are the two main calcium permeable receptor families expressed on lysosomes. However, the pharmacology of these receptors has not yet been well elucidated and still requires further studies. In addition, new findings are necessary to clarify if the Ca^{2+} efflux from lysosomes is helpful or not. In fact, contradictory data are present in the scientific literature. According to some researchers, the accumulation of calcium in lysosomes seems to be associated with lysosomal dysfunctions in neurodegenerative diseases (Bae et al., 2014). Others, instead, suggest that the inhibition of the NAADP-induced Ca^{2+} mobilization is beneficial in some experimental models of neurodegenerative diseases. It is well established that the NAADP-induced lysosomal Ca^{2+} efflux is dependent on TPCs (Brailoiu et al., 2010; Yamaguchi et al., 2011; Morgan et al., 2015; Pitt et al., 2016; Grimm et al., 2017; Patel et al., 2017). However, recent findings also indicate that TRPML1 is a target of NAADP, thus supporting the view that it plays a role in endosome/lysosome interaction, lipid trafficking and alterations in autophagy machinery (Lee et al., 2015). In fact, it has also been shown that in a *MCOLN1*$^{-/-}$ fibroblast model, NAADP action is abolished, an observation that suggests that NAADP-TRPML1 signaling plays a significant role (Zhang et al., 2011).

In neurons, the regulation of Ca^{2+} concentrations in each cellular compartment is essential for the maintenance of normal cellular functions and for neuronal plasticity (Ureshino et al., 2019). Ca^{2+} buffering is controlled by the interplay between ER, mitochondria and lysosomes that express Ca^{2+} transport mechanisms such as TRPML channels. Moreover, Ca^{2+} mobilization is regulated by several cation channels expressed in the plasma membrane involved in the cation exchange with the microenvironment. It is definitively clear that the imbalance of Ca^{2+} concentrations is strongly involved in the pathogenesis of neurodegenerative diseases, as in these pathologies there is often an evident defect in intracellular calcium storage. In fact, in many different experimental models of neurodegeneration, Ca^{2+} mobilization from organelles to cytoplasm or vice versa is impaired. However, it is still difficult to clarify whether Ca^{2+} plays the same role in the different neuronal disorders, especially because it functions as a messenger in an intricate network regulated by the ER/mitochondria/lysosome axis involving both pro-survival and death pathways (**Figure 2**; Ureshino et al., 2019). Therefore, calcium dyshomeostasis in both lysosome and cytoplasm is detrimental. In this regard, there is no doubt that channel dysfunctions are manifest in vesicular trafficking defects, and further work is required to delineate the affected processes more precisely.

As shown in this review, calcium imbalance, lysosomes and oxidative stress, as well as the function of TRPML1 seem to be highly significant in the neurodegenerative diseases described. Unfortunately, to date little data is available linking TRPML channels and neurodegeneration, and more studies are needed in order to clarify the role of these channels. In conclusion, a deeper understanding of the exact mechanism of neurodegeneration will offer a valid starting point for the development of new therapeutic strategies, and in this regard TRPML1 is turning out to be a key candidate.

AUTHOR CONTRIBUTIONS

GS and MM contributed to the conception and design. MM, FM, and CA drafted the manuscript. OM, MN, and GS contributed to the critical revision of the manuscript. MM and CA re-examined and revised the manuscript.

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TRPV1 Tunes Optic Nerve Axon Excitability in Glaucoma

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The transient receptor potential vanilloid member 1 (TRPV1) in the central nervous system may contribute to homeostatic plasticity by regulating intracellular Ca^{2+}, which becomes unbalanced in age-related neurodegenerative diseases, including Alzheimer's and Huntington's. Glaucomatous optic neuropathy – the world's leading cause of irreversible blindness – involves progressive degeneration of retinal ganglion cell (RGC) axons in the optic nerve through sensitivity to stress related to intraocular pressure (IOP). In models of glaucoma, genetic deletion of TRPV1 (*Trpv1*$^{-/-}$) accelerates RGC axonopathy in the optic projection, whereas TRPV1 activation modulates RGC membrane polarization. In continuation of these studies, here, we found that *Trpv1*$^{-/-}$ increases the compound action potential (CAP) of optic nerves subjected to short-term elevations in IOP. This IOP-induced increase in CAP was not directly due to TRPV1 channels in the optic nerve, because the TRPV1-selective antagonist iodoresiniferatoxin had no effect on the CAP for wild-type optic nerve. Rather, the enhanced CAP in *Trpv1*$^{-/-}$ optic nerve was associated with increased expression of the voltage-gated sodium channel subunit 1.6 (NaV1.6) in longer nodes of Ranvier within RGC axons, rendering *Trpv1*$^{-/-}$ optic nerve relatively insensitive to NaV1.6 antagonism via 4,9-anhydrotetrodotoxin. These results indicate that with short-term elevations in IOP, *Trpv1*$^{-/-}$ increases axon excitability through greater NaV1.6 localization within longer nodes. In neurodegenerative disease, native TRPV1 may tune NaV expression in neurons under stress to match excitability to available metabolic resources.

Keywords: glaucoma, transient receptor potential vanilloid member 1, optic nerve, compound action potential, nodes of Ranvier, NaV1.6

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INTRODUCTION

Transient receptor potential vanilloid member 1 (TRPV1) channels are activated by both physiologically relevant and pathological stimuli, conducting large Ca^{2+} currents that initiate downstream signaling cascades (Caterina et al., 1997; Hui et al., 2003; Patapoutian et al., 2009; Weitlauf et al., 2014). TRPV1 channels densely accumulate in nociceptor cells of dorsal root ganglia to transduce noxious sensory input into the electrochemical responses of the spinal nerve (Simone et al., 1989; Caterina et al., 1997, 2000; Bolcskei et al., 2005). Recent evidence shows widespread TRPV1 expression in the central nervous system (CNS) tissues, including the cortex, hippocampus, hypothalamus, and retina (Mezey et al., 2000; Roberts et al., 2004; Cristino et al., 2006; Sappington et al., 2009, 2015; Jo et al., 2017; Lakk et al., 2018). TRPV1 has also been implicated in neurodegenerative disorders such as Alzheimer's disease (Jayant et al., 2016;

Balleza-Tapia et al., 2018), Parkinson's disease (Marinelli et al., 2003; Morgese et al., 2007; Nam et al., 2015; Chung et al., 2017), Huntington's disease (Lastres-Becker et al., 2003), and glaucomatous optic neuropathy, or glaucoma (Ward et al., 2014; Weitlauf et al., 2014). Glaucoma is the leading cause of irreversible blindness (Quigley and Broman, 2006), involving sensitivity to intraocular pressure (IOP) that stresses retinal ganglion cell (RGC) axons as they form the optic nerve (Calkins, 2012). Many RGCs express TRPV1 channels (Jo et al., 2017; Lakk et al., 2018), localizing to dendrites, unmyelinated axon segment, and cell body, where it increases with short-term elevations in IOP (Sappington et al., 2009, 2015; Weitlauf et al., 2014) but is negligible within the optic nerve itself (Choi et al., 2015). Even so, *Trpv1*$^{-/-}$ accelerates optic nerve degeneration with elevated IOP and increases the depolarization necessary for RGCs to produce action potentials (Ward et al., 2014; Weitlauf et al., 2014). To better understand the early stages of this acceleration, we investigated how *Trpv1*$^{-/-}$ influences physiological signaling along the optic nerve with short-term elevations in IOP. We found that enhanced excitability in *Trpv1*$^{-/-}$ optic nerve was associated with longer axonal nodes of Ranvier with greater levels of the voltage-gated sodium channel, NaV1.6. These results suggest a role for TRPV1 in native tissue to regulate NaV in response to disease-relevant stressors. The absence of this tuning in *Trpv1*$^{-/-}$ mice suggests that accelerated axonopathy could arise from excessive excitation even as elevated IOP stresses match available metabolic resources in the optic projection to the brain (Baltan et al., 2010; Calkins, 2012).

MATERIALS AND METHODS

Animal Experiments

Adult male *Trpv1*$^{-/-}$ (B6.129 × 1-Trpv1^{TM1Jul}/J) mice (1.5–2 months old) were obtained from The Jackson Laboratory, whereas the appropriate wild-type (WT) background strain C57Bl/6 mice were purchased from Charles River Laboratories (male, 1.5–2 months old). The *Trpv1*$^{-/-}$ mice have a targeted mutation causing a non-functional truncated form of TRPV1 (Caterina et al., 2000; Ren et al., 2019; Stanford et al., 2019). *Trpv1*$^{-/-}$ animals were genotyped prior to performing experiments, following our protocol (Ward et al., 2014; Weitlauf et al., 2014; Sappington et al., 2015) using primers recommended by the vendor. The mutant forward primer was TAA AGC GCA TGC TCC AGA CT compared with the WT forward primer of TGG CTC ATA TTT GCC TTC AG. The common primer was CAG CCC TAG GAG TTG ATG GA. DNA gel electrophoresis of *Trpv1*$^{-/-}$ animals showed a single band at 176 bp, indicative of truncated TRPV1 (Caterina et al., 2000; Ren et al., 2019; Stanford et al., 2019), whereas WT showed a single band at 289 bp indicative of the native protein. We verified this pattern in each animal utilized.

Mice were maintained in a 12 h light/dark cycles, and animals were allowed water and standard rodent chow as desired. All animal experiments were approved by The Vanderbilt University Medical Center Institutional Animal Care and Use Committee. Baseline IOP was measured bilaterally in anesthetized (2.5% isoflurane) mice using Tono-Pen XL (Medtronic Solan) for 1–2 days prior to experimental manipulation. Baseline IOP measurements were averaged (day 0). After baseline IOP measurements, unilateral elevation of IOP was induced by injecting 1.5 μl of 15 μm polystyrene microbeads (Invitrogen) into the anterior chamber; the fellow eye received an equal volume of sterile saline to serve as control. We measured IOP 2–3 times per week for 2 weeks as described previously (Crish et al., 2010; Weitlauf et al., 2014; Risner et al., 2018).

Optic Nerve Compound Action Potential Electrophysiology

Animals were euthanized by cervical dislocation and decapitated. The skull was cut along the sagittal suture and removed, and the optic nerves were sectioned from the brain. Optic nerves were cut at the optic chiasm and posterior to the optic nerve head, and nerves were placed in carbogen-saturated (95% O_2, 5% CO_2) ice-cold (4°C) artificial cerebrospinal fluid (aCSF) for 30 min (Wang et al., 2012). The aCSF contained (in mM/L) 124 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 23 $NaHCO_3$, and 10 glucose (Baltan et al., 2010). The pH of the aCSF was 7.4.

Optic nerves were incubated in ice-cold aCSF to slow metabolism because we recorded from optic nerves one at a time. The first nerve recorded from (saline- or microbead-injected eyes) was alternated daily to avoid any possible order effects. After incubation, one optic nerve was transferred into a physiological chamber (Model PH1, Warner Instruments) and continually perfused at a rate of 2 mL/min using a peristaltic pump (Model 7518, Masterflex) and maintained at 35°C (Model TC-344C, Warner Instruments). Optic nerves adjusted to physiological conditions for 30 min prior to recording. After adjustment to physiological conditions, the rostral end of the optic nerve was positioned into a bipolar recording suction electrode (Model 573040, A-M Systems), and the caudal end of the optic nerve was positioned into a custom-made bipolar stimulating suction electrode. The syringe section of each electrode was attached to separate micromanipulators (Model MM33, WPI) to allow fine positioning of the electrodes. The electrode section of the suction electrodes was fabricated from borosilicate glass (Model TW150-4, WPI) that was heat-pulled (Model P2000, Sutter Instruments) to form an average opening of ∼350 μm in diameter. The stimulating electrode contained a Ag wire, and the recording pipette contained a Ag/AgCl wire; both pipettes were filled with aCSF.

Evoked potentials were bandpass filtered (0.0001–10 kHz), amplified (100 × gain, DAM-60, WPI), digitized (Digidata 1440A, Molecular Devices), and sampled at 50 kHz (Clampex 10.6, Molecular Devices). Afterward, we measured the resistance between the nerve and recording pipette by stimulating the nerve with 10-μs 100-μA pulses at a minimum of three positions along the optic nerve and measuring the compound action potential (CAP) (Model ISO-STIM 01-DPI, NPI). The resistance of the optic nerve and pipette at each spatial position along the nerve was computed using Ohm's law.

Current-evoked CAPs were obtained for at least three spatial positions along each optic nerve. Thus, at each spatial position, the resistance between the recording pipette and nerve was unique. We then plotted the resultant CAP area obtained at each spatial position as a function of resistance. Then, we obtained the slope of the linear regression of these data. The slope of the data represents an approximation of the current-induced voltage output of the nerve (Stys et al., 1991).

In a subset of experiments, CAPs were evoked with brief, 10 μs, square pulses, ranging from 10 to 200 V, every 30 s until a maximal response was produced. Maximal response was defined by the peak of the CAP. Once we determined the voltage required to produce a maximum response, we challenged optic nerve excitability by bath application of 300 and 600 nM of 4,9-anhydrotetrodotoxin (aTTX; Alomone Labs) or 100 nM of iodoresiniferatoxin (IRTX; Tocris). After 5 min of drug application, an evoked CAP was obtained using the max-response stimulus previously determined under normal bath conditions. To assess excitability within the optic nerve, we computed the percent decrease or percent of baseline of the evoked CAP based on before and after drug responses.

At the end of each recording session, optic nerves were placed in 4% paraformaldehyde overnight at −4°C. Afterward, we placed nerves on slides, imaged nerves on a microscope slide micrometer, and quantified length and width using the "segmented line" tool in ImageJ [Version 1.51i, National Institutes of Health (NIH)]. The average optic nerve width for WT and *Trpv1*$^{-/-}$ mice was 0.329 ± 0.004 and 0.333 ± 0.003 mm, respectively. There was no difference in optic nerve length between genotypes (p = 0.45) or between experimental condition (p = 0.56).

Optic Nerve Immunohistochemistry, Imaging, and Analysis

For optic nerve sections, mice were first perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Optic nerves were placed separately into optimal cutting temperature (OCT) compound (Fisher Scientific). Optic nerves were sectioned longitudinally every 7 μm, taking care to keep the nerves as flat as possible. Sections were first blocked with 5% normal donkey serum for 2 h and then incubated in primary antibodies for 3 days at 4°C with gentle shaking. Primary antibodies used for optic nerve sections were mouse-contactin-associated protein 1 (Caspr1, 1:300, Millipore) and rabbit-NaV1.6 (1:200, Alomone). Confocal micrographs of all sections were acquired using an Olympus FV1000 inverted microscope with 100 × objective and 2 × zoom.

Optic nerve node–paranode complexes were assessed using similar methods as Arancibia-Cárcamo et al. (2017). To determine the length of the node and paranode segments for each node–paranode complex, the following analysis was performed for each complex using a series of custom-written MATLAB functions: First, the most prominent trough of the Caspr1 staining intensity profile was noted, and the location of its minimum point identified. Next, the most prominent peak to both the right and left of this minimum point was identified. These maxima were averaged, and half of the average value was used to define a threshold intensity value to distinguish node and paranode segments. For each of the two identified peaks, the contiguous region surrounding the peak and above the threshold was considered paranode, whereas the region between the two paranode segments and under the threshold was considered node. The length of these segments and their average staining intensity (Caspr1 for paranode and NaV1.6 for node) were calculated.

All data are presented as mean ± SEM. Graphs were made using Sigma Plot Version 14 (Systat, San Jose, CA, United States). Statistical analyses were performed using Sigma Plot and Matlab (R2019a, Natick, MA, United States). Parametric statistics were performed (t-tests, ANOVAs) if data passed normality and equal variance tests; otherwise, we performed non-parametric statistics (Mann–Whitney, ANOVA on ranks).

RESULTS

Trpv1$^{-/-}$ Following Short-Term Intraocular Pressure Elevation Increases Optic Nerve Excitability

Following our protocol for conformational genotyping (Ward et al., 2014; Weitlauf et al., 2014; Sappington et al., 2015), *Trpv1*$^{-/-}$ mice showed a single product band at 176 bp, indicative of a non-functional truncated form of *Trpv1* (Caterina et al., 2000; Ren et al., 2019; Stanford et al., 2019), whereas WT C57 mice had a prominent band at 289 bp characteristic of the native protein (**Figure 1A**).

Recently, we discovered that short-term (2 weeks) elevations in IOP enhance excitability in multiple types of RGCs and their axons (Risner et al., 2018). Following the same procedure for unilateral microbead injection, IOP significantly increased for the 2-week duration of the experiment for both WT and *Trpv1*$^{-/-}$ mice (**Figure 1B**). In WT mice, IOP increased by 33% (20.5 ± 1.3 mmHg) compared with saline-injected eyes (15.4 ± 1.1 mmHg, $^{*}p < 0.01$). Similarly, in *Trpv1*$^{-/-}$ mice, IOP increased by 29% in microbead-injected eyes (19.6 ± 1.1 mmHg) relative to saline controls (15.2 ± 1.0 mmHg, $p < 0.01$, **Figure 1C**). Genotype had no influence on IOP for either saline- or microbead-injected eyes ($p \geq 0.96$).

To determine whether IOP modulates electrical activity in the myelinated optic nerve as it does for the retina, we measured the current-evoked CAP (Baltan et al., 2010). Optic nerve CAP typically demonstrated a single voltage peak following depolarizing current stimulation (**Figure 2A**), which could be eliminated by blocking voltage-gated sodium channels with tetrodotoxin (TTX; 1 μM; **Figure 2B**). In the retina, RGC excitability can be modulated directly by TRPV1 activation and inhibition (Weitlauf et al., 2014). This is not so for optic nerve. Application of the TRPV1-specific antagonist IRTX at sub-micromolar concentrations known to inhibit TRPV1 (Wahl et al., 2001) did not significantly affect the evoked CAP for naïve WT optic nerve (p = 0.91, **Figure 2C**).

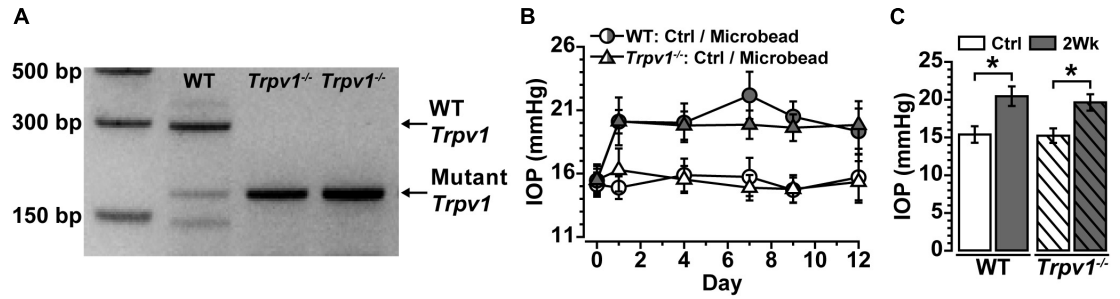

FIGURE 1 | (A) Genotype confirmation shows the band for wild-type (WT) *Trpv1* at 289 bp (lane 1, C57 background strain) vs. the 176 bp mutant *Trpv1* (lanes 2 and 3). **(B)** Intraocular pressure (IOP) for WT and $Trpv1^{-/-}$ mice following unilateral injection of microbeads (vs. saline injection control, Ctrl) was similar between genotypes. **(C)** IOP significantly increased in WT (33%) and $Trpv1^{-/-}$ (29%) eyes compared with their respective saline-injected control eyes (WT: $^*p < 0.01$, $Trpv1^{-/-}$: $^*p < 0.01$). Statistics: Independent samples *t*-tests. $n = 16$ (WT Ctrl), 16 (WT 2Wk), 15 ($Trpv1^{-/-}$ Ctrl), and 15 ($Trpv1^{-/-}$ 2Wk).

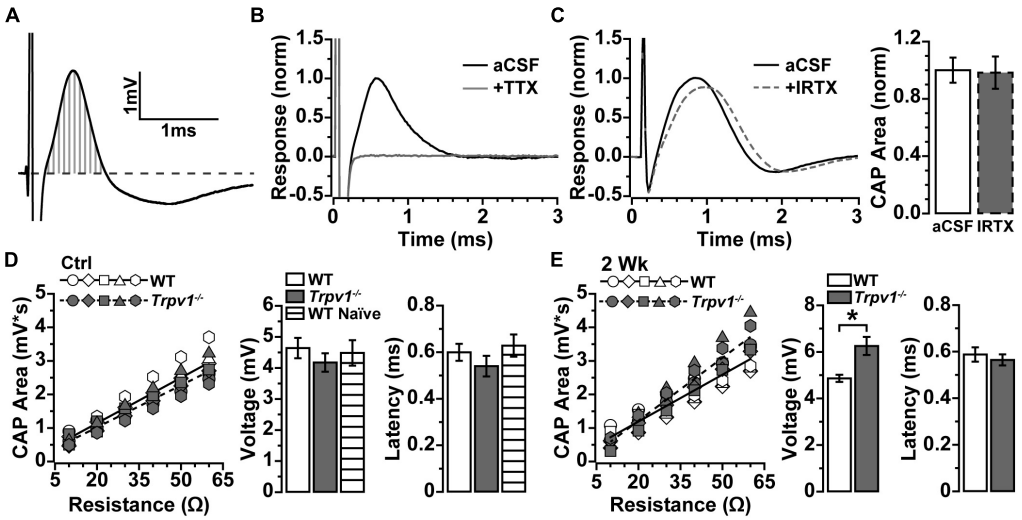

FIGURE 2 | (A) Compound action potential (CAP) area measured as integral (vertical gray lines) above baseline (dashed line) for current-evoked voltage changes over time. **(B)** Example of CAP from wild-type (WT) naïve optic nerve in artificial cerebral spinal fluid (aCSF) and with 1 μM of tetrodotoxin (TTX) added, which eliminated the CAP. **(C)** Example CAP from WT naïve optic nerve before and after bath application of 100 nM of iodoresiniferatoxin (IRTX) (left), which did not influence area when normalized to aCSF ($p = 0.34$, $n = 5$). **(D)** Integrated CAP calculated as in **(A)** increases with nerve resistance for individual WT ($n = 7$) and $Trpv1^{-/-}$ ($n = 5$) nerves from control eyes. Slope of best-fitting regression line indicates CAP voltage (right), which did not differ between WT, $Trpv1^{-/-}$, and WT naïve ($n = 4$; $p = 0.62$). Latency too did not differ ($p = 0.40$). **(E)** Integrated CAP for individual WT ($n = 7$) and $Trpv1^{-/-}$ ($n = 5$) nerves following 2 weeks of elevated IOP (left). For $Trpv1^{-/-}$ nerves, elevated IOP increased slope of best-fitting line compared with that of corresponding control (CAP voltage, right; $^*p = 0.001$). Latency did not differ for either WT or $Trpv1^{-/-}$ nerves compared with control nerves ($p = 0.59$). Statistics: **(C,E)** independent samples *t*-tests; **(D)** one-way ANOVA.

Resistance to stimulating current varies with axon density and diameter, extra-axonal space and glia, and positioning of the recording electrode, all of which alter the measured response (Stys et al., 1991). To compare optic nerve CAP between animals more accurately, we obtained multiple measurements while varying the positioning of the recording electrode. As resistance increased, so too did the integral of the CAP response (**Figures 2D,E**), with the slope of the best-fitting line yielding a more precise measure of CAP voltage (Stys et al., 1991). In addition, we assessed the amount of time required for axons to conduct action potentials by measuring the response latency as the time from stimulus onset to peak of the CAP. For control nerves, $Trpv1^{-/-}$ did not influence the CAP voltage ($p = 0.16$) or latency ($p = 0.40$) as compared with WT (**Figure 2D**). In contrast, following 2 weeks of elevated IOP, $Trpv1^{-/-}$ significantly increased the CAP voltage relative to control nerves (6.3 ± 0.4 vs. 4.2 ± 0.3 mV; $p = 0.001$) but did not modulate latency. Elevated IOP did not affect the WT CAP voltage or latency as compared with control nerves (**Figure 2E**).

$Trpv1^{-/-}$ Optic Nerve Is Less Sensitive to NaV1.6 Antagonism

Action potentials are propagated in myelinated nerve by activation of the voltage-gated sodium (NaV) channel 1.6, which densely accumulates within nodes of Ranvier (Craner et al., 2003). Because IRTX did not significantly

modulate optic nerve CAP (**Figure 2C**), we tested whether the increase in *Trpv1*$^{-/-}$ optic nerve CAP with elevated IOP (**Figure 2E**) is due to NaV1.6 activity. We again measured optic nerve CAP following bath application of 300 and 600 nM of aTTX, a selective inhibitor of the NaV1.6 subunit (Hargus et al., 2013). For WT optic nerve, the CAP was suppressed by 300 nM and further reduced by 600 nM of aTTX (**Figure 3A**). In contrast, the *Trpv1*$^{-/-}$ optic nerve CAP appeared relatively insensitive to aTTX of either concentration (**Figure 3B**). We quantified the influence of aTTX as the percent decrease in CAP area following drug administration, normalized to baseline area for each nerve. In WT nerve, regardless of IOP elevation or aTTX concentration, CAP area declined significantly with time after drug application (**Figure 3C**), as indicated by the slope of the best-fitting regression line. The CAP for WT control nerves decreased by 50% following 300 nM of aTTX and by 91% following 600 nM of aTTX compared with baseline (**Figure 3D**). Elevated IOP had little influence for either aTTX concentration, as compared with control nerves ($p \geq 0.53$). For *Trpv1*$^{-/-}$, aTTX had little influence on CAP over time, with the slope of the best-fitting regression line significantly declining only for 600 nM of aTTX treatment of control nerves (**Figure 3E**). With 300 nM, only the CAP for 2-week nerves declined compared with baseline, whereas only control nerves declined further with 600 nM compared with treatment with 300 nM (**Figure 3F**).

Trpv1$^{-/-}$ Alters NaV1.6 Density and Node Length With Elevated Intraocular Pressure

The results in **Figure 3** indicate that *Trpv1*$^{-/-}$ optic nerve is relatively insensitive to aTTX suppression of NaV1.6 activation

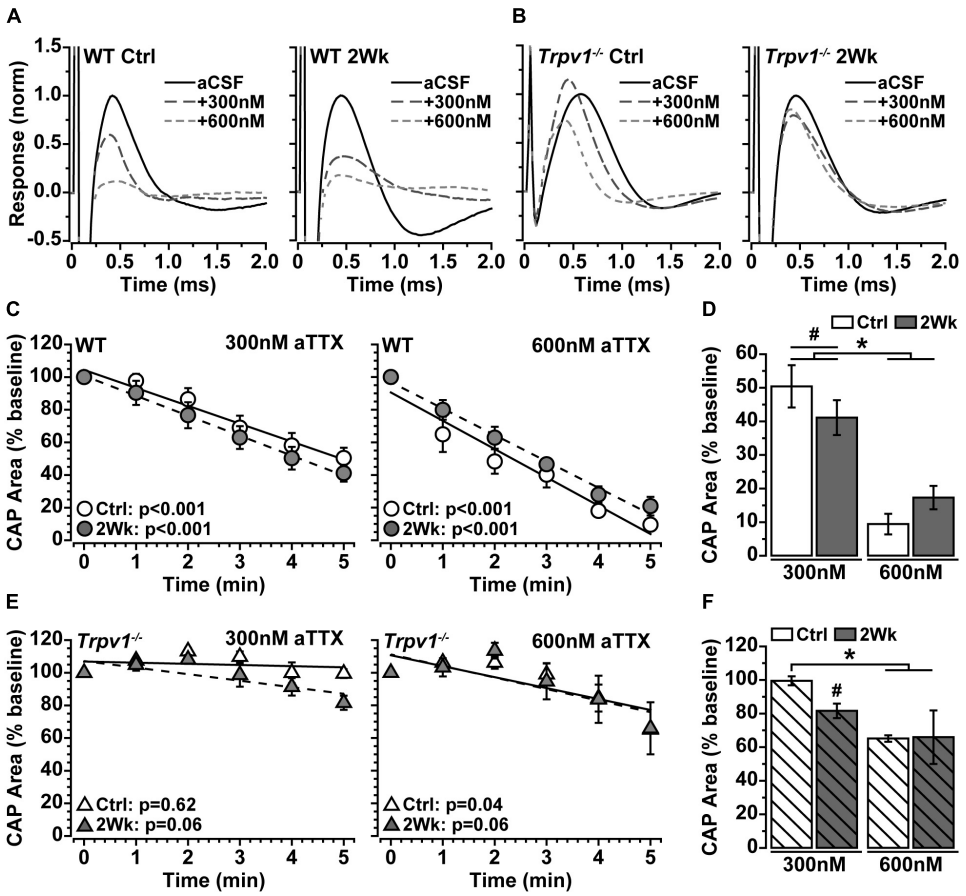

FIGURE 3 | (A,B) Example compound action potential (CAP) responses of nerves from Ctrl eyes and following 2 weeks of elevated intraocular pressure (IOP) from wild-type (WT) and *Trpv1*$^{-/-}$ mice with bath application of 300 and 600 nM of aTTX. **(C)** Mean WT CAP area for control and 2-week nerves decreases over time following bath application of 300 and 600 nM of aTTX. Individual recordings normalized to corresponding baseline (pre-drug) response. Slopes of best-fitting regression lines indicated significant decline (*p*-values indicated). **(D)** Final CAP area for WT decreases significantly following 300 nM of aTTX for both control ($n = 7$, 50% decrease) and 2-week ($n = 6$, 59% decrease) nerves compared with baseline for each ($^{\#}p \leq 0.03$). CAP area decreased further from baseline for control ($n = 6$, 91% decrease) and 2-week nerves ($n = 6$, 83% decrease) following application of 600 nM of aTTX, both significant declines compared with 300 nM ($^{*}p < 0.001$). **(E)** Mean *Trpv1*$^{-/-}$ CAP area following bath application of 300 and 600 nM of aTTX; for slopes of best-fitting regression lines, only control nerves with 600 nM of aTTX showed significant decline (*p*-values indicated). **(F)** Final CAP area for *Trpv1*$^{-/-}$ control nerves were minimally affected by 300 nM of aTTX ($n = 5$, 0.5% decrease), whereas area for 2-week nerves declined compared with baseline ($n = 5$, 18% decrease; $^{\#}p = 0.02$). Like WT, 600 nM of aTTX caused a greater reduction in CAP area compared with 300 nM for Ctrl nerves (35% decrease; $^{*}p = 0.02$). Statistics: **(C,E)** linear regressions; **(D,F)**: one-way ANOVAs, Tukey *post-hoc*.

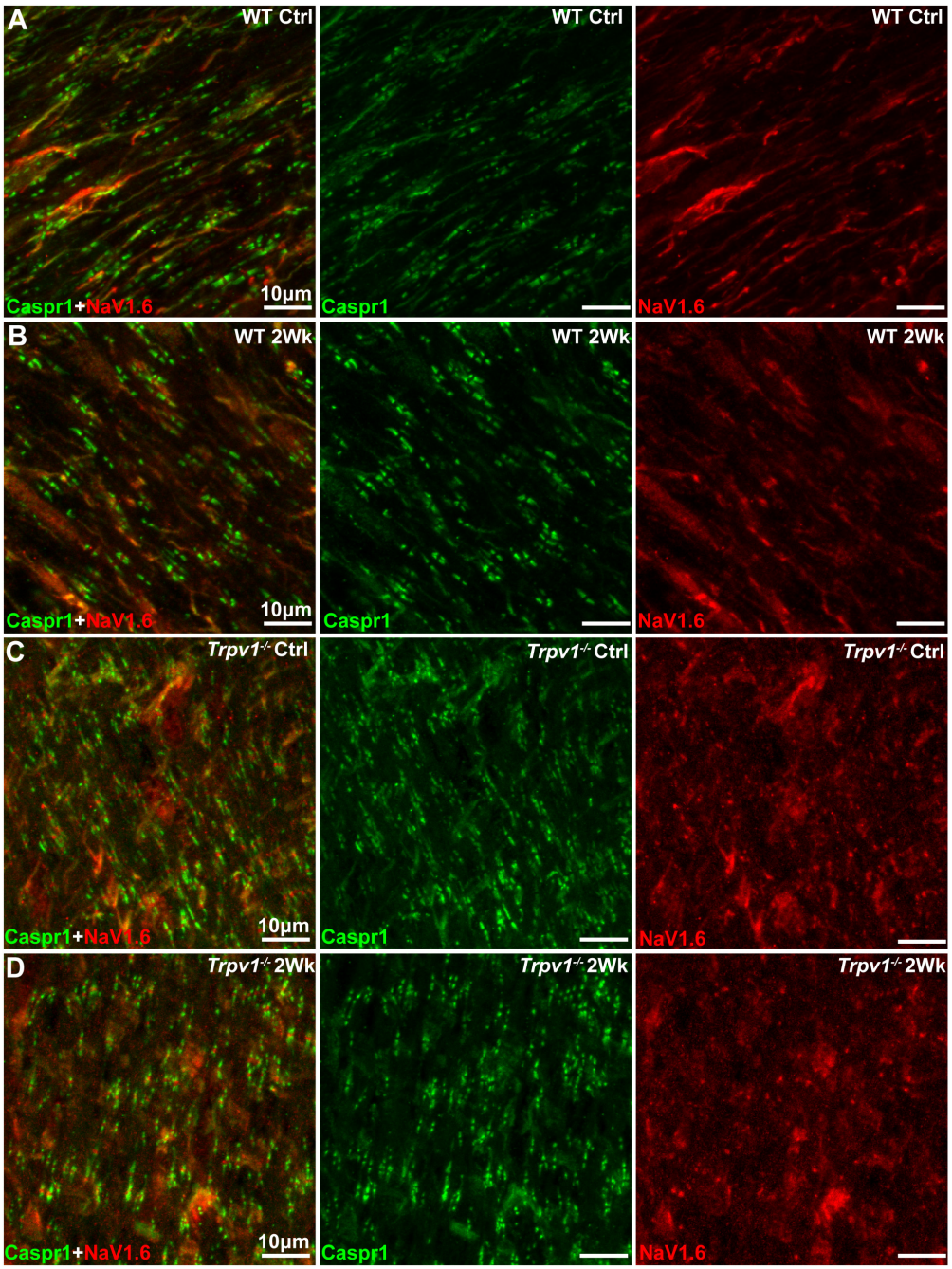

FIGURE 4 | (A–D) Representative confocal micrographs of Caspr1 (green) and NaV1.6 (red) immunostaining of longitudinal optic nerve sections from wild-type (WT) **(A,B)** and *Trpv1*$^{-/-}$ **(C,D)** mice. Scale bar = 10 μm.

than are WT nerves. In the myelinated optic nerve, NaV1.6 localizes to nodes of Ranvier flanked by paranodes defined by the membrane protein Caspr1 (contactin associated protein 1; Craner et al., 2003). Immunolabeling for NaV1.6 and Caspr1 in longitudinal sections confirmed this fundamental configuration in both WT and *Trpv1*$^{-/-}$ optic nerves (**Figure 4**). Compared with WT nerves from control and IOP-stressed eyes (**Figures 4A,B**), the node–paranode complex appeared smaller in *Trpv1*$^{-/-}$ optic nerves with more intense location of NaV1.6 (**Figures 4C,D**).

To quantify these apparent differences, we measured paranode and node length and intensity of Caspr1 and NaV1.6 localization within well-defined paranode–node complexes (**Figure 5A**). For WT optic nerve, elevated IOP had no effect on levels of paranodal Caspr1 compared with control (p = 0.76) nor on paranode length (p = 0.81; **Figure 5B**). However, for *Trpv1*$^{-/-}$ optic

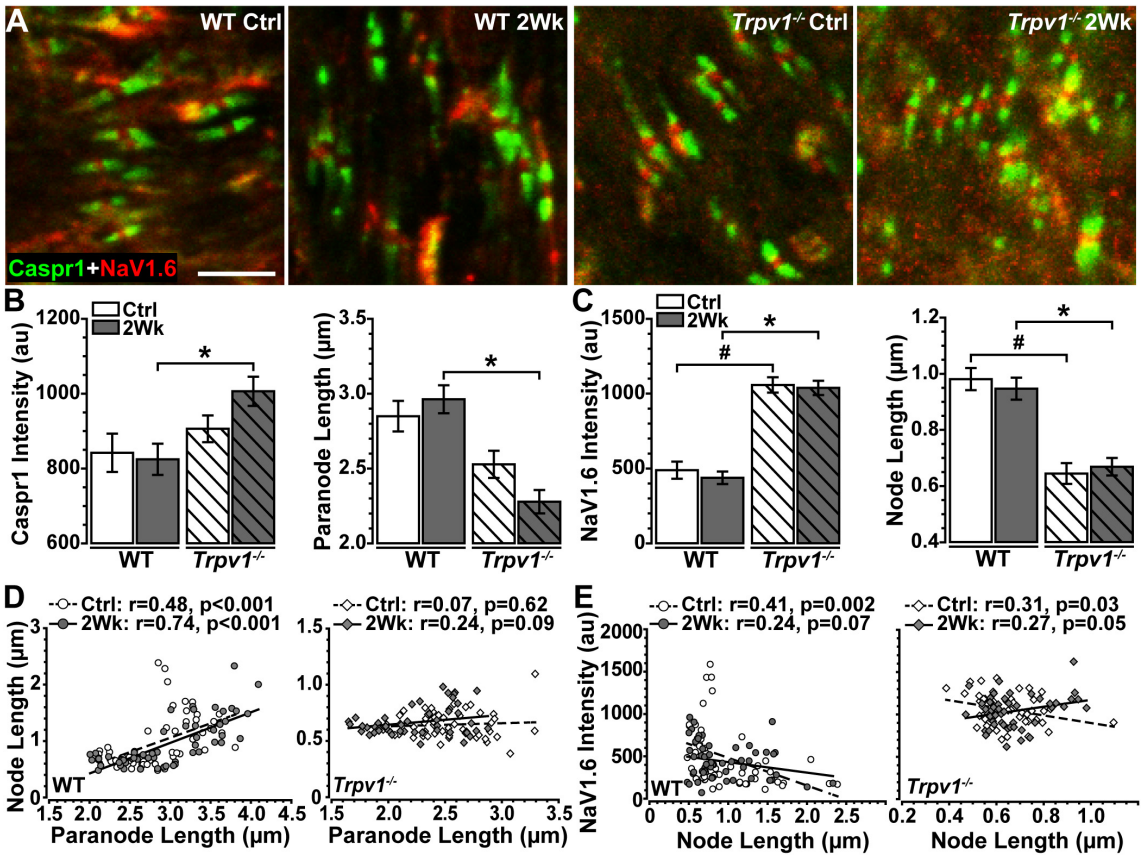

FIGURE 5 | (A) High magnification confocal micrographs of longitudinal sections through Ctrl and 2 Wk wild-type (WT) and *Trpv1*$^{-/-}$ optic nerves show Caspr1-labeled paranodes (green) flanking NaV1.6 (red) within nodes of Ranvier. **(B)** *Trpv1*$^{-/-}$ 2 Wk paranodes contain increased Caspr1 than did WT 2 Wk nerves (left, $^{*}p = 0.012$) and are shorter (right, $^{*}p < 0.001$). **(C)** NaV1.6 is higher in nodes of Ranvier of *Trpv1*$^{-/-}$ nerves (left) compared with WT Ctrl ($^{\#}p < 0.001$) and 2 Wk ($^{*}p < 0.001$) optic nerve (left), although node length is significantly shorter for both *Trpv1*$^{-/-}$ Ctrl ($^{\#}p < 0.001$) and 2 Wk ($^{*}p < 0.001$) nerves. Elevated intraocular pressure (IOP) had no effect on either measure ($p \geq 0.88$). **(D)** There is a positive relationship between node and paranode lengths in WT optic nerves (left), however, this relationship is lost in *Trpv1*$^{-/-}$ (right) optic nerves. Elevated IOP had no effect on this relationship for WT or *Trpv1*$^{-/-}$ optic nerves. **(E)** NaV1.6 intensity decreases as node length increases in WT (left) and *Trpv1*$^{-/-}$ (right) control optic nerves. Following IOP elevation, this relationship is lost in WT nerves, whereas the relationship becomes positive in *Trpv1*$^{-/-}$ nerves. Scale = 5 μm **(A)**. Statistics: **(B,C)**: one-way ANOVAs, Tukey *post-hoc*; **(D,E)** linear regressions. Total nodes analyzed: WT Ctrl, 3,942; WT 2 Wk, 3,890; *Trpv1*$^{-/-}$ Ctrl, 2,024; *Trpv1*$^{-/-}$ 2 Wk, 2,191. Five animals per condition. Ten images per animal.

nerve, elevated IOP increased Caspr1 significantly compared with that for WT ($p = 0.012$; **Figure 5B**, left) whereas significantly shortening paranode length compared with that for WT ($p < 0.001$; **Figure 5B**, right). Within the nodes themselves, NaV1.6 was significantly higher for *Trpv1*$^{-/-}$ compared with WT for both control and 2-week nerves ($p < 0.001$; **Figure 5C**, left). As with Caspr1-labeled paranodes, *Trpv1*$^{-/-}$ significantly shortened the nodes compared with WT ($p < 0.001$; **Figure 5C**, right). Thus, NaV1.6 concentrates at a higher level in truncated paranode–node complexes in *Trpv1*$^{-/-}$ optic nerve. We found significant positive correlations between node and paranode length in WT control and 2-week nerves ($p < 0.001$, **Figure 5D**, left). For *Trpv1*$^{-/-}$ optic nerve, there was no correlation (Ctrl, $p = 0.62$; 2Wk, $p = 0.09$, **Figure 5D**, right). For both WT and *Trpv1*$^{-/-}$ control nerves, NaV1.6 intensity decreased significantly with increasing nodal length, so that NaV1.6 was more concentrated in shorter nodes ($p \leq 0.03$, **Figure 5E**). However, for *Trpv1*$^{-/-}$ nerves with elevated IOP, the relationship was reversed so that NaV1.6 concentrated in *longer* nodes ($p = 0.05$, **Figure 5E**, right); this was not so for WT nerves ($p = 0.07$). These results suggest that the combined increase in NaV1.6 localization with decreased length of the paranodal complex strengthens the *Trpv1*$^{-/-}$ CAP, rendering these nerves far less sensitive to aTTX antagonism (**Figure 3**). That elevated IOP increases NaV1.6 with increasing node length likely explains the increased CAP for *Trpv1*$^{-/-}$ nerves under these conditions.

DISCUSSION

Previously, we found that *Trpv1*$^{-/-}$ accelerates optic nerve axonopathy with elevated IOP, reducing nerve area, axon density, and axon transport to the brain (Ward et al., 2014). The deleterious influence of *Trpv1*$^{-/-}$ on nerve health and axon function with IOP-related stress likely can be linked to cationic

activity. Here, we sought to determine the impact of *Trpv1*$^{-/-}$ with short-term IOP elevation on optic nerve signaling to the brain, using the evoked CAP. As expected (Baltan et al., 2010), the optic nerve CAP demonstrated a single-peaked voltage inflection in response to depolarizing current that was eliminated by application of the voltage-gated sodium channel antagonist, TTX (**Figures 2A,B**), underscoring the dependence of CAP on these channels. Under control conditions, in the absence of IOP-related stress, the CAP for WT and *Trpv1*$^{-/-}$ optic nerves was identical, and naïve CAP was unaffected by specific pharmacological antagonism of TRPV1 (**Figures 2C,D**).

Our key physiological result is that modest, short-term IOP elevation significantly increases *Trpv1*$^{-/-}$ optic nerve CAP. On the surface, this finding is paradoxical. We have recently shown TRPV1 expression and RGC excitability concurrently increase following 2 weeks of elevated IOP (Weitlauf et al., 2014). In that study, *Trpv1*$^{-/-}$ eliminated the stress-related enhancement of RGC excitability, and *Trpv1*$^{-/-}$ RGCs required larger depolarizing currents to generate action potentials with elevated IOP. On the basis of this collective evidence, one would expect IOP elevation to reduce *Trpv1*$^{-/-}$ optic nerve CAPs. How then do we explain our results? *Trpv1*$^{-/-}$ nerves were relatively impervious to NaV1.6 antagonism by aTTX, which suppressed the WT CAP (**Figure 3**). This difference accompanies shorter nodes of Ranvier with far greater NaV1.6 localization in *Trpv1*$^{-/-}$ but not WT nerves (**Figures 4**, **5**). In fact, NaV1.6 in WT optic nerve nodes is unaltered following up to 5 weeks of microbead-induced IOP elevation (Smith et al., 2018). This novel finding suggests that TRPV1, which is typically associated with presynaptic potentiation of glutamatergic action (Marinelli et al., 2003; Medvedeva et al., 2008), can also tune channel expression within axons – even though localization of TRPV1 in the optic nerve head is negligible (Choi et al., 2015).

Our data show that *Trpv1*$^{-/-}$ causes a compensatory aggregation of NaV1.6 protein expression within nodes of Ranvier and a significant decrease in nodal length (**Figure 5C**). This may serve as a cautionary note that genetic excision of a single gene, *Trpv1* in this case, can lead to unexpected effects on neuronal structure and expression levels of other channels. Here, we observed that *Trpv1*$^{-/-}$ led to increased NaV1.6 expression, which conferred greater resistance to the NaV1.6 antagonist, aTTX (**Figure 3**). The general observation that overexpression of a drug target correlates with a higher resistance to inhibition is a fundamental assumption for drug target identification. This assumption is often true when inhibition of the target only reduces target activity. However, if inhibition of the target also catalyzes harmful downstream effects, drug efficacy cannot be predicted (Palmer and Kishony, 2014). Although it is unknown if inhibition of NaV1.6 by aTTX impacts off-target sites, here, we find that for WT control nerves, 300 nM of aTTX caused a 50% reduction of the CAP and 600 nM of aTTX decreased the CAP near 100%, suggesting that NaV1.6 resistance to aTTX is linear (**Figures 3D,F**).

Finally, we found that *Trpv1*$^{-/-}$ with elevated IOP causes a modest but significant shift in the relationship between NaV1.6 expression and node length, where NaV1.6 accumulates more in longer nodes (**Figure 5E**). Interestingly, others have found that increased nodal length and ectopic expression of NaV1.6 in aged optic nerves are related to larger CAP despite decreased levels of ATP (Stahon et al., 2016). We previously found that elevated IOP in *Trpv1*$^{-/-}$ mice accelerates ATP-dependent anterograde axon transport deficits and optic nerve axon degeneration (Ward et al., 2014). Ultimately, our results indicate that IOP-related stress, like aging, requires a redistribution of energy resources at the expense of axon transport to preserve voltage-dependent axon signaling. In the absence of TRPV1, this demand is increased, further taxing a vulnerable system. Thus, in glaucoma and other age-related neurodegenerative diseases, TRPV1 may reconfigure NaV expression in neurons under stress to normalize excitability to existing metabolic resources.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Vanderbilt University IACUC.

AUTHOR CONTRIBUTIONS

NM, MR, and DC designed research and wrote the manuscript. NM and MR performed research. NM, MR, VV, and DC analyzed data.

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REVIEW
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TRP Channels as Emerging Therapeutic Targets for Neurodegenerative Diseases

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The development of treatment for neurodegenerative diseases (NDs) such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis is facing medical challenges due to the increasingly aging population. However, some pharmaceutical companies have ceased the development of therapeutics for NDs, and no new treatments for NDs have been established during the last decade. The relationship between ND pathogenesis and risk factors has not been completely elucidated. Herein, we review the potential involvement of transient receptor potential (TRP) channels in NDs, where oxidative stress and disrupted Ca^{2+} homeostasis consequently lead to neuronal apoptosis. Reactive oxygen species (ROS) -sensitive TRP channels can be key risk factors as polymodal sensors, since progressive late onset with secondary pathological damage after initial toxic insult is one of the typical characteristics of NDs. Recent evidence indicates that the dysregulation of TRP channels is a missing link between disruption of Ca^{2+} homeostasis and neuronal loss in NDs. In this review, we discuss the latest findings regarding TRP channels to provide insights into the research and quests for alternative therapeutic candidates for NDs. As the structures of TRP channels have recently been revealed by cryo-electron microscopy, it is necessary to develop new TRP channel antagonists and reevaluate existing drugs.

Keywords: TRP, Ca^{2+}, ROS, cell death, neurodegeneration, AD, PD, HD

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INTRODUCTION

Neurodegenerative disorders (NDs) are one of the most devastating types of chronic diseases and lead to a significant social and medical burden on society. With the growing elderly population, the number of patients with NDs is also increasing. Although many pharmaceutical companies are struggling to develop novel therapeutics for neurological diseases, some of the world's leading pharmaceutical companies have declared their abandonment of the development of therapeutics for NDs. Alzheimer's disease (AD) is the most common ND, which accounts for 60–70% of all dementia (Association, 2016). Nevertheless, no new treatments for AD have been developed in over a decade. Some of the reasons for the difficulty in treating NDs are the combination of complex causative factors and irreversible structural and functional damage of neurons.

Neurodegenerative disorders are typically progressive, late-onset disorders, and aging is the greatest risk factor (Wakabayashi et al., 2014). In addition, genetic and environmental factors not only contribute to their pathogenesis independently but also interact with each other to increase their effects. The pathogenesis of NDs involves an initial toxic insult and consequences of the secondary pathological damage. The most primary causative hypothesis of AD is the intraneuronal accumulation of amyloid-beta (Aβ) and hyperphosphorylated tau protein (Iqbal et al., 2010; Murphy and LeVine, 2010). Parkinson's disease (PD) is caused by the degeneration of dopaminergic neurons in the substantia nigra par compacta (SNpc), with subsequent dopamine deficiency (Michel et al., 2013; Kalia and Lang, 2015). A type of hereditary ND, Huntington's disease (HD) is an autosomal dominant disorder caused by CAG repeat expansion within the Huntington (*HTT*) gene (Kremer et al., 1994). The third common ND after AD and PD is amyotrophic lateral sclerosis (ALS) that is characterized by the deterioration of motor neurons.

Neurodegenerative disorders such as AD, PD, HD, and ALS are distinguished by clinical symptoms and specific neuronal sites with distinct pathology. However, apparent clinical symptoms are manifested only after extensive pathological damage, with significant neuronal and synaptic loss. Eventually, the contribution of individual insults reaches a common end state, which causes severe impairments in the function and plasticity of neuronal and glial cells (Rasband, 2016). Over the past few decades, there has been considerable effort to understand the pathogenesis of NDs. To date, a number of studies have reported that oxidative stress, ER (endoplasmic reticulum) stress, abnormal Ca^{2+} homeostasis, protein misfolding, aggregation, neuroinflammation, and mitochondrial dysfunction are highly related to neuronal damage. The relationship between them, however, has not been completely elucidated. Besides, even though Aβ is still a compelling candidate in the pathogenesis of AD, the latest experiments raise doubts about the Aβ hypothesis and Aβ -based drug development for AD (Du et al., 2018). Therefore, it is necessary to find the missing links amongst the risk factors of NDs and to discover new therapeutic targets based on novel mechanisms.

Ion channels are key determinants of brain function, since the physiological function of neurons is to carry information or impulses via electrical signals (action potentials) to communicate with each other at synapses. Thus, neurological channelopathies have been identified mainly in voltage-gated and ligand-gated channels or receptors that result from genetically determined defects in their function. However, based on patients with progressive NDs with adulthood manifestations, we have focused on the age-related susceptibility to environmental toxins and chemicals. Recently, emerging evidence has indicated that transient receptor potential (TRP) channels, ubiquitously expressed throughout the brain (Sawamura et al., 2017), play a significant role in the regulation of physiological functions, as well as in reactive oxygen species (RO)-related human diseases. Based on the polymodal activation of TRP channels acting as cellular sensors, many researchers are investigating their activation mechanisms (Takada et al., 2013). Here, we review the potential involvement of TRP channels in NDs, where oxidative stress and disrupted Ca^{2+} homeostasis have been characterized with respect to pathological consequences in neuronal apoptosis. Second, we discuss the latest findings in the field of TRP to provide insight into the research and quest for alternative therapeutic candidates for the treatment of NDs.

THE CRITICAL ROLE OF Ca^{2+} IN THE PATHOGENESIS OF NEURODEGENERATIVE DISEASES

Ca^{2+} homeostasis is crucial to the normal physiological functions of neurons, such as neuronal survival, growth, and differentiation. Hence, long-lasting Ca^{2+} dyshomeostasis can eventually lead to neuronal loss. Accumulated evidence strongly implicates that abnormal Ca^{2+} levels stimulate dysregulation of intracellular signaling, which consequently induces neuronal cell death (Barnham et al., 2004). Therefore, disruption of Ca^{2+} homeostasis in neuronal cells leads to ROS generation and ATP depletion, following the mitochondrial dysfunction in NDs such as AD, PD, HD, and ALS. Interestingly, a close correlation between the increase in $[Ca^{2+}]_i$ and other pathogenic mechanisms has been reported, such as Aβ deposition (Demuro et al., 2010), imbalance between ROS and antioxidant function (Gorlach et al., 2015), and mitochondrial dysfunction (Contreras et al., 2010; Pivovarova and Andrews, 2010).

Some reports have shown bidirectional crosstalk between amyloid pathology and the Ca^{2+} pathway. Most studies reported that Aβ increases intracellular Ca^{2+} levels by inducing ER Ca^{2+} depletion (Suen et al., 2003; Abramov et al., 2004b; Ferreiro et al., 2008). Abramov et al. (2004a) identified that Aβ causes Ca^{2+}-dependent oxidative stress by the activation of NADPH oxidase in astrocytes and that the reduced antioxidant activity induces neuronal death. Recently, Calvo-Rodriguez et al. (2019) suggested that Aβ oligomers exacerbate Ca^{2+} remodeling from ER to mitochondria in aged neurons but not in young neurons. Conversely, Itkin et al. (2011) argued that Ca^{2+} stimulates the formation of Aβ oligomers, leading to neuronal toxicity in AD.

The imbalance between ROS production and antioxidant defenses results in the excessive accumulation of ROS and oxidative stress. Since aged neurons with low antioxidant capacity are more vulnerable to oxidative insults (Chen et al., 2012), ROS overproduction can chronically lead to irreversible oxidation (Ivanova et al., 2016). Oxidative stress also causes mitochondrial dysfunction, which itself aggravates ROS generation. Moreover, the opening of the mitochondrial permeability transition pore (mPTP) and the release of cytochrome c into cytoplasm activate pro-apoptotic caspases (Guo et al., 2013). Oxidative stress and mitochondrial dysfunction are well known to be related to an increase in cytosolic Ca^{2+} levels that underlies the pathogenesis of AD (Cenini and Voos, 2019), PD (Blesa et al., 2015), HD (Zheng et al., 2018), and ALS (Carri et al., 2015). Interestingly, the mitochondrial metabolic state can affect the Mg^{2+} concentration of both the matrix and the cytoplasm, where Mg^{2+} interferes with mitochondrial Ca^{2+} transport and mitochondrial ATP generation (Llorente-Folch et al., 2015). Since the efficient removal of $[Ca^{2+}]_i$ requires ATP, impairment of mitochondrial

ATP generation prevents Ca^{2+} pumps from operating both in the plasma membrane and in the ER (Ott et al., 2007). Thus, dysregulation of Ca^{2+} signaling is one of the key processes in early stage neuronal loss. In spite of its significance, the mediator of such aberrant Ca^{2+} increase and its source are not fully understood. To test the effectiveness of preventing Ca^{2+} overload for ND therapy, a variety of appropriate channel candidates should be further examined by developing channel-specific drugs for new channel targets. Last but not least, experimental data on existing drugs also need to be reevaluated.

Oxidative stress can directly modulate the gating properties of ion channel proteins. Pathological mechanisms underlying the dysregulation of ion channels by oxidation have been previously proposed in a variety of diseases, especially cancer (Reczek and Chandel, 2018) and NDs (Gorlach et al., 2015). Under normal conditions, defensive antioxidants can protect or repair the damage caused by oxidation. However, the target proteins are retained in their oxidized forms and are activated as long as the antioxidant activity is reduced. To date, several studies have shown that oxidative stress is involved in the modulation of activities of voltage-gated Ca^{2+} (Gorlach et al., 2015; Ramirez et al., 2016), Na^{+}, and K^{+} (Sesti et al., 2010) channels and ligand-gated receptors such as NMDA (Kamat et al., 2016), AMPA (Joshi et al., 2015), GABA (Bradley and Steinert, 2016), and RyR (Zima and Mazurek, 2016). However, there is limited evidence that directly identifies the oxidative modification of a channel protein based on molecular mechanisms.

Since TRP channels are non-selective, Ca^{2+}-permeable channels that can be opened at resting membrane potential in response to various stimuli, we focused on TRP channels. The activation of TRP channels consequently changes membrane depolarization toward the action potential threshold. When TRP channels open, they allow sodium and calcium into the cytoplasm, which subsequently triggers the opening of voltage-dependent Ca^{2+} channels. This is why TRP channels are upstream risk factors, ahead of voltage-dependent channels (Numata et al., 2011). Therefore, the hyperactivation of TRP channels is responsible for neuronal excitotoxicity, which is closely associated with NDs. In the following section, we will address the physiological and pathological roles of TRP channels in neurons through the recent studies related to TRP channels and our study of the TRPC5 channel.

THE PHYSIOLOGICAL AND PATHOLOGICAL ROLE OF TRP CHANNELS IN NEURONS

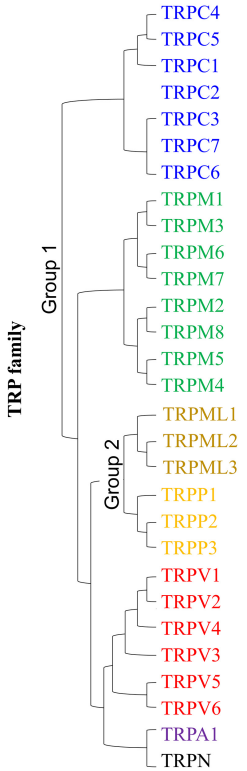
As mentioned above, TRP channels are widely expressed in almost every mammalian cell, predominantly in the brain. TRP channels can be activated by diverse stimuli ranging from temperature, mechanical or osmotic stress, chemical compounds, and redox modification (Sawamura et al., 2017; Samanta et al., 2018). Based on sequence homology, the TRP superfamily is divided into six subfamilies in mammals: TRPC (classical or canonical; seven sub-members), TRPM (melastatin; eight sub-members), TRPV (vanilloid; six sub-members), TRPA (ankyrin; one sub-member), TRPP (polycystin; three sub-members), and TRPML (mucolipin; three sub-members) (**Figure 1**). Notably, most TRP channels (except TRPM4 and TRPM5) are non-selective channels with consistent Ca^{2+} permeability (Guinamard et al., 2011). TRP channels are tetrameric protein complexes that can be assembled into homomeric or heteromeric channels, either with the same subfamily members or with the other subfamily members. Thus, when TRP channels assemble with different subunits, further heterogeneity diversifies their functions. In addition to the physiological roles of TRP channels in neurons, a number of studies regarding the pathological functions have been reported. Intracellular Ca^{2+} influx through TRP channels is involved either in neuronal survival or death and is discussed with respect to the different TRP channel families in the following sections (Bollimuntha et al., 2011).

TRPC (Classical or Canonical)

TRPC was the first group of TRPs to be discovered in a mammal (Wes et al., 1995), and it shows the highest amino acid similarity to the *Drosophila* TRP channel. The TRPC subfamily is divided into seven subtypes, namely, TRPC1–7. Depending on amino acid similarities, the subtypes are divided into four groups: TRPC1, 2, 3/6/7, 4/5 (Venkatachalam and Montell, 2007). There is still disagreement over the mechanism of action of TRPC; TRPC has been reported to be involved in ion permeation as receptor operated channel (ROC) or to influence intracellular mechanisms of store-operated calcium entry (SOCE) (Vazquez et al., 2004). Recently, as the TRPC channel has been found to have regulation, structure, and novel small molecular probes, research is being actively conducted on it as a therapeutic target for various diseases (Wang et al., 2020).

TRPC1

In particular, there has been debate about the role or opening mechanisms of TRPC1. Initially, TRPC1 was claimed to take the role of a SOCE in regulating Orai1-mediated Ca^{2+} entry (Ambudkar et al., 2017). Consistent with this claim, the role of TRPC1 in AD has been reported by Linde et al. (2011). Knock-down (KD) of the amyloid precursor protein (APP) gene decreased store-operated Ca^{2+} channel-mediated Ca^{2+} entry and expression of TRPC1 and Orai1 in cultured astrocytes. However, overexpression of APP in TG5469 did not alter TRPC1/4/5 and stored Ca^{2+} level in astrocytes. In SH-SY5Y human neuroblastoma cells, TRPC1 has been reported to reduce expression levels by MPP^{+} (Bollimuntha et al., 2006). Activation of TRPC1 by TRPC1 overexpression or by ER depletion using thapsigargin (TG) ameliorates neurotoxicity. Selvaraj et al. (2009, 2012) showed that Ca^{2+} entry through the activation of store-operated channels (SOC) is important for the survival of dopaminergic neurons (**Figure 2C**). In the MPTP-induced PD model, TRPC1 expression was suppressed and induced the death of dopaminergic neurons in the substantia nigra. The authors suggested that the cause was reduced interaction with the SOCE modulator stromal interaction molecule 1 (STIM1) and decreased Ca^{2+} entry into the cell. However, our recent study showed that TRPC1 functions as a negative regulator of TRPC4 and TRPC5 (**Figure 2C**; Kim et al., 2019). Heterodimers



TRP	Therapeutic approach		For NDs
	using KO mice	using agents	
TRPC4	(Riccio et al., 2014)		AD
TRPC5	(Phelan et al., 2013)		
		ML204 (Hong et al., 2015)	HD
TRPC1	(Selvaraj et al., 2009)		PD
TRPC2			
TRPC3	(Hartmann et al., 2008)		
	(Zhou et al., 2008)		PD
TRPC7			
TRPC6	(Wang et al., 2015)		AD
	(Lu et al., 2018)		PD
TRPM1			
TRPM3			
TPRM6			
TRPM7	(Ryazanova et al., 2010)		
	(Hashimoto et al., 2008;Paravicini et al., 2012)		PD
	(Hermosura and Garruto, 2007;Hermosura et al., 2008;Tedeschi et al., 2019)		ALS
TRPM2	(Jang et al., 2015)		
	(Ostapchenko et al., 2015)		AD
	(Belrose et al., 2012;Huang et al., 2018)	Clotrimazole (Sun et al., 2018)	PD
	(Hermosura and Garruto, 2007)		ALS
TRPM8			
TRPM5			
TRPM4			
TRPML1			
TRPML2			
TRPML3			
TRPP1			
TRPP2			
TRPP3			
TRPV1	(Gibson et al., 2008, Balleza-Tapia et al., 2018)	Capsazepine (Balleza-Tapia et al., 2018)	AD
	(Kim SR, 2005;Nam et al., 2015)		PD
		Capsazepine (Lastres-Becker et al., 2003)	HD
TRPV2			
TRPV4		HC-067047 (Suresh et al., 2015) Gd^{3+} & ruthenium red (Bai and Lipski, 2014)	AD
TRPV3			
TRPV5			
TRPV6			
TRPA1	(Lee et al., 2016)		AD
TRPN			

FIGURE 1 | Summary of TRP studies using knockout mice or antagonists to investigate therapeutic targets of neurodegenerative diseases. ND, neurodegenerative diseases; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis.

of TRPC1/4 and TRPC1/5 suppressed inward current, which may reduce Ca^{2+} influx and Ca^{2+}-dependent apoptosis in neurons. We identified that the expression level of endogenous TRPC1 in striatal cells of the HD model was decreased compared to wild-type cells, indicating that HD cells could be more susceptible to oxidative stress due to the activity of the dominant homomeric TRPC5 (**Figure 2D**; Hong et al., 2015).

TRPC3

The important roles of TRPC3 in the hippocampus have been implicated in ND more than in other TRP channels with higher expression levels (Neuner et al., 2015). TRPC3 notably contributes to the maintenance of Ca^{2+} homeostasis and cell growth, such as differentiation and proliferation. In a study conducted by Wu et al. (2004) it was reported that switching proliferation to differentiation is related to TRPC3-induced Ca^{2+} influx and TRPC3-mediated SOCE in the H19-7 hippocampal cell line. Under cell differentiation conditions, TRPC3 expression and TRPC3-induced SOCE levels were increased. The differentiation was blocked by siRNA KD of TRPC3. In addition, TRPC3 is indirectly activated by BDNF. In a study conducted by Li et al. (1999) TRPC3 was activated by neurotrophin receptor TrkB, which is affected by BDNF. A non-selective cationic current was observed in CA1 pyramidal neurons treated with BDNF, although the current was inhibited by siRNA-mediated TRPC3 KD and TrkB-lgG (Amaral and Pozzo-Miller, 2007). This study suggests that BDNF-induced membrane current is due to stimulation of TRPC3 by TrkB (**Figure 2A**).

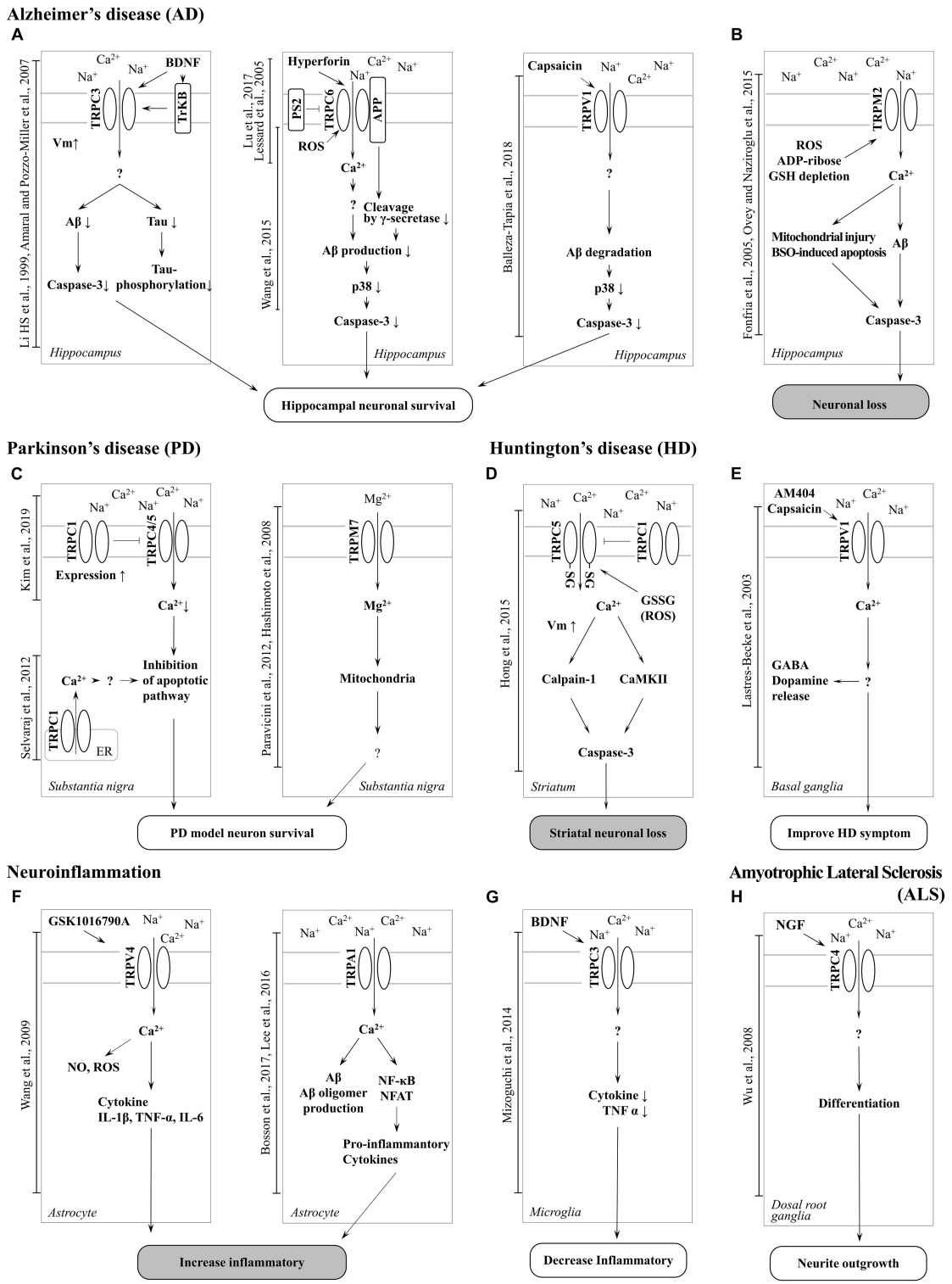


FIGURE 2 | Schematic of TRP channel-mediated mechanisms in neurodegenerative diseases. **(A)** Activation of TRPC3, TRPC6 and TRPV1 channel increase neuronal survival in AD. **(B)** Neuronal loss can be induced by Aβ toxicity, ROS generation, and mitochondrial damage resulting from TRPM2 channel-mediated Ca^{2+} entry in AD. TRPA1 is also involved in neuroinflammation in AD. **(C)** Inhibition of TRPC4/5 by TRPC1 contribute to inhibition of apoptotic pathways and TRPM7-mediated Mg^{2+} influx is involved in neuronal survival in PD. **(D)** Increased activity of TRPC5 by oxidative stress induces striatal neuronal loss via Ca^{2+}-dependent pathways in HD. **(E)** Activation of TRPV1 by an agonist improves HD symptoms. **(F)** Activation of TRPV4 and TRPA1 induces a proinflammatory response in astrocytes **(G)** whereas upregulation of surface TRPC3 induced by BDNF regulates microglial functions and reduces inflammation. **(H)** Upregulation of TRPC4 promotes neurite outgrowth and differentiation in DRG (GTEx Consortium, 2013).

In a recent study by Mizoguchi et al. (2014) TRPC3 was found to be involved in the function of microglia, such as the release of cytokines and nitric oxide (NO). Treatment with BDNF rapidly increased the surface expression levels of TRPC3 in rodent microglial cells. In addition, pre-treatment with BDNF inhibited the release NO-induced tumor necrosis factor α (TNFα), which was rescued by treatment with TRPC3 inhibitor. This report suggests that Ca^{2+} influx and concentration maintenance by TRPC3 plays an important role in the improvement of NDs (**Figure 2G**).

Another characteristic of TRPC3 related to neurodegeneration is directly activated by oxidative stress. The change in Ca^{2+} influx by TRPC3 is associated with neuronal cell death (Selvaraj et al., 2010). Treatment with tertiary butyl hydroperoxide (tBHP) increased the Na^{+} current in HEK293T cells overexpressing TRPC3. Further, Rosker et al. (2004) reported the increase of Na^{+} influx by TRPC3-regulated Ca^{2+} influx in overexpressed HE293T cells. When the Na^{+} concentration of extracellular solution decreased to 5 mM, Ca^{2+} influx was increased by TRPC3 agonist. In addition, treatment of the inhibitor of the Na^{+}/Ca^{2+} exchanger strongly inhibited Ca^{2+} influx but Na^{+} did not. This suggests that Ca^{2+} influx by TRPC3 is accompanied by Na^{+}/Ca^{2+} exchange. Pesticides, such as rotenone and paraquat, are neurotoxins that induce PD by increasing intracellular oxidative stress. Moreover, both of these pesticides induce the loss of dopaminergic neurons in the SNpc. In a recent study by Roedding et al., chronic treatment of rotenone and paraquat dose-dependently reduced expression levels of TRPC3 and TRPC3-mediated Ca^{2+} influx in primary rat cortical neurons and astrocytes. In another report, OAG-induced Ca^{2+} transients were inhibited in MPP^{+}-treated murine striatal astrocytes, and the same was observed in HEK293 cells overexpressing TRPC3 (Streifel et al., 2014). These studies suggest that an increased Na^{+} influx of TRPC3 due to oxidative stress may reduce Ca^{2+} influx and contribute to the treatment of PD. Inhibition of TRPC3 was shown to depolarize GABA neurons in the substantia nigra pars reticulate (SNpr), which are associated with parkinsonism (Zhou et al., 2008). In summary, AD symptoms are recovered by TRPC3 activation. In a previous study, BDNF protected neurons from the neurotoxicity of Aβ and tau (Arancibia et al., 2008; Jiao et al., 2016). TRPC3 activation may decrease Ca^{2+} concentration due to a change in the influx ratio of Na^{+}/Ca^{2+}. Insufficient Ca^{2+} concentration by TRPC3 activation may be involved in BDNF-induced interference of Aβ plaque formation and tau hyperphosphorylation (**Figure 2A**).

TRPC4 and TRPC5

TRPC4 and TRPC5, which share similar amino acid sequence identity, have important roles in the neuron (**Tables 1**, **2**), in particular with respect to memory in the hippocampus. TRPC5 regulates synaptic plasticity by changing the presynaptic Ca^{2+} homeostasis of hippocampal neurons (Schwarz et al., 2019). TRPC1/4/5 knockout (KO) mice show reduced action potential-triggered excitatory postsynaptic currents (EPSCs) in hippocampal neurons and deficits in spatial working memory (Broker-Lai et al., 2017). However, comprehensive studies of TRPC4 in ND are yet to be undertaken. Only axonal regeneration is associated with TRPC4 expression in the dorsal root ganglia (DRG) (Wu et al., 2008). Neuron growth factor (NGF) and dibutyryl cAMP increase the expression level of TRPC4 in DRG differentiation. Improvement of TRPC4-siRNA reduces the length of neuritis. These results suggest a role for TRPC4 in ALS (**Figure 2H**). TRPC5, together with TRPC1, has high expression levels in the SN and an important role in dopaminergic neurons (De March et al., 2006). In rat PC12 cells, overexpression of TRPC5 inhibited the neurite outgrowth induced by NGF, and shRNA-mediated KD of TRPC5 enhanced outgrowth (Kumar et al., 2014). TRPC5 has also been reported to regulate neuronal growth cone morphology and nervous system development. In the downstream processes involving semaphorin 3A, growth cone collapse is induced through the cleavage and activation of TRPC5, using calpain (Kaczmarek et al., 2012). In neural progenitor cells, KD of TRPC5 using siRNA reduced the elevation of SOCE and blocked the switch between proliferation and neuronal differentiation (Shin et al., 2010). TRPC5 activity also inhibited neural migration and neurite extension (Tian et al., 2010). Similarly, in the striatum of both YAC128 HD transgenic (Tg) mice and patients, we identified that altered glutathione homeostasis, or increased oxidative potential, resulted in Ca^{2+}-dependent apoptosis of striatal neurons, consistent with increased TRPC5 S-glutathionylation and hyperactivation (**Figure 2D**). Thus, downregulation of TRPC5 activity by siTRPC5 KD and ML204-specific blocker improved the survival of striatal neurons and behavioral motor symptoms (Hong et al., 2015). Furthermore, we recently reported that TRPC5 instability induced by depalmitoylation protects against neuronal death of HD striatal cells (Hong et al., 2019). S-palmitoylation is a reversible covalent lipid modification that promotes membrane trafficking and stability by anchoring the palmitoylated protein to the membrane (Greaves et al., 2009).

TRPC6

It is known that early onset dominant AD is caused by mutations in the APP (Dahlgren et al., 2002) or presenilin 1 (PS1) genes (Dillen and Annaert, 2006). APP is a precursor protein of Aβ, and PS1 is a key enzyme family that cleaves APP in complex with γ-secretase and cleavage of Notch. TRPC6 regulated the mechanism of the PS gene to prevent the progression of AD (Lu et al., 2017). Previous studies have reported that tetrahydrohyperforin, an agonist of TRPC6, lowers Aβ levels and ROS generation, also preventing learning and memory deficits in the AD model. In the research of Dinamarca and colleagues, tetrahydrohyperforin reduced amyloid deposition in rats injected with amyloid fibrils into the hippocampus, inhibited the neurotoxicity of amyloid fibrils and Aβ oligomers in hippocampal neurons, and improved neuropathological behavior in an amyloidosis rat model (Dinamarca et al., 2006). It has also been shown that interaction between TRPC6 and APP leads to inhibition of its cleavage by γ-secretase and a reduction in Aβ production (Wang et al., 2015). The authors also reported that expression of TRPC6 interferes with the

TABLE 1 | Expression levels of TRP channels in human brain, as reported by the Human Protein Atlas.

	0	0.1	1	10	<
pTPM					

	TRPA	TRPC								TRPM								TRPV				
	AI	C1	C2	C3	C4	C5	C6	C7	MI	M2	M3	M4	M5	M6	M7	M8	VI	V2	V3	V4	V5	V6
Amygdala	0.0	5.5	0.1	0.8	0.6	0.1	0.5	0.0	0.1	4.5	3.1	3.3	0.0	0.7	2.0	0.0	5.4	2.0	2.8	0.1	0.2	0.7
Basal ganglia																						
Cerebellum	0.0	19.2	0.0	5.3	0.3	0.2	0.6	0.0	0.1	19.1	20.0	8.6	0.0	0.7	15.8	0.0	23.6	3.0	1.8	0.2	0.1	0.8
Cortex	0.0	8.2	0.0	1.4	0.5	0.3	0.7	0.0	0.1	16.7	3.3	6.2	0.0	0.7	3.3	0.0	6.9	5.6	3.6	0.2	0.2	4.9
Hippocampal formation	0.0	6.8	0.1	0.8	0.8	0.2	0.5	0.0	0.2	5.3	1.8	2.7	0.0	1.1	2.5	0.0	5.8	2.5	2.8	0.2	0.4	1.1
Hypothalamus	0.0	9.8	0.1	1.3	1.1	0.3	0.7	0.5	0.0	7.8	4.0	4.3	0.0	0.8	2.9	0.1	6.8	7.2	2	0.2	0.2	0.9
Mid brain (Substantia nigra)	0.0	7.0	0.1	1.1	0.6	0.1	0.7	0.0	0.5	5.9	4.9	4.3	0.0	1.6	2.8	0.0	6.8	4.1	2.7	0.2	0.5	2.4
Pituitary gland																						
Spinal cord	0.0	11.9	0.1	0.6	0.3	0.5	0.3	0.0	0.6	5.1	1.9	3.3	0.0	4.5	4.2	0.0	8.3	3.5	4.4	0.3	1.7	8.3

The table represents the expression levels of each TRP channel in nine regions of the human brain. For ease of understanding, we have used different cell colors depending on the level of protein expression from a TPM value of zero (white) to a value greater than 10 (black). The data used for the analyses described in this manuscript were obtained from the Human Protein Atlas[1]. We refer to the TPM calculation method of τi and νi, paragraph 1.1 in Li et al. (2010). pTPM values were rounded to two decimal places (Uhlen et al., 2015).

TABLE 2 | Expression levels of TRP channels in the human brain, as reported by the GTEx project.

	0	0.1	1	10	<
pTPM					

	TRPA	TRPC								TRPM								TRPV				
	AI	C1	C2	C3	C4	C5	C6	C7	MI	M2	M3	M4	M5	M6	M7	M8	VI	V2	V3	V4	V5	V6
Amygdala	0.0	4.6	1.7	0.7	0.6	0.1	0.4	0.0	0.1	2.7	3.8	1.8	0.0	0.3	2.1	0.1	1.8	2.8	0.0	0.1	0.2	0.1
Basal ganglia	0.0	5.7	2.6	3.8	0.4	0.0	0.6	0.1	0.1	2.6	3.1	1.9	0.0	0.3	3.1	0.1	1.7	1.7	0.1	0.1	0.2	0.1
Cerebellum	0.0	17.2	9.8	5.1	0.3	0.2	0.5	0.0	0.0	9.8	20.1	4.5	0.0	0.4	15.0	0.0	3.0	2.5	0.0	0.2	0.1	0.2
Cortex	0.1	8.7	8.0	1.5	0.6	0.4	0.8	0.0	0.0	8.0	3.4	2.9	0.0	0.3	3.3	0.0	1.7	5.0	0.0	0.1	0.1	0.1
Hippocampal formation	0.0	5.8	2.4	0.7	0.9	0.1	0.5	0.0	0.2	2.4	3.2	1.8	0.0	0.5	2.6	0.0	1.8	2.5	0.0	0.2	0.4	0.3
Hypothalamus	0.0	8.2	3.6	1.2	1.2	0.4	0.7	0.2	0.0	3.6	5.0	2.6	0.0	0.4	3.0	0.1	2.4	7.7	0.0	0.1	0.3	0.3
Mid brain	0.0	5.7	2.9	1.0	0.5	0.1	0.9	0.0	0.5	2.9	5.0	2.5	0.0	0.7	2.8	0.0	2.7	4.9	0.0	0.1	0.6	0.9
Pituitary gland	0.0	14.6	0.8	10.8	0.3	0.0	0.3	0.0	0.0	0.8	4.2	6.5	0.4	0.3	8.3	0.8	2.3	2.4	0.0	3.2	0.0	0.7
Spinal cord	0.0	8.7	3.2	0.5	0.3	0.0	0.2	0.5	0.7	3.2	3.3	2.9	0.0	1.8	4.4	0.0	1.7	4.5	0.2	0.3	1.5	2.9

The table represents the expression levels of each TRP channel in seven regions of the human brain. For a better understanding, we have used different cell colors depending on the level of expression from a TPM value of zero (white) to a value greater than 10 (black). The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 12/08/2019. We refer to the TPM calculation method of τi and νi, paragraph 1.1 in Li et al. (2010). pTPM values were rounded to two decimal places.

interaction of APP (C99) with PS1 but does not interact with Notch. Crossing TRPC6 Tg mice and APP/PS1 model mice reduced plaque load and Aβ levels and improved cognition (**Figure 2A**). Lessard et al. (2005) reported the effect of PS2 on TRPC6-mediated Ca^{2+} entry. In this study, PS2 inhibited the influx of Ca^{2+} from TRPC6. Induction of Ca^{2+} was higher when FAD-linked PS2, a dominant negative form, was co-expressed with TRPC6 than with wild-type (WT) PS2 and TRPC6 (**Figure 2A**). However, TRPC6 was still activated by 1-oleoyl-2-acetyl-sn-glycerol (OAG), suggesting that it does not impair channel function[1].

[1]http://www.proteinatlas.org

TRPM (Melastatin)

The TRPM subfamily has the highest expression level in the brain amongst the TRP channels, and there are many reports in relation to ND. One of the most distinctive features of TRPM ion channels is the high permeability to Ca^{2+} and Mg^{2+} (Hashimoto and Kambe, 2015). The important role of Mg^{2+} signaling in neuroprotection and neurodevelopment has been reported on (Lingam and Robertson, 2018). Thus, studies of TRPM in ND relate pathogenesis to Mg^{2+} signaling.

TRPM2

TRPM2 is an ion channel that is abundantly expressed in the brain (**Tables 1**, **2**). TRPM2 has been reported to be activated

by a wide range of factors, such as oxidative stress, NAD^+-related metabolites, and ADP-ribose (Huang et al., 2018). In the study of Belrose et al. (2012) depletion of glutathione (GSH) was reported to be a factor in activating TRPM2. In hippocampal neurons, an increase in ROS due to GSH depletion activated TRPM2, and an increase in TRPM2-dependent Ca^{2+} influx induced neuronal apoptosis (Ovey and Naziroglu, 2015). In the study of Fonfria et al. (2005) an increase in intracellular Ca^{2+} and Aβ induced by TRPM2 activity induced neuronal cell death in rat striatal. Treatment with TRPM2 blocker or SB-750139, which inhibits the production of ADP-ribose, inhibited intracellular Ca^{2+} concentration and cell death via H_2O_2 and Aβ. In the study of Ostapchenko et al. (2015) an aged APP/PS1 AD mouse model showed increased ER stress and decreased presynaptic markers (**Figure 2B**). However, elimination of TRPM2 in APP/PS1 mice improved abnormal response regardless of plaque burden. Age-dependent spatial memory deficits were observed in APP/PS1 mice (Ostapchenko et al., 2015). However, the absence of TRPM2 in these mice attenuated synapse loss and spatial memory. In summary, GSH deficiency and ROS induction activate TRPM2, and Ca^{2+} influx by TRPM2 contributes to the neuronal toxicity of Aβ. TRPM2 may be an important therapeutic target for AD. In the study of PD, Ca^{2+} influx through the TRPM2 channel was induced by ROS and promoted the death of dopaminergic neurons in the SN (Sun et al., 2018). A variant of TRPM2 (P1018L) was found in a Guamanian ALS/PD patient. P1018L attenuates oxidative stress-induced Ca^{2+} influx through TRPM2 (Hermosura et al., 2008).

TRPM7

TRPM7 has the Mg^{2+} permeability to maintain the homeostasis of Mg^{2+}. In HEK293 cells overexpressing TRPM7, H_2O_2 increased Ca^{2+} concentration and TRPM7 current (Nadler et al., 2001). In mouse cortical neurons, TRPM7-siRNA KD and treatment with TRPM7 inhibitors protected against neuronal cell damage (Coombes et al., 2011). In contrast, TRPM7-overexpressing HEK293 cells aggravated cell damage from H_2O_2, which was independent of the voltage-gated Ca^{2+} channel. Interestingly, in the study of Aarts et al. (2003) blocking of Ca^{2+}-permeable non-selective cation conductance or KD of TRPM7 inhibited TRPM7 currents, anoxic Ca^{2+} uptake, ROS production, and anoxic death in cortical neurons. Mg^{2+} permeability of TRPM7 is implicated in PD (**Figure 2C**). Continuous administration of Mg^{2+} significantly inhibited the neurotoxicity of MPP^+, reduced the death of dopaminergic neurons, and improved the length of dopaminergic neurites (Hashimoto et al., 2008). In a recent zebrafish study, TRPM7 mutation suppressed dopamine-dependent developmental transitions and increased sensitivity to the neurotoxicity of MPP^+ (Decker et al., 2014), and expression of the channel-dead variant of TRPM7 in SH-SY5Y cells increased cell death. These studies suggest that the role of Mg^{2+} influx and TRPM7 in dopaminergic neurons is important and could be a therapeutic target for PD. A variant of TRPM7 (T1482I) was also found in Guamanian ALS/PD cases. Incidentally, mutant G93A-superoxide dismutase (SOD1) mice are used as an ALS model (Guo et al., 2009).

TRPV (Vanilloid)

The TRPV subfamily has been reported to have the highest number of sensory functions, such as nociception, mechano-sensing, osmolarity-sensing, and thermo-sensing. Usually, TRPV is expressed in peripheral sensory nerves, although pathological studies have also reported expression in the brain. The various antagonists of TRPV4 could protect damaged neurons and inhibit the production of ROS (Suresh et al., 2018; Wu et al., 2019).

TRPV1

TRPV1 is expressed not only in the plasma membrane but also in the ER and calcium storage vesicles (Marshall et al., 2003). TRPV1 is phosphorylated to enable translocation from the ER to the plasma membrane. TrkA activity due to NGF increases the surface expression level of TRPV1 located in the ER through PKC phosphorylation (Zhang et al., 2005). An increase in intracellular calcium levels due to TRPV1 activity may aggravate neuronal cell death. In microglia cells, TRPV1 activity by agonists such as capsaicin (CAP) and resiniferatoxin (RTX) induce apoptosis (Kim et al., 2006). Dopamine release is dependent on the mechanosensitive TRPV1 channels activated by cannabinoid receptor stimulation in dopaminergic neurons (Oakes et al., 2019). Capsaicin, a TRPV1 agonist, induces the death of mesencephalic dopaminergic neurons through the activation of TRPV1 and CB1 receptors. Activation of TRPV1 increases the release of the mitochondrial cytochrome C and caspase3 cleavage. Cell damage is attenuated by an intracellular Ca^{2+}-chelator. In a recent study, the activity of TRPV1 was reported to decrease Aβ-induced cytotoxicity (Balleza-Tapia et al., 2018). Treatment with a TRPV1 agonist rescued Aβ-induced degradation of hippocampal neuron function (**Figure 2A**). In addition, it is suggested that TRPV1 contributes to the movement of patients with HD (**Figure 2E**). In the 3-nitropropionic acid-induced HD model, hyperkinesia was attenuated by administering AM404, an endocannabinoid reuptake inhibitor (Lastres-Becker et al., 2003). This phenomenon is reversed by the TRPV1 antagonist capsazepine, suggesting that TRPV1 activity may facilitate the movement of HD patients. Depending on the pathological mechanism, the role of TRPV1 activity can be distinguished accordingly and can be an important drug development target of NDs.

TRPV4

The activity of TRPV4 causes neuronal injury in pathological conditions. In many types of cells, TRPV4 activity increases the production of ROS and NO (Hong et al., 2016). GSK1016790A, an agonist of TRPV4, increased the concentration and NO in the hippocampus. TRPV4 agonist-induced neuronal cell death in hippocampal CA1 was inhibited by treatment with ROS scavengers such as Trolox or ARL-17477. In a recent report, TRPV4 enhanced neuronal inflammatory responses and pro-inflammatory cytokine release (**Figure 2F**; Wang et al., 2019). GSK1016790A-injected mice also showed increased levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 and showed TRPV4-mediated microglial and astrocyte activation. Although direct evidence linking TRPV4 to NDs has not

been reported, these results suggest a clear association between neuronal cell death and ROS.

TRPA

The TRP ankyrin 1 (TRPA1) channel is a non-selective transmembrane cation channel with multiple ankyrin repeats at its N-terminal. TRPA1 is mainly expressed in primary sensory neurons and non-neuronal cells (Jo et al., 2013). According to RNA-seq ALTAS data (**Tables 1**, **2**), the expression level of TRPA1 in the brain is low, but various functions have been reported in recent studies. Reported TRPA1 functions are mainly the detection of pain, cold temperature, and cannabinoids, in addition to noxious compounds that elicit pain and neurogenic inflammation (Paulsen et al., 2015).

TRPA1

Until now, the role of TRPA1 in neurons has only been reported on with respect to pain and inflammation, although recent studies have revealed a potential involvement in AD pathogenesis. Deposition of Aβ is an important factor in the exacerbation of AD, and soluble Aβ oligomers mediate fast and widespread Ca^{2+} influx in astrocytes (Bosson et al., 2017). TRPA1 was first identified in mouse hippocampal astrocytes and associated with Aβ-mediated Ca^{2+} signaling (**Figure 2F**; Lee et al., 2016). The cause of Aβ oligomer-mediated fast Ca^{2+} signaling appears to be the hyperactivation of TRPA1 (Bosson et al., 2017). TRPA1-induced Ca^{2+} signaling initiates the release of inflammatory factors such as PP2B, NF-κB, and NFAT (**Figure 2F**). APP/PS1 Tg mice, an AD mouse model, have increased expression of TRPA1 in hippocampal astrocytes. Loss of function of TRPA1 channels improves spatial learning, memory and cognition, and decreases Aβ deposition in APP/PS1 Tg mouse also (Lee et al., 2016). In summary, TRPA1-induced Ca^{2+} influx in astrocytes may be evidence of the critical role of Aβ in inflammatory processes and AD progression. Drug development focused on TRPA1 could be a novel target for treating dysfunction in AD.

TRP CHANNELS: NOVEL THERAPEUTIC CANDIDATE FOR NEURODEGENERATIVE DISEASE

Various causes of NDs have been reported recently. However, most of the brain lesions in ND present alongside several

TABLE 3 | Disease-related functions of TRP channels.

Disease	*Region*	*Channel*		*Mechanism of related disease*	*References*
NDs	Microglia	TRPC	C3	Inhibit to release cytokines and NO	Mizoguchi et al., 2014
	Neuronal progenitor		C5	Reduce elevation of SOCE Regulate the switching between proliferation and differentiation	Shin et al., 2010
	Hippocampus	TRPM	M2	Activate due to ROS and increase Ca^{2+}-mediated cell death	Ovey and Naziroglu, 2015
	Cortical		M7	Aggravate cell damage by increase Ca^{2+} induced oxidative stress	Coombes et al., 2011
	Microglia	TRPV	V1	Increase neuronal cell death by agonist, such as cannabinoid, Capsaicin	Kim et al., 2005
	Microglia Astrocyte		V4	Enhance neuronal inflammatory responses Inhibit pro-inflammatory cytokine release	Wang et al., 2019
	Hippocampus	TRPC	C3	Regulate to switch between proliferation and differentiation Change influx ratio of Na^{+}/Ca^{2+} when activate by oxidative stress	Wu et al., 2004; Rosker et al., 2004
			C5	Regulate neuronal growth cone morphology and nervous system development	Kumar et al., 2014; Kaczmarek et al., 2012
			C6	Inhibit function of y-secretase and reduced Aβ level by PS1 PS2 regulate TRPC6-mediated Ca^{2+} entry Interaction between TRPC6 and APP inhibit PS1 process	Lu et al., 2017; Lessard et al., 2005; Wang et al., 2015
AD	Hippocampus Striatum	TRPM	M2	Increase Aβ-mediated and Ca^{2+}-mediated cell death Damage to neuronal cell in APP/PS mice	Fonfria et al., 2005 Ostapchenko et al., 2015
	Hippocampus	TRPV	V1	Decrease Aβ-induced cytotoxicity and apoptosis	Balleza-Tapia et al., 2018
			V4	Aggravate neuronal cell death from oxidative stress	Hong et al., 2016
		TRPA	A1	Exacerbate spatial learning, memory and cognition Increase Aβ deposition and release inflammatory factors	Lee et al., 2016
PD	Substantia nigra	TRPC	C1	Decrease neurotoxicity and unfolded protein response Regulate SOCE and increase survival of dopaminergic neuron	Bollimuntha et al., 2006 Selvaraj et al., 2009, 2012
	Dopaminergic neuron	TRPM	M7	Reduce neurons death and activate growth the length of DA neurites	Hashimoto et al., 2008; Oakes et al., 2019
HD	Striatum	TRPC	C1	Inhibit neuronal cell death by reducing TRPC5 activity	Hong et al., 2015
			C5	Increase neuronal apoptosis by activation induced oxidative stress	Hong et al., 2015
	Basal ganglia	TRPV	V1	Improve the movement of HD patient models	Lastres-Becker et al., 2003
ALS	DRG	TRPC	C4	Activate growth of neurite length and regulation of differentiation	Wu et al., 2008

ND, neurodegenerative diseases; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; SOCE, store-operated calcium entry; PS, presenilin1; ROS, reactive oxygen species; Aβ, amyloid-beta; DA, dopamine; DRG, dorsal root ganglia.

pathological environments, such as the presence of ROS, impaired antioxidant systems, and disrupted Ca^{2+} homeostasis (Di Meo et al., 2016). In consideration of the results discussed above, the regulation of TRP channels plays a key role in the Ca^{2+}-dependent neuronal death in NDs. The involvement of TRP channels in NDs is summarized in **Figure 1**. To investigate the role of TRP channels in NDs, TRP channel antagonists and TRP-KO mice have been generated and utilized. TRP KO mice exhibit behavioral and neurological phenotypes.

TRPC3 is required for slow excitatory postsynaptic potential in cerebellar Purkinje cell synapses, and consequently, severe ataxic phenotypes have been shown in TRPC3 KO mice. In contrast to TRPC1/4 double-KO or TRPC1/4/6 triple-KO mice, TRPC3 KO mice show movement deficits of the hind-paws (Hartmann et al., 2008). In addition, TRPC4 plays a role in fear and anxiety-related behaviors. TRPC4 KO mice show innate fear responses in elevated plus maze and open-field tests. These fear responses result from mGluR-mediated EPSC in the lateral nucleus of the amygdala neurons (Riccio et al., 2014). TRPC5 also plays an essential role in fear-related behavior. In addition, disruption of burst firing in the potent muscarinic antagonist pilocarpine-induced seizure in TRPC5 KO mice was reduced. Seizure-induced neuronal loss in the hippocampal region was also reduced in TRPC5 KO mice (Phelan et al., 2013). TRPM2 KO mice exhibited disturbed EEG rhythms and bipolar disease-related behavior, including impairment of social behavior and increased anxiety (Jang et al., 2015). TRPM7 KO mice exhibited clasping, tremors, and slow movement associated with Mg^{2+} deficiency (Ryazanova et al., 2010).

TRPC KO mice combined with ND models have also been generated. In models of AD, TRPC6 modulates cleavage of APP by gamma secretase and APP (C99) interaction with PS1 (Wang et al., 2015). TRPC6 overexpression in APP/PS1 mice results in a reduction of Aβ accumulation in the hippocampus (**Table 3**). Therefore, TRPPC6 overexpression improves spatial learning and memory in APP/PS1 mice. In addition, the expression of the inflammatory factors TNF-α, IL-1β, COX-2, and IL-6 is regulated by levels of TRPC6 via Aβ, and levels of TRPC6 are increased by Aβ via NF-κB in BV-2 microglia cells (Lu et al., 2018). TRPM2 expression is involved in synapse loss, microglial activation, and spatial memory deficits in APP/PS1 mice (Ostapchenko et al., 2015). Activation of TRPV1 channels is required to trigger long-term depression at interneuronal synapses (Gibson et al., 2008) and prevents Aβ-involved impairment of functional networks in the hippocampus (Balleza-Tapia et al., 2018). Astrocytic Ca^{2+} hyperactivity is induced by Aβ oligomers via TRPA1 in the hippocampus. Moreover, astrocyte hyper-excitability is replaced by CA1 neuronal activity in APP/PS1 mice (Lee et al., 2016). Moreover, TRPA1 regulates astrocyte-derived inflammation in APP/PS1 mice. TRP channel antagonists regulate the production of ROS, APP processing, and Aβ accumulation. The TRPV4 antagonist HC-067047 attenuates the H_2O_2-induced Ca^{2+} influx (Suresh et al., 2015). Aβ-mediated cell damage was attenuated by treatment with TRPV4 blockers ruthenium red and gadolinium chloride (Bai and Lipski, 2014).

TRPC1, TRPC3, TRPM2, TRPM7, and TRPV1 have been shown to be involved in PD. TRPC1 activation reduces dopaminergic neuronal death (Selvaraj et al., 2009). TRPC3-mediated Ca^{2+} influx contributes to the survival of neurons in the SN (Zhou et al., 2008). Additionally, MPP^{+}-induced oxidative stress increases intracellular Ca^{2+} via TRPM2 activation (Sun et al., 2018). TRPM7 channels regulate magnesium homeostasis in cells, and the presence of Mg ameliorates MPP^{+} toxicity (Hashimoto et al., 2008; Paravicini et al., 2012). Ca^{2+} influx via TRPV1 in dopaminergic neurons mediates mitochondrial dysfunction, microglial activation, ROS generation, and cell death (Kim et al., 2005; Nam et al., 2015). TRPV1 or TRPN-like channel-dependent dopamine release is mediated by CB1 stimulation (**Table 3**; Oakes et al., 2019). In addition, regulation of TRPV1 activity is closely related to the survival of dopaminergic neurons. TRPM2 is controlled by oxidative stress (Belrose et al., 2012; Huang et al., 2018). Therefore, the regulation of TRP channels contributes to overcoming dopamine depletion and the loss of dopaminergic neurons in PD patients. Likewise, oxidizing modulation of posttranslational modification (glutathionylation) of TRPC5 leads to apoptosis in an HD model (**Figure 2D**; Hong et al., 2015). Attenuation of TRPC5 activity by KD, blocker, or depalmitoylation shows therapeutic effects against oxidative stress by lowering TRPC5 toxicity (Hong et al., 2019). Additionally, regulation of TRPML1, TRPM2, and TRPM7 activity might also be a therapeutic strategy for ALS (Hermosura and Garruto, 2007; Hermosura et al., 2008; Tedeschi et al., 2019). The TRPV1 antagonist capsazepine has antihyperkinetic effects in a model of HD (Lastres-Becker et al., 2003).

As previously reported, TRP channels can be assembled as homo- or heteromeric complexes in nature. However, individual TRP channel KO models that lack phenotypes are limited in their ability to determine the cause of the functional compensation of each TRP channel. Moreover, *in vivo* studies regarding chronological changes in TRP channel expression patterns in the brain are needed for NDs. The regulation of TRP channels can be a novel therapeutic target for NDs. Nevertheless, a limitation to the development of TRP channel-specific antagonists is that their structures remain unknown. Therefore, structural analyses must precede pathological and clinical studies.

CONCLUSION

Transient receptor potential channels may not be the only, or main, pathogenic factors contributing to the pathogenesis of ND. Research in the field of ND is challenging; it is necessary to either conclusively prove a relationship between pathogenic factors or identify new therapeutic targets. In achieving one of these two possibilities, it is important not to underestimate the potential of TRP channels, based on the physiological and pathological functions of TRP channels discovered so far. Through the interrelationship between disruption of Ca^{2+} homeostasis and the development of NDs, lowering the activity of TRP channels is sufficient to enable expectations for new therapeutic strategies. As we have discussed, increased TRP channel activity has been widely observed in NDs, and model studies have shown that abnormal function due to upregulation of TRP channels

can be controlled by drug treatments. Since many drugs have been reported to be TRP channel inhibitors, understanding binding modes will provide deep insight for pharmacological application in NDs. However, direct evidence for drug binding to TRP channels is unavailable. To address this issue, the most effective approach for understanding drug binding would be a structural study.

POSSIBILITIES OF DRUG-BOUND STRUCTURE

For several decades, the detailed structures and topologies of TRP channels were not understood, although the first high-resolution structure of TRPV1 was recently resolved by a single particle cryo-EM (Liao et al., 2013). Since TRPV1 was the first membrane protein to be characterized from single particle cryo-EM and the biochemical methods were relatively similar for other family members, most follow-up studies concentrated on TRP channel structure. However, though high-resolution structures have been resolved for most ND-related TRP channels, the structures of the drug-bound forms are mostly unknown. Here we discuss the technical possibilities for determining the drug-bound structure of the TRP channel family.

For analysis of drug-channel binding, which would improve our understanding of the inhibition mechanisms and would provide clues for developing drug design, the determination of the high-resolution structure is absolutely necessary. In the TRP family, the resolutions of most ND-related TRP structures are above 3.5 Å, with a few exceptions such as TRPC6, TRPA1, and TRPV4 (3.8, 4.24, and 3.8 Å, respectively) (Paulsen et al., 2015; Deng et al., 2018; Tang et al., 2018). TRPC5 broke the 3 Å barrier (Duan et al., 2019), and TRPM2 also came close to hitting the barrier (3.07 Å) (Zhang et al., 2018). The structures of other members of the TRP family that are associated with NDs, such as TRPC3, TRPC4, TRPM7, and TRPV1, were determined at ∼3.3 Å (Liao et al., 2013; Duan et al., 2018; Fan et al., 2018; Liu et al., 2018). Therefore, there is still room for improvement in their resolutions, possibly by biochemical techniques such as nanodisc reconstruction. The resolution of the drug-bound form does not always guarantee higher resolution than that of the apo structure, but generally, the occupation of an inhibitor in the binding cavity facilitates conformational stabilization, resulting in higher resolution. Therefore, since there is optimism regarding drug-binding studies, we suggest that ongoing attention and efforts be focused on this area of therapeutic target research.

AUTHOR CONTRIBUTIONS

CH, BJ, HP, and IS wrote and discussed the review at almost all stages. JL professionally edited the manuscript for English language. JC, JK, and Y-CS analyzed representative tables presenting TRP channel expression data and also provided technical support. IS and CH supervised the entire writing process.

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Isoproterenol-Dependent Activation of TRPM7 Protects Against Neurotoxin-Induced Loss of Neuroblastoma Cells

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Neuronal function and their survival depend on the activation of ion channels. Loss of ion channel function is known to induce neurodegenerative diseases such as Parkinson's that exhibit loss of dopaminergic neurons; however, mechanisms that could limit neuronal loss are not yet fully identified. Our data suggest that neurotoxin-mediated loss of neuroblastoma SH-SY5Y cells is inhibited by the addition of β-adrenergic receptor (β-AR) agonist isoproterenol. The addition of isoproterenol to SHSY-5Y cells showed increased Mg^{2+} influx and cell survival in the presence of neurotoxin especially at higher concentration of isoproterenol. Importantly, isoproterenol potentiated transient receptor potential melastatin-7 (TRPM7) channel activation that leads to an increase in intracellular Mg^{2+} levels. The addition of 2APB, which is a known TRPM7 channel blocker, significantly decreased the TRPM7 function and inhibited isoproterenol-mediated protection against neurotoxins. Moreover, neurotoxins inhibited TRPM7 expression and function, but the restoration of TRPM7 expression increased neuroblastoma cell survival. In contrast, TRPM7 silencing increased cell loss, decreased Mg^{2+} homeostasis, and inhibited mitochondrial function. Moreover, isoproterenol treatment prevented neurotoxin-mediated loss of TRPM7 expression and inhibited Bax expression that induces cell survival. These effects were dependent on the neurotoxin-induced increase in oxidative stress, which inhibits TRPM7 expression and function. Together, our results suggest a positive role for β-AR in activating TRPM7 channels that regulate Mg^{2+} homeostasis and are essential for the survival of SH-SY5Y cells from neurotoxin.

Keywords: Mg^{2+} influx, TRPM7 activation, β-AR, cell survival, apoptosis, neurodegeneration

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INTRODUCTION

Parkinson's disease (PD) is an age-related movement disorder that is mainly due to selective degeneration of nigrostriatal dopaminergic (DA) neurons (Venderova and Park, 2012). The major clinical symptoms observed in PD are rigidity, bradykinesia, and resting tremor, which are caused by the deficiency in neurotransmitter dopamine-mediated signaling (Javitch et al., 1985;

Venderova and Park, 2012). Additionally, non-motor symptoms are also present in these patients, including cognitive and autonomic functions, olfactory, sleep, mood disorders, and gut physiology, which may not be directly due to the loss of other neurons (Chen et al., 2019). Although several mutations have been identified, majority of them (90%) of these PD cases identified, are idiopathic or sporadic in nature, and only a small percentage of patients exhibit genetic mutations, suggesting that exogenous factors makes these DA neurons vulnerable (Surmeier et al., 2017). Environmental factors such as neurotoxins have been one of the major inducers of PD, thus toxin-induced animal models have been crucial in elucidating the pathophysiology of PD. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an exogenous neurotoxin that induces Parkinson's like symptoms in humans, monkeys, and other small animals. MPTP is metabolized by monoamine oxidase type B (MAO-B) that is present in microglia/astrocytes cells, into 1-methyl-4-phenyl-pyridinium ion (MPP^+), which selectively destroys the nigrostriatal DA neurons (Javitch et al., 1985). Although protection against neurotoxins-mediated loss of DA neurons has been a major focus in preventing PD, the exact mechanisms involved in DA neuronal loss are not known. Recent studies have shown that imbalance in divalent cations that could lead to ER/oxidative stress and/or mitochondrial dysfunction could contribute to PD (Tatton and Olanow, 1999; Dawson and Dawson, 2003; Selvaraj et al., 2012; Sukumaran et al., 2018). Moreover, divalent cations such as calcium (Ca^{2+}) or magnesium (Mg^{2+}), which modulates cellular processes such as cell proliferation, mitochondrial function and energy metabolism, gene regulation, and synthesis of biomacromolecules (Selvaraj et al., 2012) has gained much attention as this could be a possible target for understanding PD.

Lower Mg^{2+} concentrations have been observed in the brain samples of PD patients as compared with non-PD subjects (Uitti et al., 1989; Bocca et al., 2006). Importantly, these decreases in Mg^{2+} concentrations were present in the substantia nigra region/mid brain region especially nucleus accumbens and the caudate nucleus (Uitti et al., 1989; Bocca et al., 2006). Furthermore, intracellular Mg^{2+} concentrations showed a significant correlation with the severity of PD and the extend of the disease phenotype observed (Uitti et al., 1989). Consistent with these reports, mice that had decreased Mg^{2+} concentrations exhibited an increase in the loss of neurotoxin-mediated cell death, especially in the DA neurons (Muroyama et al., 2009). Similarly, animals treated with another neurotoxin, 6-hydroxydopamine also exhibited decreased intracellular Mg^{2+} concentrations when compared with control mock-treated mice (Sturgeon et al., 2016). Although the channels that modulate Mg^{2+} influx are not well identified, Transient receptor potential Melastatin 6 and 7 (TRPM 6 and 7) channels are the main channels that modulate intracellular Mg^{2+} levels in various cells. Interestingly, *TRPM7* has been observed to be mutated in Guamanian ALS/PD patients (Hermosura et al., 2005) and TRPM7 expression is observed to be blunted in PD patients along with a similar decrease in neurotoxin models of PD (Sun et al., 2015). Similarly, TRPM7 mutants in zebrafish have decreased DA neurons (Decker et al., 2014), suggesting that changes in the Mg^{2+} influx could induce neurodegeneration. Consistent with this observation, decreased Mg^{2+} intake induced DA neuron loss, whereas Mg^{2+} supplementation prevented neurotoxin-mediated decrease in DA neurons (Oyanagi and Hashimoto, 2011; Sun et al., 2019). These results suggest that TRPM7-mediated regulation of intracellular Mg^{2+} could promote neuronal survival, however, its regulation, specifically TRPM7 activation in DA cells is not fully identified.

Increased intracellular levels of cAMP have also been shown to increase DA neurons survival and protect them from MPP^+-mediated degeneration (Scarpace et al., 1991; Hartikka et al., 1992). Importantly, β-adrenergic receptors (β_1-, β_2-, and β_3-AR subtypes) mediate the action of catecholamines via the classical adenylyl cyclase/cAMP/protein kinase A (PKA) cascade to modulate important biological responses (Hishida et al., 1992). Previous studies utilizing small groups of PD patients have demonstrated that co-administration of salbutamol (a β_2-AR agonist) with levodopa helps reduce parkinsonian symptoms (Alexander et al., 1994; Uc et al., 2003). Furthermore, longitudinal analyses of PD incidents in Norway demonstrated that the use of salbutamol is associated with a decreased risk of developing PD while treatment with β-AR antagonist (beta-blocker) propranolol increased the risk of suffering from PD (Mittal et al., 2017). Similarly, β_2-AR agonist clenbuterol reduced the levels of α-synuclein protein and protected against neurotoxin-induced degeneration of dopaminergic neurons (Mittal et al., 2017). Importantly, TRPM7 has been shown to be activated by β-AR in non-excitable cells, however, is similar mechanisms are observed in DA neurons is not yet defined. Thus, the purpose of this study was to establish if TRPM7 activation via β_2-AR agonist modulates neuronal survival. Our data suggest that β-AR agonist protects against neurotoxin-mediated loss of neuroblastoma cells, which was mediated through TRPM7. β-AR agonist potentiated TRPM7 function and maintained Mg^{2+} homeostasis that is essential for the survival of neurotoxin-induced loss of neuroblastoma SH-SY5Y cells. Furthermore, knockdown of TRPM7 abolished the protective effect of β-AR agonist, whereas TRPM7 overexpression increased intracellular Mg^{2+} levels and prevented MPP^+-induced cellular death. These results suggest that β-AR-mediated activation of TRPM7 could be essential in the survival of neurons especially in neurotoxin-induced degeneration.

MATERIALS AND METHODS

Cell Culture and Chemicals

Neuroblastoma cells (SH-SY5Y) were previously obtained from the American Type Culture Collection (Manassas, VA, United States), which were cultured as suggested and differentiated into dopaminergic like cells using retinoic acid (10 μM) for 7 days as previously described (Bollimuntha et al., 2005) prior to be used for all the experiments. The chemicals used were: 1-Methyl-4-phenylpyridinium, 2-Aminoethoxydiphenyl borate, Isoproterenol (+)-bitartrate salt which were purchased from Sigma-Aldrich. ISO was freshly prepared and dissolved in PBS and used for the experiments.

Transient Transfections and Cell Viability Assays

For the silencing of TRPM7 expression, shRNA plasmids that specifically targets the coding sequence of human TRPM7 was obtained from Origene (Rockville, MD, United States). All transfections were transient and differentiated SH-SY5Y cells were used for all experiments using lipofectamine as previously described (Sun et al., 2018). For TRPM7 overexpression, full length HA-TRPM7 plasmids was used to transiently overexpress TRPM7 in these cells. Briefly, 5 μg of the plasmid DNA was used to transform differentiated SH-SY5Y cells using Lipofectamine in the Opti-MEM medium for 24 h as indicated. To measure cell viability SH-SY5Y cells were trypsinized, counted, and seeded equally on 96-well plates at a density of 0.5×10^5 cells/well. The cultures were grown for 24 h with appropriate treatments as labeled in the figure and cell viability under various conditions was measured using the MTT regents as previously described by us (Selvaraj et al., 2012). Cell viability was expressed as a percentage of the control (untreated) when compared with neurotoxin treatment. The methods described here are modified from our previous publication (Sun et al., 2018).

Electrophysiology

For patch-clamp experiments, differentiated SH-SY5Y cells were grown on glass coverslips and single coverslips were placed in the recording chamber. The cells perfused with an external Ringer's solution that has the following composition (in mM): NaCl, 145; CsCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; Glucose, 10; pH 7.3 (NaOH). Whole-cell currents were recorded using an Axopatch 200B (Axon Instruments, Inc.) (Singh et al., 2000). The patch pipette used for each experiment had a resistance between 3 and 5 MΩ, which was measured after filling the standard intracellular solution, which contained the following (in mM): Cesium methanesulfonate, 150; NaCl, 8; Hepes, 10; EDTA, 10; pH 7.2 (CsOH). After whole cell configuration was established, the voltage ramp protocol was initiated that ranged from −100 mV to +100 mV and 100 ms duration were delivered at every 2 s intervals formed. Currents observed in each condition were recorded at 2 kHz, digitized followed by analysis using the pClamp 10.1 software that was used for data acquisition as well. The data presented is from an average of 6–10 cells in each condition. Basal leak currents were subtracted from the final currents (when current reach the peak) and average currents are shown. The methods used for this study are taken from our previous publication (Sun et al., 2018).

Magnesium/Calcium Imaging

For Imaging experiments, differentiated cells that were grown on glass bottom coverslips were incubated with 2 μM Mag-Fura 2-AM (Invitrogen) for the measurement of intracellular Mg^{2+} or with Fura-2 (Molecular Probes for 45 min) for the measurement of intracellular Ca^{2+}. After loading cells were washed twice with SES (Standard External Solution that includes: 10 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, pH 7.4) buffer. For fluorescence measurements, the fluorescence intensity of Fura-2 or Mag-FURA-loaded cells was monitored with a CCD camera-based imaging system linked with an Olympus XL70 inverted fluorescence microscope. Fluorescence traces from individual cells imaged were obtained and the data shown represent $[Mg^{2+}]_i$ or Ca^{2+} values that are average from at least 30–40 cells. Also, the data presented are representative of at least 3–4 individual experiments performed in duplicate. Mg^{2+} or Ca^{2+} concentrations in individual cells were estimated by evaluating the 340/380 ratio as described before in (Sun et al., 2018).

Western Blot Analysis

Cell lysates (differentiated SH-SY5Y cells) under different conditions (as labeled in the figures) were obtained using NP40 or 0.5% SDS treatment for 15 min on ice followed by centrifugation at 10,000 × *g* for 15 min at 4°C. Protein concentrations from all treatments as labeled in the figures were evaluated using the Bradford reagent (Bio-Rad) and 25 μg of total lysates from individual samples were resolved on NuPAGE 4–12% Bis-Tris gels. Western blotting were performed using specific antibodies (Singh et al., 2000; Selvaraj et al., 2009). The antibodies used were the following monoclonal or polyclonal antibodies: anti-TRPM7 (Abcam, MA; Cat# 109438; 213 kDa; Dilution in 1:500), anti-Bcl_2 (Cell Signaling, MA; Cat# 209039; Dilution used were 1:1000), anti-Bax (Cell Signaling, MA; Cat# 5023; Dilution used was 1:1000), anti-β-Actin (Cell Signaling, MA; Cat# 4970, at 1:2000 dilution) and anti- β_2-AR (Abcam, MA; Cat# ab36956; Dilution used was 1:1000). The methods described here are taken from our previous publication (Sun et al., 2018).

Mitochondrial Membrane Potential

Rhodamine 123 was used to measure the Mitochondrial transmembrane potential as described in Selvaraj et al. (2009). To quantify the membrane potential, fluorescence signals observed in different conditions were measured (excitation wavelength used was 488 nm and an emission wavelength used was 510 nm) using a fluorescence microplate reader (biotex) and plotted as percentage.

Statistical Analysis

Origin 9.0 (Origin Lab) was used for all data analysis. Statistical significance was established either using Student's *t*-test or one-way ANOVA (*post hoc* using Tukeys or Fisher test when compared between more than 2 variables). All values indicated in the figure are shown as means ± SEM or ± SD as stated in the figure legends. *p*-value are also indicated and $0 < 0.05$ or lower were considered significant.

RESULTS

β-Adrenergic Receptor Agonist Protects Against Neurotoxin-Dependent Loss of Cells

Neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) metabolite MPP^+, induce a loss of DA neurons in most vertebrate animals including

sub-human primates lower animals that show Parkinson's disease (PD) like symptoms (Burns et al., 1983). We thus used differentiated neuroblastoma SH-SY5Y cells that were treated with MPP^+ to examine the effect of this neurotoxin on its survival. Consistent with our previous results (Sun et al., 2017), addition of neurotoxin, MPP^+ showed an increase in cell death in differentiated SH-SY5Y cells (**Figure 1A**). Importantly, pretreatment of SH-SY5Y cells with β-adrenergic receptor (β-AR) agonist, isoproterenol showed a dose-dependent increase in cell survival (**Figure 1A**). Interestingly, even low doses (20 μM) of isoproterenol (ISO) showed a significant decrease in cell death when compared with cells that were treated with MPP^+ alone. Furthermore, increase in the doses used for ISO treatment (more than 40 μM) further increased cell survival, which was much higher than control untreated cells in 100 μM of ISO treatment. β-AR activation has been shown to mobilize intracellular Ca^{2+} via the non-canonical cAMP-independent pathway (Galaz-Montoya et al., 2017), thus we evaluated if isoproterenol treatment induces Ca^{2+} entry. However, as shown in **Figure 1B**, the addition of isoproterenol did not increase cytosolic Ca^{2+} levels in these cells. In contrast, an increase in Mg^{2+} concentration was observed upon addition of isoproterenol, which was significantly decreased upon the addition of 2APB (**Figures 1C,D**). Together these results suggest that isoproterenol stimulation modulates intracellular Mg^{2+} concentration that could protect against neurotoxin-induced cell death.

Recent studies have shown that intracellular Mg^{2+} concentration is mediated through TRPM6/7 channels (Sun et al., 2019). Whole-cell current recordings were used to further establish the channel identity that is responsible for Mg^{2+} influx. In differentiated SH-SY5Y cells decrease in intracellular Mg^{2+} generated a current, which was both inward and outward rectifying and reversed around zero mV (**Figures 2A–C**). The properties of the currents were similar as observed with TRPM6/7 channels. 2APB has been previously used to differentiate TRPM6/7 currents as addition of 2APB potentiates TRPM6 function, but inhibits TRPM7 currents (Mishra et al., 2009; Sun et al., 2019). Thus, to differentiate between these two Mg^{2+} channels we further studied the effects of 2-APB that further decreased these currents, suggesting that these currents

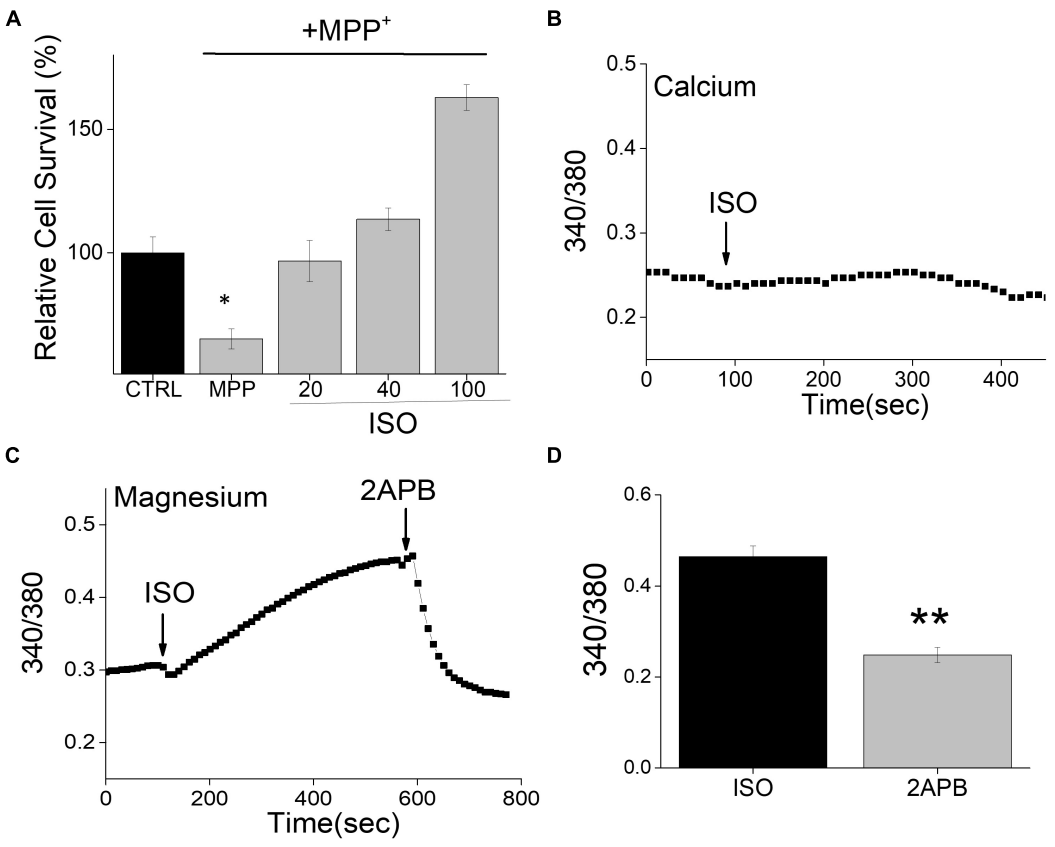

FIGURE 1 | Isoproterenol treatment induces survival of neuroblastoma cells: **(A)** Cell survival under conditions as labeled was performed using MTT assays on control and MPP^+ (500μM for 24 h) treated SH-SY5Y cells. The conditions used were control (mock treatment), ISO 20, 40, and 100 μM, which was added 15 min prior to the addition of MPP^+. Individual columns show the means ± SD of 5 separate experiments performed in triplicates (*$p < 0.05$, **$p < 0.01$; One-way ANOVA, Tukey *post hoc* test). **(B)** Ca^{2+} imaging in Fura 2 loaded cells was performed by the application of ISO (100 μM) in normal SES (1 mM Ca^{2+}, 1 mM Mg^{2+}) solution in SH-SY5Y cells. **(C)** Mg^{2+} imaging was performed using mag-Fura in normal SES (1 mM Ca^{2+}, 1 mM Mg^{2+}) solution using differentiated SH-SY5Y cells. Application of ISO (100 μM) in the external solution induces Mg^{2+} influx and addition of 2APB (100 μM) inhibits Mg^{2+} influx in these cells. **(D)** Quantification (mean ± SE) of intracellular Mg^{2+} concentrations are also included as bar graph.

are mainly through TRPM7 channels (**Figures 2A–C**). Also, the characteristics of the current observed was consistent with TRPM7 channels, which have been previously reported (Sun et al., 2013). Importantly, addition of isoproterenol further increased TRPM7 currents at both positive and negative membrane potentials, which was again inhibited by the addition of 2APB (**Figures 2D–F**).

TRPM7 Expression and Function Modulate Cell Survival in SH-SY5Y Cells

The data presented thus far suggest that isoproterenol activates TRPM7 currents. Hence, we further investigated this relationship, and cells overexpressing TRPM7 showed increased TRPM7 protein levels, without altering the expression of β-actin, which was used as a loading control (**Figure 3A**). Furthermore, an increase in the TRPM7 currents was observed in SH-SY5Y cells overexpressing TRPM7 (**Figures 3A,B**). Importantly, overexpression of TRPM7 inhibited MPP^+-mediated cell death of neuroblastoma cells (**Figure 3C**). Moreover, addition of low doses of isoproterenol (20 μM) in TRPM7 overexpressing cells did not increase cell protection any further (**Figure 3C**). In contrast, inhibition of TRPM7 currents using 2APB further increased MPP^+-mediated cell death even at low doses (20 μM) of isoproterenol, which was again blocked in TRPM7 overexpressed cells (**Figure 3C**). Similar results were obtained where MPP^+-inhibited cell survival of control untreated cells that do not overexpress TRPM7 (**Figure 3D**). In contrast, ISO treatment (20 μM) blunted the effects of MPP^+; however, pretreatment with 2APB inhibited ISO-mediated protection in control cells (**Figure 3D**). Importantly, the addition of non-specific Ca^{2+} channel blocker (SKF 96365) failed to block isoproterenol-mediated protection of differentiated SH-SY5Y cells (**Figure 3E**). Furthermore, addition of MPP^+ decreased TRPM7 expression, whereas addition of isoproterenol (even at lower doses, 20 μM) was able to prevent MPP^+-induced loss of TRPM7 (**Figure 3F**). We next evaluated the expression of various proteins upon exposure of SH-SY5Y cells to MPP^+ in the absence or presence of isoproterenol. Importantly, a significant decrease in DA neuron marker [tyrosine hydroxylase (TH)] was observed upon addition of MPP^+, which was partially rescued upon prolonged treatment with isoproterenol (**Figure 3G**). Consistent with TH expression, MPP^+-treatments also significantly increased expression of Bax, which is a pro-apoptotic protein along with a subsequent decrease in Bcl_2, an anti-apoptotic protein, without any change in actin levels (**Figures 3G,H**). Furthermore, treatments with isoproterenol decreased the expression of Bax and increased expression of Bcl_2 (**Figures 3G,H**), suggesting that TRPM7 expression and function are modulated via isoproterenol,

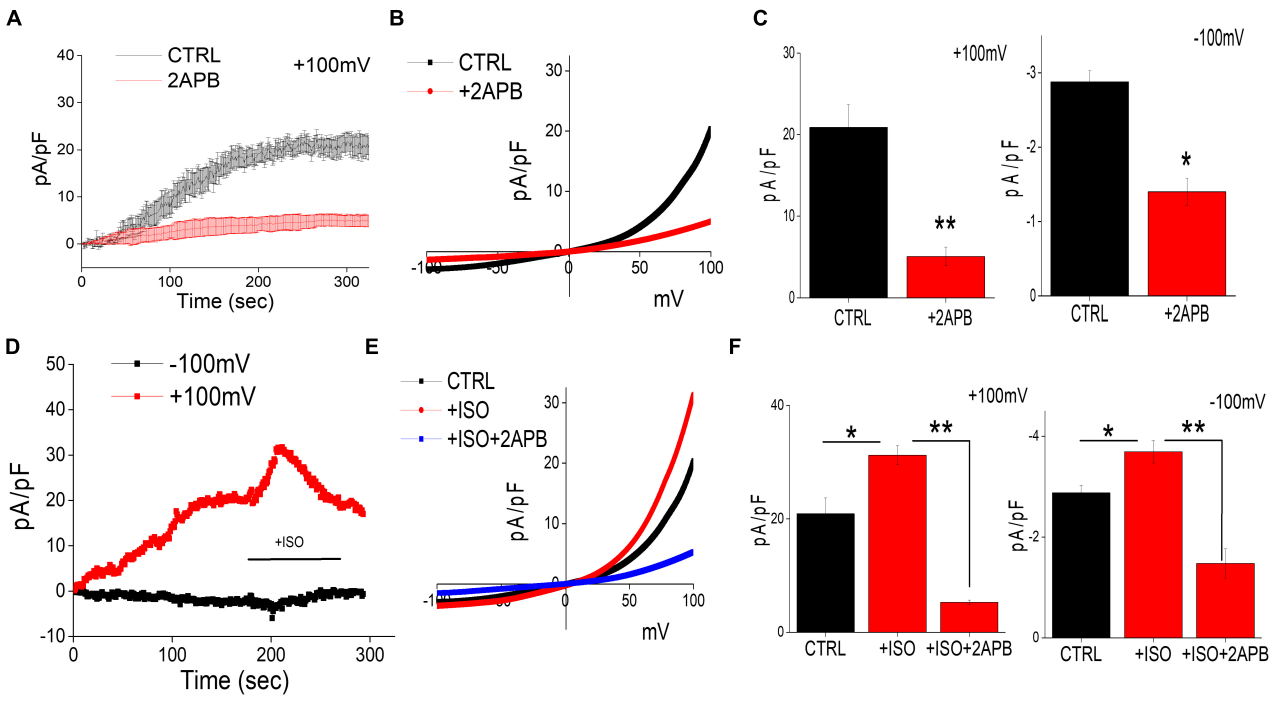

FIGURE 2 | Isoproterenol-Induces TRPM7 activation in neuroblastoma cells: **(A)** Representative trace showing whole cell recording (outward currents) in control cells and cells pretreated with 2APB (100 μM). The external solution used was normal SES solution that had 1 mM Ca^{2+} and Mg^{2+} respectively was used to obtain the currents (outward/inward) at +100 mV/–100 mV in differentiated SH-SY5Y cells. **(B)** IV curves under these conditions (control and bath pre application of 100 μM 2APB) as labeled in the figure were obtained using peak currents. Quantitation of current density at ±100 mV is shown in **(C)**. * Indicate significance ($p < 0.05$) and ** Indicate significance ($p < 0.01$). **(D)** Traces showing whole cell recording in control cells at ±100 mV were recorded followed by stimulation with ISO (bath application of 20 μM ISO, with and without pretreatment of 100 μM 2APB) as labeled in the figure. IV curves shown are acquired when currents reach their peak in each condition and are shown in **(E)** Quantitation of current density at ±100 mV is shown in **(F)**. The columns show the means ± SD of 6 experiments.

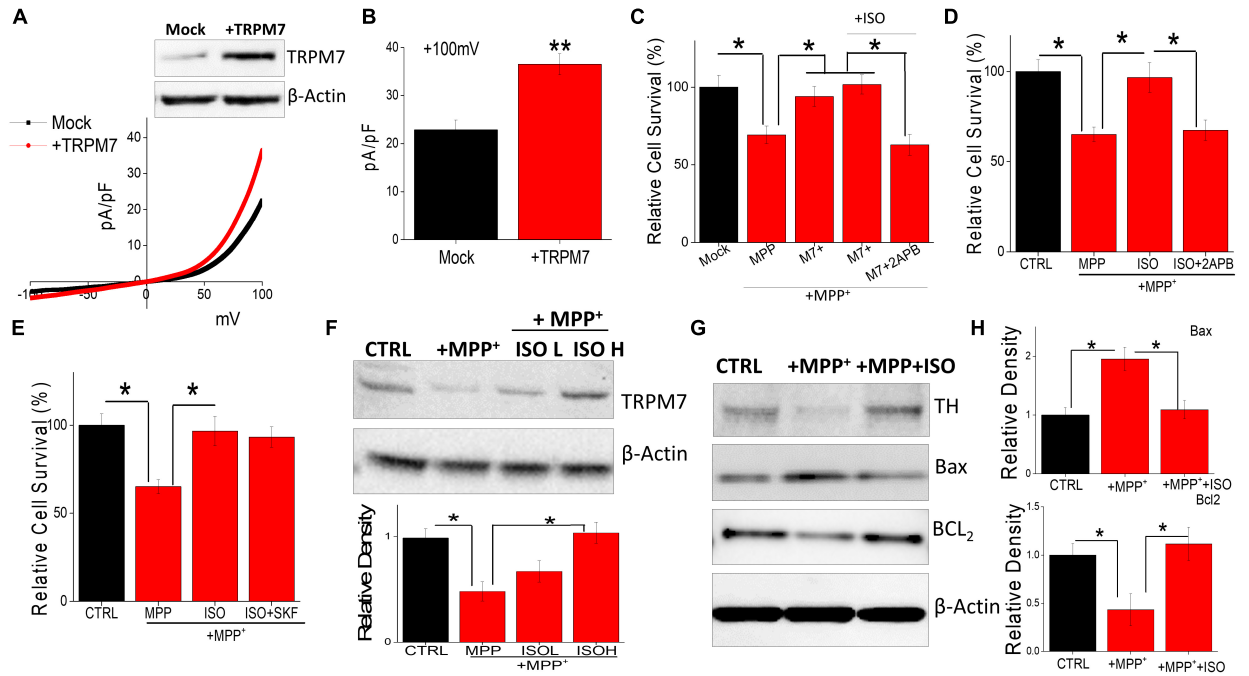

FIGURE 3 | Restoration of TRPM7 expression inhibits neurotoxin-induced loss of SH-SY5Y cells: IV curves in control and TRPM7 overexpressing cells were measured and shown in **(A)**. Western blots were also performed in these cells to look at TRPM7 expression under these conditions. Quantitation of current density at +100 mV is demonstrated in **(B)**. **(C–E)** MTT assays were performed to observe cell survival on TRPM7 overexpressed cells **(C)** and control cells **(D,E)** with and without MPP^+ (500 μM for 24 h) under various conditions as labeled (+ISO 20 μM, or +SKF 10 μM). The columns show the means ± SD of 5 individual experiments performed in triplicates. (*$p < 0.05$; One-way ANOVA, Tukey *post hoc* test). Sample from differentiated SH-SY5Y [treated for 24 h with MPP^+ 500 μM, with and without ISO 20 μM (L) and 40 μM (H)] were resolved and protein expression was evaluated by western blotting, antibodies use is labeled in the figures **(F)**. Quantification of TRPM7 is shown as bar graph. **(G)** Also shows western blots under different conditions, antibodies used are labeled in the figure. **(H)** Shows quantification where the columns represent mean ± SD of 3 independent experiments that were normalized by β-actin expression. (*$p < 0.05$; **$p < 0.01$; One way ANOVA, Tukey *post hoc* test).

which is essential for the neurotoxin-induced survival of neuroblastoma cells.

It is also possible that neurotoxin treatment could alter the expression of β-AR subtype, which could contribute toward cell death. Interestingly, neurotoxin treatment did not alter the expression of β_2-AR subtype (**Figure 4A**); whereas loss of TRPM7 protein was observed in MPP^+-treated neuroblastoma cells (**Figure 4B**). These results strongly suggest that neurotoxin-mediated loss of neuroblastoma SH-SY5Y cells are dependent on TRPM7 expression. To further establish the role of TRPM7, we transiently knocked down TRPM7 expression in these cells. TRPM7 silencing decreased TRPM7 protein levels, its function, and addition of neurotoxin in TRPM7 silenced SH-SY5Y cells showed a further decrease in TRPM7 activity (**Figure 4C**). Moreover, TRPM7 silencing, abolished the isoproterenol-induced increase in intracellular Mg^{2+} (**Figure 4D**). Similarly, the protective effect observed with isoproterenol was also abolished as TRPM7 knockdown prevented cell survival and inhibition of apoptosis by isoproterenol was inhibited (**Figure 4E**). As mitochondrial membrane potential is critical for cell survival, we used rhodamine 123 to elucidate the role of TRPM7 activity in regulating neurotoxin-mediated loss of mitochondrial membrane potential. As expected, MPP^+ treatment resulted in a reduction of mitochondrial membrane potential as compared with control untreated cells (**Figure 4F**). Moreover, silencing of TRPM7 further decreased MPP^+-mediated mitochondrial membrane potential, which was not restored even upon isoproterenol treatment (**Figure 4F**). These results further show that isoproterenol-mediated protection is dependent on TRPM7 expression and function.

Neurotoxin Treatment Induces Reactive Oxygen Species That Modulates TRPM7 Expression and Function

Oxidative stress has also been suggested to be a cause for the degeneration of dopaminergic neurons (Bollimuntha et al., 2011). Thus, H_2O_2 generation in neurotoxin-treated cells was evaluated. Addition of MPP^+ showed a time dependent increase in intracellular H_2O_2 generation (**Figure 5A**). To evaluate the consequence of endogenous H_2O_2, we evaluated the expression and function of TRPM7 channels. MPP^+ treatment significantly decreased TRPM7 protein level within 12 h of exogenous H_2O_2 treatment, without any noticeable change in the actin levels (**Figure 5B**). Consistent with western blot data, exogenous H_2O_2 application in SH-SY5Y cells decreased TRPM7 activity, which was reversed upon isoproterenol treatment (**Figures 5C,D**). Consistent with TRPM7 activity, the cell death was also

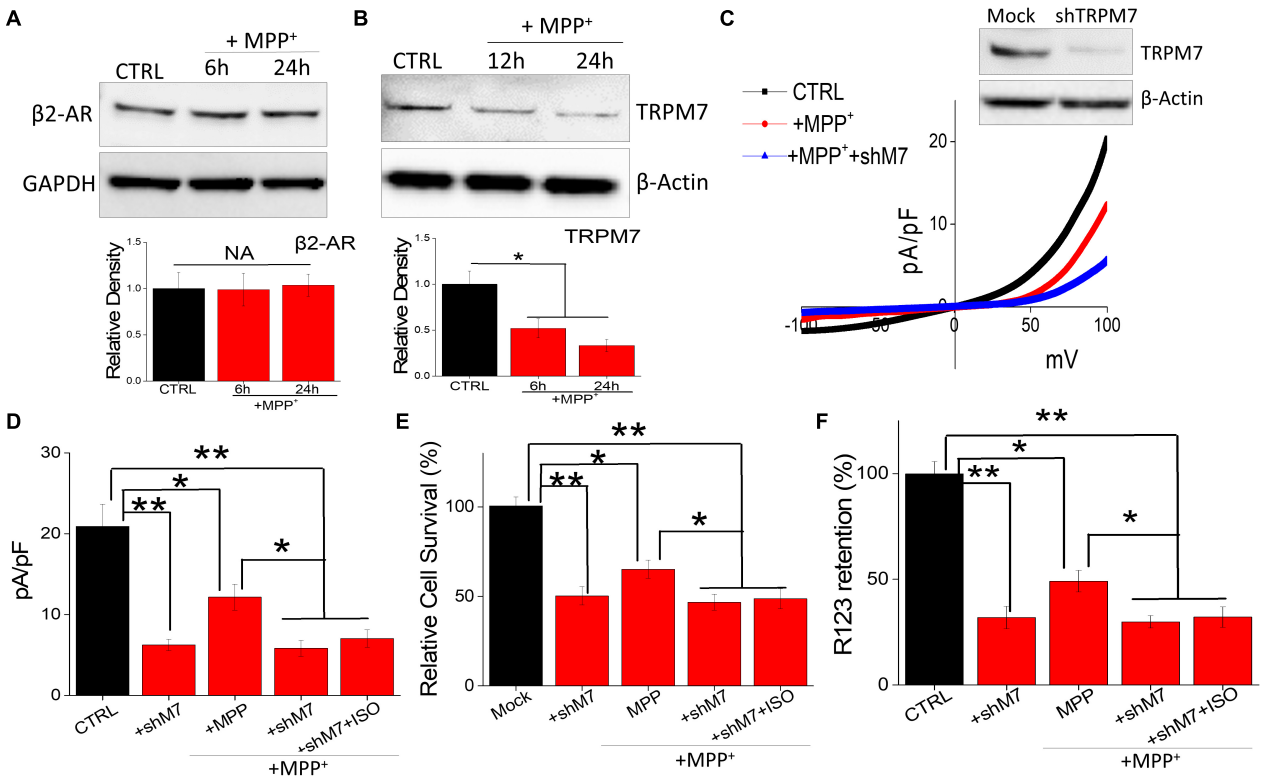

FIGURE 4 | Neurotoxin treatment inhibits TRPM7 expression/function: **(A)** Western blots showing the expression of β_2-adrenergic receptor **(A)**, TRPM7 **(B)**. β-actin was used as a loading control in CTRL and MPP^+-treated (500 μM for 24 h) cells. **(C)** IV curve and quantitation of currents observed are shown from control, MPP^+ treatment (500 μM, 24 h), and shTRPM7 cells. **(D)** The columns represent means ± SD of 6 independent experiments. (**$p < 0.01$, *$p < 0.05$; was established using one-way ANOVA, Tukey *post hoc* test). MTT assays **(E)** and mitochondrial transmembrane potential **(F)** were evaluated under various conditions as labeled in the figure. The concentration of ISO used was 20 μM and for MPP^+ 500 μM was used for each experiment. The columns show the means ± SEM of 4 independent experiments. (**$p < 0.01$, *$p < 0.05$).

significantly higher in H_2O_2 treated cells, which was further reversed upon isoproterenol treatment (data not shown). These findings implicate that H_2O_2 accumulation is observed upon neurotoxin-treatment that decreases TRPM7 expression thereby decreasing intracellular Mg^{2+} concentration essential for the survival of neuroblastoma cells.

DISCUSSION

Neurotoxin treatment has been well used as a model for PD and using this *in vitro* model we have here established the significance of β−AR-mediated activation of TRPM7 in the loss of neuroblastoma cells. We have previously shown that Mg^{2+} homeostasis prevents neurotoxin-induced cell death (Sun et al., 2019), however, the mechanisms for TRPM7 activation are not known. Importantly, increasing Mg^{2+} concentration has been shown to protect against neurotoxin-induced loss of dopaminergic cells (Hashimoto et al., 2008; Muroyama et al., 2009), suggesting that Mg^{2+} influx leads to the survival of dopaminergic neurons. Mg^{2+} is a divalent cation that regulates physiological processes such as mitochondrial function, ATP modulation, and cell survival (Romani, 2011; Zhang et al., 2012).

The data presented here indicate that loss of Mg^{2+} homeostasis (either by the addition of neurotoxin or by artificially silencing TRPM7 expression) results in a loss of mitochondrial membrane potential, which leads to apoptosis. Importantly, loss of mitochondrial integrity facilitates the translocation of Bax protein to the mitochondria activating the mitochondrial transition pore that induces apoptosis-mediated cell death (Enari et al., 1998). Apoptosis is the main mechanism that promotes the loss of DA neurons (Selvaraj et al., 2009; Venderova and Park, 2012). Consistent with these published studies, our results using differentiated SH-SY5Y cells also showed that neurotoxin treatment leads to alteration in protein expression that are involved in apoptosis. Importantly, Bax, a pro-apoptotic protein, was decreased upon the addition of isoproterenol; whereas, Bcl_2 (a member of anti-apoptotic proteins) expression was sequestered upon neurotoxin treatment (but reversed upon the addition of isoproterenol) that could initiate mitochondrial-mediated cell death.

One of the important findings presented here was that TRPM7 is the major ion channel that modulates Mg^{2+} homeostasis in neuroblastoma cells. Our results also show that MPP^+ or H_2O_2 treatments, which induces ROS, significantly decrease TRPM7 protein levels. Although the

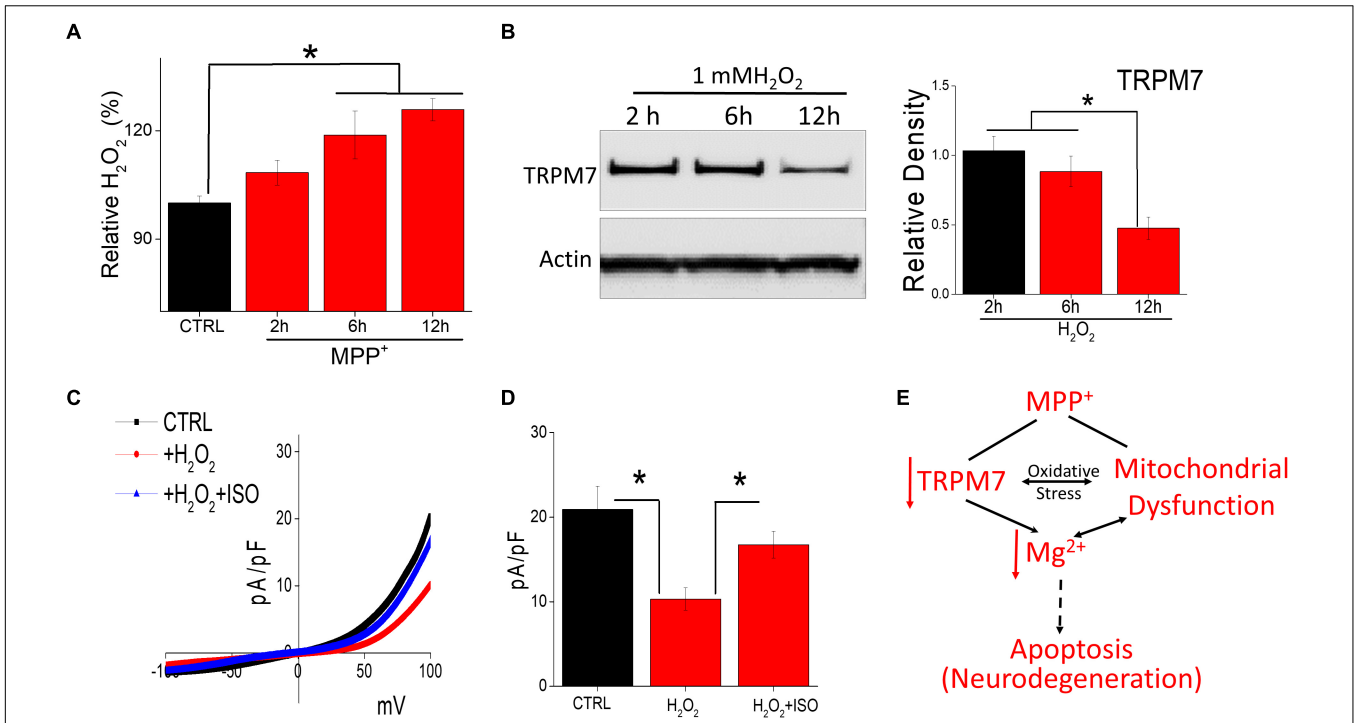

FIGURE 5 | Oxidative stress induced by neurotoxin treatment inhibits TRPM7 expression/function: **(A)** Relative H_2O_2 release was evaluated under various conditions (MPP^+-treated 500 μM) in SH-SY5Y cells. The columns show the means ± SEM of 4 experiments. (*$p < 0.05$, **$p < 0.01$; One-way ANOVA, Tukey *post hoc* test). **(B)** Western blots showing the expression of TRPM7 and loading control β-actin in CTRL and H_2O_2-treated (1 mM) in SH-SY5Y cells. **(C)** IV curves of TRPM7 currents under conditions as labeled in the figure, which are quantitation as current density at ±100 mV [mean ± SD of 6 experiments (**$p < 0.01$)] are shown in **(D)**. **(E)** Proposed model for the role of TRPM7 in MPP^+ mediated cell death.

exact mechanism as how TRPM7 expression is decreased is not known, ROS has been shown to alter gene expression. This neurotoxin-mediated loss of TRPM7 expression further decreases TRPM7 activity thereby decreasing intracellular Mg^{2+}, which leads to the loss of neuroblastoma cells. Although the mechanisms involved in TRPM7-mediated protection of neuronal cells is not clear, an increase in pro-apoptotic proteins along with a decrease in ATP levels could be the major reason for the loss of neuroblastoma cells. In addition, the presence of ROS could increase the release of Zn^{2+} from TRPM7 vesicles which could also contribute toward neurodegeneration.

Importantly, the decrease in TRPM7 expression was specific, since no change in actin levels were observed upon neurotoxin-treatment. Consistent with these studies a decrease in TRPM7 expression has also been shown in PD patients and in the samples from neurotoxin-induced substantia nigra pars compacta regions (Sun et al., 2019), which further suggests that loss of TRPM7 could lead to neurodegeneration. Similarly, mutations in TRPM7 has been reported in some familial PD patients (Hermosura et al., 2005), and although the expression and/or function of TRPM7 was not identified in this study, a decrease in TRPM7 expression and/or function could be the reason for the observed neuronal loss. Intracellular Mg^{2+} levels have been shown to rescue cell growth and increase viability (Schmitz et al., 2003), and as TRPM7 down-regulation further leads to a decrease in intracellular Mg^{2+}, it could be suggested that loss of Mg^{2+} homeostasis could be the reason for the loss of dopaminergic cells; however, activation of TRPM7 is not known. Importantly, our data showed that addition of β-adrenergic receptor agonist, isoproterenol, even at low doses that are physiologically relevant significantly increased TRPM7 activity and inhibited neurotoxin-mediated cell death. Moreover, isoproterenol-mediated activation of TRPM7 restored Mg^{2+} homeostasis in neuroblastoma cells. Another important aspect of this study was that the concentration of isoproterenol used was much lower that will limit any off target effects. Furthermore, ISO-mediated protection was dependent on TRPM7 expression, as TRPM7 silencing cells failed to show increased cell survival even in the presence of isoproterenol. Moreover, apoptosis was increased in cells treated with siTRPM7 even in the presence of isoproterenol. In contrast, restoration of TRPM7 expression increased intracellular Mg^{2+}, inhibited apoptosis, and promoted cell survival. These results further emphasize the importance of TRPM7 as expression of other Mg^{2+} transporters were unable to overcome the loss of TRPM7. β-AR agonists, have been shown to increase the survival of DA neurons (Peterson et al., 2014; Sun et al., 2018), however, the mechanism is not clear. Based on our findings, we postulated that β-AR agonist activates TRPM7 channel activity that could modulate the survival of DA cells/neurons. Importantly, TRPM7 levels were decreased in the presence of

neurotoxins, but pretreatment with β-AR agonists even at low doses increased TRPM7 levels, which restored Mg^{2+} homeostasis thereby inhibiting cell death. Although the exact mechanism involved in isoproterenol-mediated protection of dopaminergic neurons is not fully established, it could inhibit reactive oxygen species (ROS) formation, which regulates TRPM7 expression and maintains appropriate Mg^{2+} levels in dopaminergic cells. Importantly, a recent study has shown that addition of low concentrations of β-AR agonist inhibited the LPS-induced production of inflammatory mediators, such as ROS, TNFα, and nitric oxide (NO) (Izeboud et al., 1999; Sun et al., 2013), which further supports the interpretation of our results.

Mitochondrial dysfunction, as well as oxidative damage, are typical features observed in neurodegeneration including PD, which not only decline ATP production, but also increases ROS generation and induction of apoptosis. Mutations in genes that maintain mitochondrial quality control and function have been suggested as the main culprit that contributes toward familial PD (McLelland et al., 2014; Kazlauskaite and Muqit, 2015). Moreover, ROS production in neuronal cells (due to increase in ATP demand) is an important factor that leads to the demise of dopaminergic neurons. Mg^{2+} is also essential for ATP production and dysregulation of mitochondrial Mg^{2+} homeostasis has been shown to disrupt ATP production *via* the shift of mitochondrial energy metabolism and morphology (Romani, 2011; Zhang et al., 2012). Our results further provide evidence and we show for the first time that β-AR agonists activate TRPM7, which modulate Mg^{2+} homeostasis that prevents neurotoxin-induced loss of neuroblastoma cells. However, as these studies are mainly performed in isolated cells, they need to be replicated using dopaminergic cells and tissues. Nevertheless, these results identify the mechanisms involved in β-AR agonist induced protection of dopaminergic neurons by modulating TRPM7 expression thereby contributing to the survival of DA neurons.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BS and AK designed the studies that were performed by YS. YS and BS wrote the manuscript. AK edited the manuscript. All authors reviewed the results and approved the final manuscript.

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SYSTEMATIC REVIEW
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Targeting Ubiquitin-Proteasome Pathway by Natural Products: Novel Therapeutic Strategy for Treatment of Neurodegenerative Diseases

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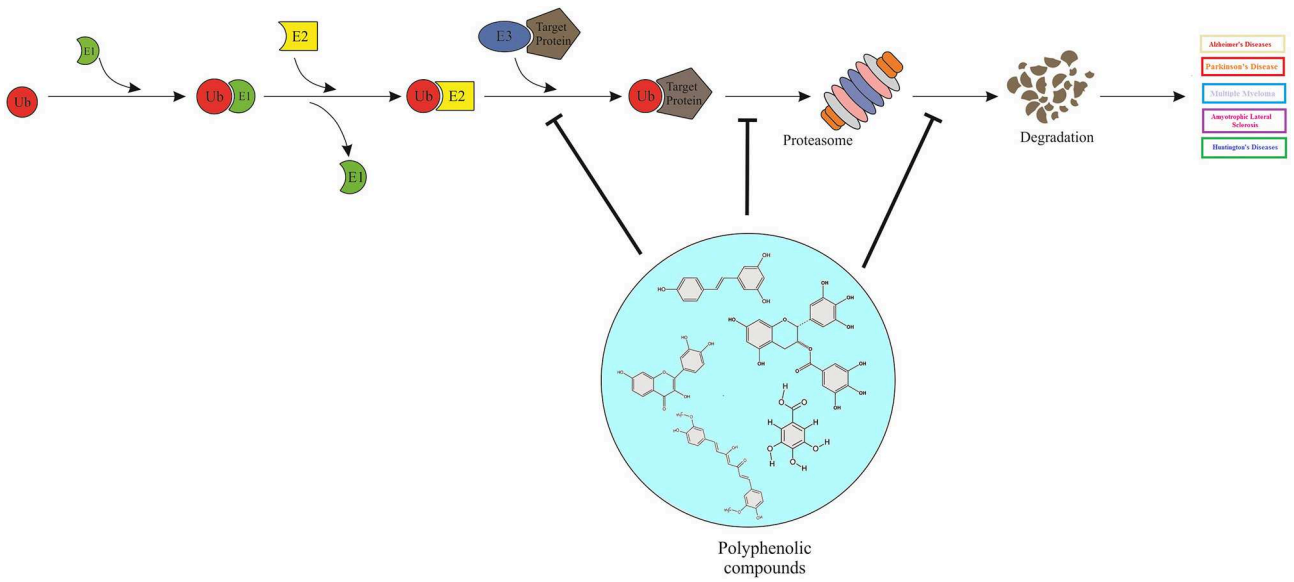
Misfolded proteins are the main common feature of neurodegenerative diseases, thereby, normal proteostasis is an important mechanism to regulate the neural survival and the central nervous system functionality. The ubiquitin-proteasome system (UPS) is a non-lysosomal proteolytic pathway involved in numerous normal functions of the nervous system, modulation of neurotransmitter release, synaptic plasticity, and recycling of membrane receptors or degradation of damaged and regulatory intracellular proteins. Aberrant accumulation of intracellular ubiquitin-positive inclusions has been implicated to a variety of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), Amyotrophic Lateral Sclerosis (ALS), and Multiple Myeloma (MM). Genetic mutation in deubiquitinating enzyme could disrupt UPS and results in destructive effects on neuron survival. To date, various agents were characterized with proteasome-inhibitory potential. Proteins of the ubiquitin-proteasome system, and in particular, E3 ubiquitin ligases, may be promising molecular targets for neurodegenerative drug discovery. Phytochemicals, specifically polyphenols (PPs), were reported to act as proteasome-inhibitors or may modulate the proteasome activity. PPs modify the UPS by means of accumulation of ubiquitinated proteins, suppression of neuronal apoptosis, reduction of neurotoxicity, and improvement of synaptic plasticity and transmission. This is the first comprehensive review on the effect of PPs on UPS. Here, we review the recent findings describing various aspects of UPS dysregulation in neurodegenerative disorders. This review attempts to summarize the latest reports on the neuroprotective properties involved in the proper functioning

of natural polyphenolic compounds with implication for targeting ubiquitin-proteasome pathway in the neurodegenerative diseases. We highlight the evidence suggesting that polyphenolic compounds have a dose and disorder dependent effects in improving neurological dysfunctions, and so their mechanism of action could stimulate the UPS, induce the protein degradation or inhibit UPS and reduce protein degradation. Future studies should focus on molecular mechanisms by which PPs can interfere this complex regulatory system at specific stages of the disease development and progression.

Keywords: ubiquitin-proteasome pathway, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, Huntington disease, Amyotrophic lateral sclerosis, Multiple myeloma, polyphenols



GRAPHICAL ABSTRACT | Polyphenols can act as proteasome-inhibitors or may modulate the proteasome activity, thereby improving neurodegenerative disorders by means of accumulation of ubiquitinated proteins, suppression of neuronal apoptosis, reduction of neurotoxicity, and improvement of synaptic plasticity and transmission.

HIGHLIGHTS

- Aberrant accumulation of intracellular ubiquitin-positive inclusions associated to neurodegenerative disorders.
- Polyphenols, can act as proteasome-inhibitors or may modulate the proteasome activity.
- Polyphenols can manage neurodegenerative impairments by targeting the ubiquitin-proteasome system (UPS).
- Polyphenols exert UPS inhibitory activity, resulting in the accumulation of ubiquitinated proteins, suppression of neuronal apoptosis, reduction of neurotoxicity, and improvement of synaptic plasticity and transmission.

INTRODUCTION

Proper production and degradation of proteins are vital for both cellular homeostasis and neuronal function. Approximately, the majority of cellular proteins are degraded by UPS, highlighting its regulatory effect on cell cycle, proliferation, and survival procedures. Generally, UPS modulates the procedure of proteolysis in neurons. In this manner, an evolutionarily conserved small protein named ubiquitin attached to the substrates (misfolded proteins); under precisely controlled conditions, via the sequential participation of several ubiquitinating and deubiquitinating enzymes, tagging them for degradation by a multi-subunit complex called the proteasome (Hegde and van Leeuwen, 2017). Later, the 26S proteasome binds to the polyubiquitinated proteins and efficiently degrades them (Grice and Nathan, 2016). In addition, proteolysis by UPS has great importance in regulation of many physiological processes, from gene transcription to posttranslational modification of proteins (Hegde and van Leeuwen, 2017).

Emerging data revealed that UPS plays an undeniable role in several neurodegenerative diseases, although, UPS impairment has been reported during the process of neurodegeneration. The

proper function of proteasome and UPS are essential factors for specific function of neurons (Jara et al., 2013). The deposition of protein aggregates and the formation of inclusion bodies are common features of most neurodegenerative diseases, since, the majority of intracellular inclusions contain ubiquitin. For instance, mutant α-synuclein (SACN) protein in PD, Amyloid-β (Aβ) and tau protein in AD, expanded polyglutamine tracts in HD and misfolded prion protein in Prion disorders (Zheng et al., 2016); besides, UPS dysregulation results in increased endoplasmic reticulum stress, and cell death in neuronal cells (Jara et al., 2013). In addition, current pharmacological standard treatments for neurodegenerative diseases, such as cholinesterase inhibitors licensed to treat AD; dopaminergic treatments for PD; antidepressants, neuroleptics, and tetrabenazine for HD; glutamate blockers to treat ALS; elicit a wide range of side effects. In this context, identification and characterization of compounds that selectively inhibit UPS and/or proteasome functions, or the substrates capable of triggering deubiquitinases, gained much attention, particularly in line of drug discovery for neurodegenerative diseases and various cancers.

Accordingly, herbal based interventions, predominantly PPs, seems to be an alternative adjuvant therapeutic approach to delay the onset of neurodegeneration and to reduce the burden of symptoms, to maximize the function and optimize the quality of patient's life. Mechanistic studies on the neuroprotective/neuroregenerative effects of PPs, exhibited that these compounds persuasively act as anti-inflammatory agents and antioxidants, either by quenching free radical species or by inhibiting pro-oxidant enzymes. PPs also function as modulators of the anti-apoptotic factors expression; intracellular neuronal signaling and metabolism; cell survival/death genes; protein aggregation and degradation pathways; and have mitochondrial function either directly or by regulating the mitochondrial signaling pathways (Mandel et al., 2011; Branquinho Andrade et al., 2016; Nabavi et al., 2018b).

Recently, both scientific and public interests shifted toward dietary regimens and nutraceuticals associated with reduced risk of neurodegenerative diseases, in a way to find molecules exploitable for prevention of the onset, progression, and severity of such impairments. Reliable evidence supports the beneficial effects of natural phytochemicals, in particular polyphenolic compounds in attenuating neurological deterioration by means of protein clearance machineries. Among these systems, UPS plays a crucial role in degradation of misfolded protein aggregates. Thereby, the scope of this review is to introduce the UPS in neurodegenerative diseases, to assess the favorable effects of PPs in UPS inhibition, and to discuss their potential application in clinical trials to target neurodegeneration pathologies in the quest for a disease modifying therapy.

UBIQUITIN-PROTEASOME SYSTEM

The ubiquitin proteasome system (UPS), a 76-amino acid complex, is a key regulator of protein catabolism in the mammalian nucleus and cytosol. The UPS is essential for the regulation of almost all vital processes including, organelles biogenesis, cell cycle, differentiation and development, immune response and inflammation, neural and muscular degeneration, as well as response to stress and extracellular modulators. Under extremely controlled conditions, UPS affects a wide variety of cellular substrates and molecular pathways; furthermore, UPS defects could result in the pathogenesis of numerous devastating human diseases (Leestemaker and Ovaa, 2017).

Protein degradation via UPS involves two separate and consecutive phases named conjugation and degradation. Throughout the conjugation phase, the substrate protein is tagged by the covalent attachment of multiple ubiquitin molecules, thereafter, 26S proteasome (composed of the catalytic 20S core and the regulatory 19S part) degrades the tagged protein, such process is called the degradation phase. This conventional function of UPS is usually associated with antigenic peptide generation, regulation of protein turnover, and housekeeping functions. Recently, it has been shown that protein modification by UPS also has unconventional (non-degradative) functions that is dictated by the number of ubiquitin units covalently tagged to proteins (poly vs. mono-ubiquitination), and by the type of ubiquitin chain linkage that is present (Akutsu et al., 2016). Ubiquitin is tagged to the ε-amine of lysine residues of target proteins via a series of ATP-dependent enzymatic steps named; E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes. Moreover, the C-terminal Gly75-Gly76 residues of ubiquitin are the key residues that play critical roles in the diverse chemistry of ubiquitin reactions. Ubiquitin could be conjugated to itself through particular lysine residues (K6, K11, K27, K29, K33, K48, or K63), resulting in various types of chain linkages. The isopeptide binds to a target protein and ubiquitin, thereby; specific deubiquitinating enzymes (DUBs) can reverse the linkages between several ubiquitins in a chain. Recent studies have revealed that many DUBs are parts of ubiquitin ligase complexes, which enables DUBs to regulate the activity and abundance of both the ligase and the substrate (Stolz and Dikic, 2018).

Substantial evidence has clarified the determinative role of UPS dysfunction in neurodegenerative disorders, involving abnormal accumulation of inclusion bodies or insoluble protein aggregates in neurons. Furthermore, dysregulation of UPS could impede the degradation of aberrant or misfolded proteins and negatively upset synaptic transmission (Zheng et al., 2014). Eventually, unsuccessful removal of damaged proteins could result in the aggregation of these proteins and neuronal apoptosis (Hyttinen et al., 2014). On the other hand, in neurodegenerative disorders, defectiveness in synaptic plasticity is attributed to dysregulation of ubiquitin-mediated proteolysis (Selkoe, 2002).

DEUBIQUITATION

DUBs play pivotal roles in hemostasis of biological processes such as cell cycle, proliferation, programmed cell death apoptosis, and DNA repair mechanisms. Ubiquitin-specific-proteasome-7 is a DUB enzyme. Its overexpression has been detected in numerous types of cancers (Hu et al., 2002; Li et al., 2004; Nicholson et al., 2007), in particular, stabilization of

MDM-2 (murine double minute). USP7 deubiquinates MDM2, therefore, maintaining the tumor suppressor p53 ubiquitinated, it also degraded by proteasome under normal condition (Everett et al., 1997; Cummins et al., 2004). In MM patients, deletion or mutation in p53 was detected, thereby; the inhibition of USP7 could be a useful therapeutic target for accumulation of functional p53.

Both USP14 ubiquitin-specific-protease and the ubiquitin C-terminal hydrolyase (UCHL5) are cysteine proteases. DUBs are associated with 19S proteasome regulatory subunit; hence, they may modulate the capability of proteasome for target proteins to be degraded (Borodovsky et al., 2001; Al-Shami et al., 2010; Lee et al., 2010). They are able to regulate the signaling pathways such as nuclear factor (NF)-κB (Al-Shami et al., 2010), transforming growth factor (TGF)-β (Wicks et al., 2005), and CXCR4 chemotaxis (Mines et al., 2009). USP14 and UCHL5 expression levels are upregulated in different types of tumors such as colorectal cancer (Shinji et al., 2006), ovarian cancer and MM (Tian et al., 2013). Selective USP14 and UCHL5 inhibitor b-AP15, induced apoptosis in MM cell lines and in primary MM cells via downregulation of cell division cycle 25C (CDC25C), CDC2, and cyclin-B1, as well as the activation of caspases and unfolded protein response pathways (p-IREα, p-eIF2α, and CHOP) (Tian et al., 2013). VLX1570 is another USP14 inhibitor, which induced apoptosis in MM cells (Wang et al., 2016).

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is recognized as a highly common neurodegenerative disease with visual-spatial confusion and loss of short-term memory. It is known that memory loss exacerbates over time, leading to cognitive dysfunction and reduced intellectual capacity in AD patients. The pathology of AD is related to misfolded-protein aggregation, inflammatory changes, and oxidative damage, resulting in neuronal loss (Querfurth and LaFerla, 2010). In an AD brain, the most important pathognomonic lesions include the intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques (ESPs). Senile or neuritic plaques [composed of Aβ containing 39 to 42 amino-acid peptides, a product of the sequential cleavage of the β-amyloid precursor protein (APP)] and neurofibrillary tangles (filamentous bundles comprised of hyperphosphorylated tau proteins) are typical characteristic lesions in affected tissues (Haass and Selkoe, 2007). To date, two types of medications including cholinesterase inhibitors (Donepezil, Rivastigmine, Galantamine), and *N*-methyl-D-aspartate (NMDA) receptor antagonist (memantine) were approved by the U.S. Food and Drug Administration (FDA) to treat moderate to severe AD symptoms (Briggs et al., 2016).

Although the major cause of AD remains unknown, the familial type of autosomal dominant inheritance is involved in nearly 0.1% of cases. Mutations in genes encoding presenilin 1 (PS1), PS2, and amyloid precursor protein (APP), are incorporated to this type of AD (Waring and Rosenberg, 2008). Nearly 95% of all AD patients suffer from sporadic AD, which is associated with the late onset of symptoms (above 65 years) (Minati et al., 2009). According to a recent investigation, UPS either is damaged or appears inadequate in some regions of the brain of young mice (Liu et al., 2014). In this regard, the interaction of ubiquitin C with different AD factors was reported by a proteomic study; accordingly, UPS dysregulation was introduced as a mechanism underlying AD (Manavalan et al., 2013). UPS dysregulation can prevent calmodulin degradation and block Ca^{2+}/calmodulin-dependent signaling pathways in AD (Esteras et al., 2012). Some UPS and AD-related proteins, such as C-terminus of Hsc70-interacting protein (CHIP) and ubiquitin carboxyl terminal esterase L1 (UCHL1), may be expressed aberrantly. These proteins along with a mutant form of ubiquitin, can inhibit the UPS and cause proteasomal dysfunction in AD patients (Oddo, 2008; Bilguvar et al., 2013).

Ubiquitin-Proteasome System and Amyloid Beta

Aβ is identified as a peptide from APP, cleaved by β- and γ-secretases. Following cleavage of APP in its ectodomain by β -secretase 1 (BACE1), γ-secretase splits the transmembrane domain of carboxy-terminal fragments and discharges Aβ peptides into the extracellular environment (Wang et al., 2018). Overexpression of APP increased the activity of UPS in the frontal cortex of transgenic AD mice model (Seo and Isacson, 2010). According to the literature, Lysine (Lys)-203 and Lys-382 are indispensable to proteasomal degradation of BACE1 (Wang et al., 2012). On the other hand, BACE1 proteasomal degradation is accelerated by ubiquitin carboxylterminal hydrolase L1 (UCHL1) (Zhang et al., 2012). It was shown that due to the accumulation of Aβ in neurons, the activities of proteasomes and the deubiquitinating enzymes reduced (Almeida et al., 2006). Clearance of Aβ can significantly diminish the early pathogenesis of tau (Budd Haeberlein et al., 2017). However, Aβ accumulation may damage proteasome function and promote tau accumulation (Tseng et al., 2008). In addition, mutant or wild-type APP in neural cells is known to affect downstream protease inhibition (Cecarini et al., 2014).

Ubiquitin-Proteasome System and Tau

Tau, which is described as a soluble protein in neurons, is concentrated in axons and stabilizes the microtubule network (Lee et al., 2013). In adult human brain, six tau isoforms are expressed. Although, the mechanism of tau fibrillization is still indefinite in pathological disorders, formation of paired helical filaments (PHFs) is triggered by hexapeptide motifs. Overall, diverse posttranslational modifications such as glycosylation, ubiquitination, hyperphosphorylation, and proteolysis could occur in tau (Hernandez and Avila, 2007; Martin et al., 2011). Besides, tau hexapeptide motifs, ubiquitin and apolipoprotein E are among other NFT components. Stepwise fragmentation happens in tau to generate cleaved molecules with proaggregation features, such as neurodegeneration (Wang et al., 2010). In a study by Dolan and Johnson, the autophagy system removed truncated tau, while UPS was not involved (Dolan and Johnson, 2010). On the other hand, Grune et al. (2010) reported that ATP/ubiquitin-independent 20S proteasome catalyzed tau degradation. Valosin-containing proteins (part of UPS; the

machinery that degrades damaged, misshapen, and excess proteins within cells) in AD synapses are negatively correlated with the buildup of hyperphosphorylated tau oligomers, and UPS dysfunction may progress concomitantly with tau hyperphosphorylation (Tai et al., 2012).

Ubiquitin-Proteasome System and Ubiquitin Carboxyl Terminal Esterase L1

UCHL1 enzyme attributed to the removal of ubiquitin from unfolded proteins and disassembly of polyubiquitin chains for recycling of ubiquitin. The enzyme is also capable of eliminating abnormal proteins, as it stabilizes monoubiquitinated proteins (Setsuie and Wada, 2007). In a model of APP/PS1 mice, UCHL1 transduction restored normal cognition and synaptic function in hippocampal slices treated with Aβ (Gong et al., 2006). The direct correlation of neuronal UPS with sporadic AD has been proven (Oddo et al., 2006). In a study on Chinese Han patients, AD was associated with serine-to-tyrosine substitution at codon 18 in exon 3 of UCHL1 gene; the genotypes were also more resistant in females (Xue and Jia, 2006). According to a recent study by Poon et al., UCHL1 was recognized vital for the regulation of neurotrophin receptors and supporting retrograde transport. It is also known that Aβ downregulates the UCHL1 in AD, thereby, compromising synaptic plasticity, as well as neuronal survival (Poon et al., 2013).

Ubiquitin-Proteasome System and Ubiquilin-1

Polyubiquitinated proteins are delivered to proteasomes for degradation by several proteins, including ubiquilin-1 with ubiquitin-like domains. The increased risk of AD is associated with the ubiquilin-1 gene (*UBQLN1*) allelic variant (Li et al., 2017). In a study by Stieren et al., reduction of ubiquilin-1 activity, led to the production of APP fragments, along with neuronal cell death (Stieren et al., 2011). Ubiquilin-1 seems to also contribute to the pathogenesis of other neurodegenerative disorders (Safren et al., 2015).

Ubiquitin-Proteasome System and Sequestosome 1 (p62)

Most NFTs contain p62, which is a UPS-related protein (Morawe et al., 2012). P62 serves as a receptor to bind ubiquitinated proteins and to shuttle proteins to proteasome for the purpose of degradation (Zaffagnini et al., 2018). Similarly, p62 shuttles polyubiquitinated tau to proteasome. In AD, p62 is detected in neuronal inclusion bodies, containing aggregates of ubiquitinated protein (Salminen et al., 2012).

PARKINSON DISEASES

PD is associated with severe motor symptoms, attributing to dopaminergic neuron death in the substantia nigra (Kaur et al., 2018). A number of medications have been approved to treat PD symptoms, of which Levodopa is the most effective pharmacologic treatment for severe motor symptoms, moreover, monoamine oxidase type B (MAO-B) inhibitors, dopamine agonists (i.e., Bromocriptine, Pergolide, Pramipexole, Ropinirole) are effective for patients with mild symptoms at a younger age (Jankovic and Poewe, 2012). Of course, patients using such drugs are facing a verity of complicated adverse effects. Aside from reduced function of UPS, oxidative stress, and mitochondrial metabolism impairment seem to be involved in PD pathogenesis (Winklhofer and Haass, 2010). PD is associated with 10 different mutations, some of which is correlated with UPS, such as alpha-synuclein (α-SNCA), protein deglycase DJ-1 (or PARK7), UCHL1, PTEN-induced kinase 1 (PINK1), and PD protein 2 (PARK2 or parkin). Overall, parkin, PINK1, and DJ-1 mutations are involved in the autosomal recessive familial type of PD (Lunati et al., 2018; Zeng et al., 2018).

Ubiquitin-Proteasome System and α-synuclein

α-SNCA is described as the major constituent of Lewy bodies (LBs) in the brain of PD patients. LBs contain ubiquitinated proteins, such as α-SNCA. The LB protofibrils exert inhibitory effects against the degradation of 26S proteasome-mediated proteins (Chen et al., 2006; Zhang et al., 2008). α-SNCA is encoded by SNCA gene. Several mutations in SNCA at A53T, and A30P are directly linked to the familial form of PD and α-SNCAopathies (Kaur et al., 2018). It was shown that proteasome inhibitors cause α-SNCA aggregation and formation of LBs (Banerjee et al., 2014). Moreover, rats exposed to proteasome inhibitors displayed PD-like behavior and damage to the central nervous system similar to that observed in PD patients (Lorenc-Koci et al., 2011). Meanwhile, the α-SNCA aggregations may in turn selectively bind to the 6S subunit of the 26S proteasome to inhibit the proteasome activity, and to further induce neurons cytotoxicity, including mitochondrial damage and apoptosis (Tanaka et al., 2001; Snyder et al., 2003). Therefore, proteasome inhibition and α-SNCA may reciprocally regulate a feed forward mechanism and exacerbate the development of PD (Xie and Wu, 2016).

Ubiquitin-Proteasome System and Protein Deglycase DJ-1

Although DJ-1 protein is majorly expressed in the cytosol, it can also be detected in the nucleus. According to a study by Khasnavis et al., astrocytes produce DJ-1 in mice brain (Khasnavis and Pahan, 2014). Similarly, in a normal human brain, the astrocytes express DJ-1 (van Horssen et al., 2010). As suggested in literature, patients with sporadic PD have reduced levels of DJ-1 protein in the substantia nigra (Nural et al., 2009; Cookson and Bandmann, 2010). Familial forms of PD is associated with DJ-1 mutations (Giguere et al., 2018). In a study by Xiong et al., DJ-1 deficiency reduced parkin ubiquitination and improved aggregation of misfolded parkin substrates (Xiong et al., 2009). Nonetheless, to maintain the mitochondrial function, DJ-1 acts along with the PINK1/parkin pathway. Therefore, the association between DJ-1 and PINK1/parkin should be confirmed in further studies. Moreover, DJ-1 is described as a substrate for small ubiquitin-like modifier-1 (SUMO-1) conjugation, and its simulation is crucial (Shinbo et al., 2006).

Ubiquitin-Proteasome System and PTEN-Induced Kinase 1

PINK1 is expressed in different brain regions, including the hippocampus and substantia nigra (Blackinton et al., 2007; Heeman et al., 2011). Degradation of heat-induced misfolded proteins, mediated by parkin, is increased by the PINK1 expression. On the contrary, parkin and PINK1 mutations in PD, are less potent in promoting parkin substrate degradation. In fact, PINK1 leads to the clearance of aberrant proteins via proteasomal degradation (Clements et al., 2006). PINK1 mutations are involved in some cases of autosomal recessive and sporadic PD (Blackinton et al., 2007). The symptoms of PINK1 knockout mice, including mitochondrial dysfunction and reduced corticostriatal synaptic plasticity in dopaminergic neurons, are similar to PD patients (Kitada et al., 2007, 2009). Liu and colleagues reported that PINK1 deficiency interrupts proteasome activity and leads to α-SNCA aggregation. They also suggested a relationship between PINK1 and UPS in PD (Liu et al., 2009).

Ubiquitin-Proteasome System and Parkin

Considering the direct correlation between UPS and PD, the parkin gene mutations have been suggested to be involved. The amino acid sequence of parkin contains an ubiquitin-like domain at the N-terminus, as well as a RING box at the C-terminus with E3 ubiquitin-ligase activity (Kaur et al., 2018). Respecting T240R mutations in parkin gene, the association between autosomal recessive familial PD and parkin was identified. In addition, parkin can be found in PD-affected brain regions. Parkin exhibits neuroprotective functions in PD, which can be attributed to its E3 ubiquitin-ligase activity (Song et al., 2009). Wild-type α-SNCA is the most important target of ubiquitin degradation (Li et al., 2018). Research on the possible association of parkin with neurodegeneration reveals that parkin can regulate the aggresome-autophagy pathway (Lim et al., 2006). In addition, parkin triggers ubiquitination, as well as polyglutamine-expanded ataxin-3 degradation, resulting in reduced toxicity in cells (Kumar et al., 2012; Zheng et al., 2014). The deubiquitinating enzyme activity of ataxin-3, as a deubiquitinating enzyme, is improved through ubiquitination (Todi et al., 2009). To eliminate misfolded proteins, ataxin-3 and parkin contribute to aggresome formation (Olzmann et al., 2008). Parkin gene mutations lead to abnormal toxic substrate aggregation due to UPS dysfunction. Parkin might also be associated with the pathogenesis of PD, considering its role in mitochondrial functioning (Kumar et al., 2012). In addition, many mitochondrial processes in PD, involving parkin, are interrupted.

MULTIPLE MYELOMA

Multiple myeloma (MM) is a hematologic malignancy of bone marrow characterized by the accumulation and infiltration of mature plasma cells in the bone marrow (cells that produce high level of antibodies) (Morgan et al., 2012). The cancer incidence is low around 1, in every 132 individuals (0.76%). According to American cancer society, around 30,770 new cases will be diagnosed (16,400 in men and 14,370 in women) with MM and around 12,770 deaths will be expected (6,830 in men and 5,940 in women).

The offered treatment regimens for MM patients mainly include chemotherapy with a response range of 40 to 70%. Unfortunately, most patients suffer from relapsing due to the recurrence of the disease. Bone marrow transplantation combined with chemotherapy (Attal et al., 1996) is another regimen; still patients suffer from the recurrence of the cancer (Attal et al., 1996; Mitsiades et al., 2002). The urine and serum of patients contains high level of monocolonal immunoglobulins called M-protein or paraprotein, which is consisted of a heavy (most often IgG or IgA but also IgM, IgD, or IgE) and a light chain of kappa or lambda. In some patients, the plasma cells only produce light chain immunoglobulins, which dose not bind to the heavy chain. The light chain immunoglobulins are normally excreted in the urine, although their levels in the urineare considered as a prognostic marker for the MM patient.

Proteasome in Multiple Myeloma

Production of high amount of immunoglobulins require functional endoplasmic reticulum (ER) and ER quality control system (Ibba and Söll, 1999; Wickner et al., 1999), which prevents the processing of misfolded proteins and their translocation to its distinct location. Unfolded proteins induce the activation of unfolded protein response (UPR) pathway, leading to inhibition of further protein synthesis, and the expression of chaperones and enzymes required for folding proteins. Once, proteins cannot be refolded, misfolded proteins will be tagged for proteasome degradation by 26S proteasome (Ellgaard et al., 1999), however, unfolded protein stress is intolerable and unfixable, the cells will undergo apoptosis (Zinszner et al., 1998; Brewer and Diehl, 2000). Consequently, inhibition of UPR is a chemotherapeutic target in MM cells.

It was found that MM cells and the primary cells from MM patients express high level of UPR genes (i.e., chaperones glucose-regulated protein 78/binding-immunoglobulin protein (GRP78/Bip) and GRP94/gp96) (Obeng et al., 2006). Proteasome 26S is playing a vital role in maintaining cellular hemostasis via degradation of misfolded proteins, regulation of stress responses, cell cycle, DNA repair pathway, and apoptosis (Ciechanover, 2005). Proteasome 20S is a multi-catalytic enzyme with multi subunits. Each subunit performs one of the classical proteolytic activities; either chymotrypsin-like (ChT-L) activity localized in β5 subunit, trypsin-like (T-L) in β2 subunit, or peptidylglutamyl-peptide hydrolyzing (PGPH) activity in β1 subunit (Orlowski and Wilk, 2000).

There are two isoforms of proteasome, constitutive and immunoproteasome. The constitutive proteasome is distributed in most cells, while the immunoproteasome is expressed in cells of lymphoid organs. Immuoproteasome has great importance in antigen presentation by major histocompatibility class I (Rock et al., 1994, 2002), and also in proteolysis of proteins (Rivett and Hearn, 2004). The level of circulating proteasome was examined in 50 patients with MM, which showed a positive correlation with the advanced stages of the disease (Jakob et al., 2007);

representative of chemotherapeutic potential of proteasome in MM. The NFκB signaling pathway plays a major role in hemostasis of B-cells progenitor, where it inhibits the assembly of Igκ gene in prematured B-lymphocytes (Scherer et al., 1996), protects B cell lymphoma from apoptosis (Wu et al., 1996), induces the stimulus-dependent proliferation (William et al., 1995), and B cell receptor (BCR) responses (Bendall et al., 1999).

The cancer cells are characterized by high proliferative rate coupled with high levels of misfolded proteins, DNA damage and stress in comparison with normal cells, thus, cancer cells highly require a functional proteasome system. Bortezomib is a proteasomal inhibitor of MM, approved by FDA (Richardson et al., 2003). Bortezomib suppresses the NFκB signaling pathway through inhibition of proteasome degradation of iκB, which can maintain the NFκB sequestered and latent in the cytoplasm (Brockman et al., 1995; Hideshima et al., 2001; Russo et al., 2001; Sunwoo et al., 2001; Ma et al., 2003), activation of p53-mediated apoptosis, cell cycle arrest, and induction of both intrinsic and extrinsic apoptotic pathways. It also activates apoptosis via the c-Jun amino-terminal kinase (JNK)-dependent induction of mitochondrial release of second mitochondria-derived activator of caspase (SMAC) (Chauhan et al., 2003). The resistance of MM cells toward bortezomib, initiates an urgent need for either alternative or combinatorial therapy for MM patients (**Figure 1**).

Ubiquitin in Multiple Myeloma

CRL4 (Cullin-RING ubiquitin ligase) is an E3 ubiquitin ligase, composed of three subunits DDB1, cullin 4 (CUL4), and regulator of cullins 1 (RBX1/ROC1). This enzyme has great importance in ubiquitination of different cellular proteins such as c-MYC, interferon regulatory factor 4 (IRF4), Ikaros (IKZF1), and Aiolos (IKZF3) transcription factors. Lenalidomide treatment led to targeted degradation of IKZF1 and IKZF3 in different MM cell lines and primary cells from patients, via inhibition of CLR4 E3 ligase enzyme (Krönke et al., 2013).

Cereblon is a substrate recognizing subunit in E3 ligase enzyme, which binds to the DNA damage binding protein-1 (DDB1), CUL4, and Roc1 to form a functional enzyme complex (Fischer et al., 2014; Sang et al., 2015). Cereblon (442 amino acid protein) is ubiquity expressed in plants, rats, mice as well as humans, and is known to be responsible for memory and intelligence in humans (Higgins et al., 2010). Deletion of C-terminal of cereblon due to a non-sense mutation at amino acid 419 (R419X), resulted in intellectual disability syndrome. Cereblon binds to potassium (Higgins et al., 2008) and chloride channels in the brain and retina (Jo et al., 2005; Hohberger and Enz, 2009; Aizawa et al., 2011), respectively. It also inhibited the activation of adenosine monophosphate (AMP) kinase via binding to its subunit α1 (Lee et al., 2011) (**Figure 2**). Cereblon was found to be a selective target for thalidomide PS-341 (Ito et al., 2010) and lenalidomide (Krönke et al., 2013) activities in MM, leading to the degradation of IKZF3 and IKZF1. The degradation of IKZF3 transcription factor by lenalidomide led to reduction in mRNA and protein levels of IRF4 in multiple myeloma cells. IRF4 is a transcription factor required for the activation of lymphocytes (Mittrücker et al., 1997) and the plasma cells differentiation and maturation (immunoglobulin producing cells) (Klein et al., 2006; Sciammas et al., 2006). Shaffer et al. (2008) showed that IRF4 is not genetically altered in myeloma cells, but it is addicted for the maturation and activation of B cells. Such data highlighted the role of CRL4 and Cereblon in regulating the pathogenesis of MM and could be used as further objects for MM treatment.

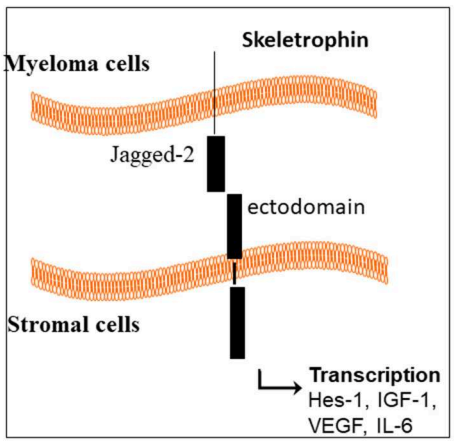
Skeletrophin is an E3 ligase, required for Notch signaling pathway activation (Saurin et al., 1996; Freemont, 2000). The Notch extracellular domains are composed of epidermal growth factor (EGF) repeats (29-36), Lin-12/Notch repeat (LIN), linker to the transmembrane and an intracellular domain. The Notch ligands are the transmembrane proteins and divided into two classes, Delta or Delta-like (Dll) and Serrate (Jagged-1 and Jagged-2 in mammals). Upon binding of the ligand to the Notch, a mechanical force leads to the cleavage of the Notch ectodomain, which is followed by endocytosis. Skeletrophin facilitates the Jagged-2-bound to Notch endocytosis, leading to a second cleavage in the intracellular domain of Notch and its translocation to the nucleus (**Figure 3**).

T-cell acute lymphoblastic leukemia (T-ALL) is characterized by chromosomal translocation, resulting in expression of truncated Notch characterized by constitutive Notch signaling activation. Moreover, myeloma cells as well as primary MM cells express high levels of Jagged-1 and−2, and play a role in the interaction between stromal and myeloma cells (Houde et al., 2004; Jundt et al., 2004). Such data introduce a new ubiquitin ligase, favorable for MM treatment.

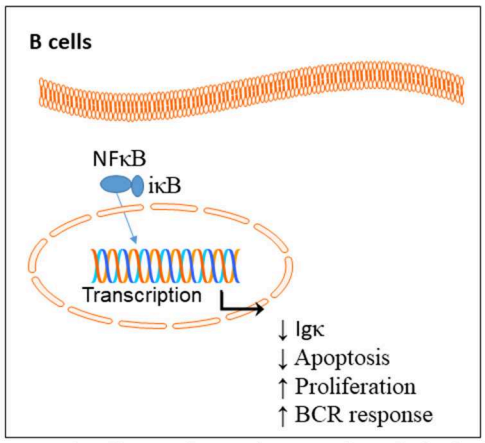
CKS1B (Cyclin-Dependent Kinases Regulatory Subunit 1) is an accessory protein in SCF SKP2. F-box (40 amino acids motif)-containing protein S-phase kinase-associated protein 2 (SKP2) is one of the four subunits of ubiquitin ligase complex [SCF (Skp1, Cullin 1, F-box protein)]. SKP2 potentially regulates the transition of cells from G1 to S phase, through a phosphorylation-dependent degradation of cell cycle inhibitor p27 (Cyclin-dependent kinase inhibitor 1B) (Tsvetkov et al., 1999), which is considered as an oncoprotein. The inhibitor p27 downregulation, led to the inhibition of p21 (Yu et al., 1998), p27 (Tsvetkov et al., 1999), and p57 (Kamura et al., 2003) degradation, and prevention of other tumor suppressors such as c-MYC (Kim et al., 2003; Von Der Lehr et al., 2003; Song et al., 2008), transducer of ERBB2, 1 (Tob 1) (Hiramatsu et al., 2006), and Forkhead transcription factors FOXO1 (Huang et al., 2005). The P27 cell cycle inhibitor is also downregulated in numerous cancers including MM (Filipits et al., 2003). In the same time, the CKS1B is overexpressed in oral, gastric, breast and colon cancers, spectating the possible role of CKS1B in regulating the p27 degradation. (Zhan et al., 2007) showed that the silencing of CKS1B led to stabilization of p27 in 4 different MM cell lines.

AMYOTROPHIC LATERAL SCLEROSIS

ALS is a neurodegenerative disorder, sporadic in most cases, involved in progressive motor neuron degeneration in the brain and spinal cord, associated with muscle weakness (Zheng et al., 2014). ALS characterized by paralysis and death within 3 to 5 years from day of appearance of symptoms due to the impairment



Skeletrophin facilitate the Jagged-2-bound to Notch endocytosis leading to a second cleavage in the intracellular domain of Notch and its translocation to the nucleus leading to the overexpression of Hes-1, IGF-1, VEGF and Il-6



NFκB signaling pathway plays a major role in the hemostasis of B-cells progenitor where it inhibits the assembly of Igκ gene in pre-matured B-lymphocytes, protects B cell lymphoma from apoptosis, induces the stimulus-dependent proliferation and B cell receptor (BCR) response

FIGURE 1 | Clinically tested drugs against MM and their targets.

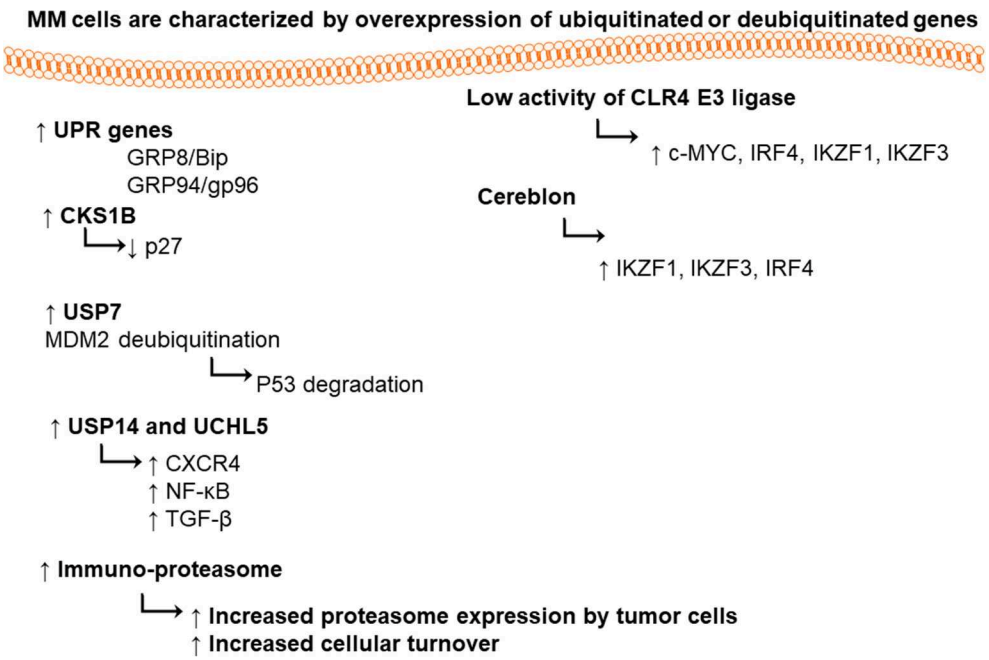


FIGURE 2 | Ubiquitinated or deubiquitinated genes in MM cells.

of respiratory systems (Hardiman et al., 2011). The loss of bulbar and limb function are the main features of ALS. According to Logroscino et al. (2010) study, the incidence rate in the Europe continent is 2.16/100,000 per year. The incidence rate was higher in men than women. Around 10% of patients inherited the disease (autosomal dominant) and 90% of patients have no family history of ALS (FALS), although they still show the pathologic features of FALS. Until now, there is no effective treatment for ALS, so the survival rate is low for the affected patients (Kim et al., 2009). In this context, only 2 drugs have FDA approval; Riluzole (glutamate inhibitor) and Edaravone (free radical and peroxynitrite scavenger with anti-inflammatory

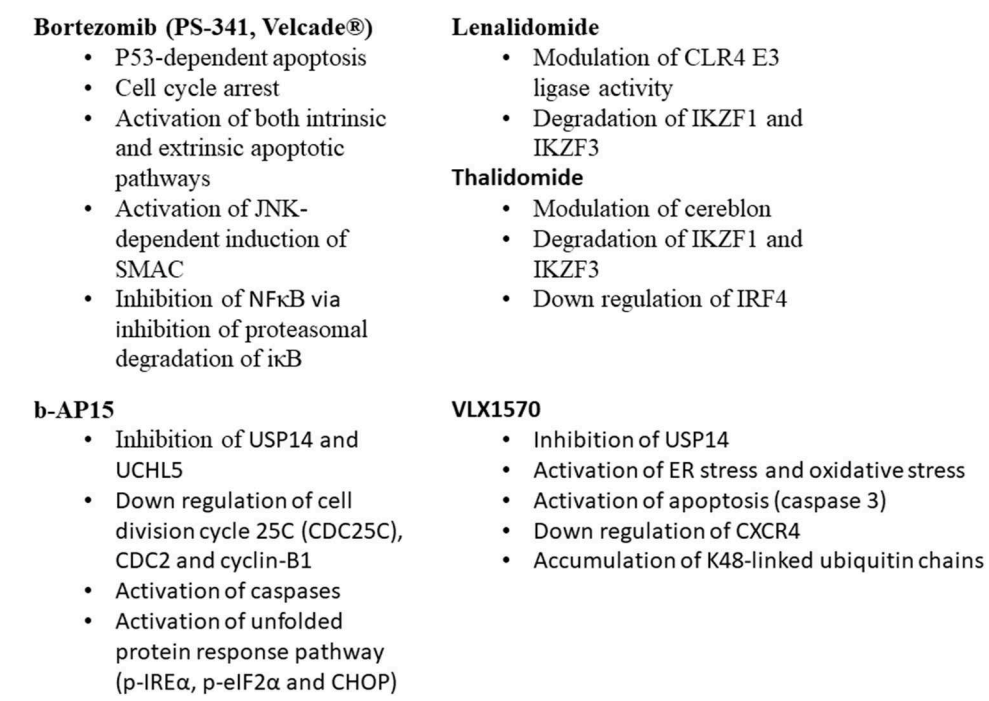


FIGURE 3 | Signaling pathways involved in MM.

properties), although they might cause side effects such as bruising, gait disturbance, hives, dizziness, gastrointestinal, and liver dysfunctions, as well as allergic reactions (Jaiswal, 2018). Although the cause of ALS is unknown in most cases, several mutations are related to familial ALS, including superoxide dismutase 1 (SOD1), ubiquilin 2 (UBQLN2), and RNA-binding protein fused in sarcoma (FUS) protein (Kwiatkowski et al., 2009; Vance et al., 2009; Deng et al., 2011). Twenty percentage of the familial ALS is linked to a genetic modification in chromosome 21 long arm q (Rosen et al., 1993).

Amyotrophic Lateral Sclerosis and Superoxide Dismutase 1

Based on several studies, UPS is associated with superoxide dismutase 1 (SOD1) turnover; however, the exact mechanism remains unclear. UPS degrades mutant SOD1 proteins faster than wild-type SOD1, and the proteasome inhibition improves the SOD1 stability (Bendotti et al., 2012). ALS-like pathologies are found in mice with conditional knockout of proteasome subunit, Rpt3 (Tashiro et al., 2012). ALS is also related to the induction of immunoproteasome subunits (Bendotti et al., 2012). In a study using a mutant SOD1-G93A model, pyrrolidine dithiocarbamate treatment reduced the survival of ALS (Ahtoniemi et al., 2007), therefore, the immunoproteasome expression may increase the coping of the nervous system with SOD1 mutations- induced ALS (Rao et al., 2015). In ALS, the function of SOD1 does not decrease, and mice with increased or decreased levels of SOD1 do not show ALS-like pathologies. On the other hand, SOD1 mutation is known to trigger a toxic gain of function, causing SOD1 aggregation. Moreover, in some cases of sporadic ALS, wild-type SOD1 aggregates support the gain of function (Bosco et al., 2010). Mutant SOD1 can transfer the misfolded SOD1, which is followed by degradation (Crippa et al., 2010). In this regard, an increase was reported in the expression level of immunoproteasome (Cheroni et al., 2008). However, according to the literature, autophagy has greater significance in SOD1 turnover, compared to UPS (Dennissen et al., 2012).

In FALS, virtually 11 missense mutations in cytosolic Cu/Zn SOD1 linked to the accumulation of free radicals to the neurodegenerative diseases, where it leads to a damage in the mitochondrial hemostasis, axon transport, and glutamate transporter function (Rosen et al., 1993). It was found that the mutated form of SOD 1 inhibits the chymotrypsin-like activity of proteasome in Neuro2A cells and depresses the induction of motor neuron death in the transgenic mouse model (Urushitani et al., 2002). Kabashi et al. (2004) found that the mutant SOD 1 caused inhibition in chymotrypsin-like, caspase-like and trypsin-like activities of proteasome, without decreasing its level in lumbar spinal cord of the transgenic mice. These data indicated the importance of functional proteasome system in preventing the ALS development.

Amyotrophic Lateral Sclerosis and Fused in Sarcoma Protein

It was evidenced that genetic modifications such as angiogenin 3 (*ANG*), transactive response (TAR) DNA-binding protein

TDP-43 (*TARDP*), sarcoma/translated in liposarcoma and optineurin (OPTN) are linked to FALS. Ubiquitination of misfolded proteins is a pre-step in degradation by proteasome system. The accumulation of misfolded proteins in the hippocampus, neocortex and spinal cord is a pathologic feature of neurodegenerative diseases, TDP-43 protein is composed of glycine-rich C-terminal with two RNA-recognition motifs and it is the main pathologic manifestation for ALS (Wang et al., 2004). Its phosphorylation, ubiquitination and cleavage into two peptide fragments have been linked to the poor prognosis of ALS patients (Leigh et al., 1991; Okamoto et al., 1991; Neumann et al., 2006). It was found that ubiquilin 1 (UBQLN) (proteasome targeting cochaperone factor) binds to the ubiquitinylated TDP-43 aggregates and targets them either to autophagy or proteasomal degradation. The binding of the mutated form of TDP-34 (D169G) to UBQLN was greatly decreased in comparison with the wild-type TDP-43. More studies are required to confirm such hypothesis in the pathogenesis of ALS.

ALS has major similarities to the frontotemporal dementia (FTD) spectrum. FUS and TAR DNA- TDP-43 inclusions are detected in sporadic ALS (Deng et al., 2010). According to the literature, inclusions co-localize with ubiquitin similar to FTD. Moreover, VCP/P97, C9ORF72, and optineurin polymorphisms can produce FALS (Johnson et al., 2010; Maruyama et al., 2010; Deng et al., 2011; Renton et al., 2011). *TARDP* is localized in chromosome 1p36.2. The mutations in *TARDP* gene (p.Gly290Ala and p.Gly298Ser mutations) were found to be linked to the sporadic and FALS (Kabashi et al., 2008). The mutation either leads to gain- or loss-of-function in TDP-43, and may be essential in binding to hnRNPs (heterogeneous nuclear riboproteins) (Van Deerlin et al., 2008). However, the main molecular mechanism of ALS remains undetermined.

Amyotrophic Lateral Sclerosis and Ubiquilin-2

UBQLN2 disorders involved in the pathogenesis of different neurodegenerative disorders, as this protein regulates ubiquitinated protein degradation. Besides, UBQLN-2 mutations result in FALS, and UBQLN-2 accumulation co-localizes with skein-like inclusions (Deng et al., 2011). UBQLN-2 proteins contribute to the transfer of ubiquitinated proteins to proteasomes. The UBQLN-2 overexpression reduces PS1 and PS2 ubiquitination (Massey et al., 2004). The influence of ALS on lysosomal degradation has been confirmed in a previous study, as UBQLN proteins can increase the binding of early autophagosomes to the lysosomes (N'Diaye et al., 2009).

Amyotrophic Lateral Sclerosis and Optineurin

Optineurin (*OPTN*) was found to have three different types of mutations in familial and sporadic ALS. The heterozygous Glu478Gly missense mutation ubiquitin-binding region, homozygous Gln398X non-sense mutation and a homozygous deletion of exon 5 (Maruyama et al., 2010). Both missense and non-sense mutations prevented the inhibition of NF-κB (Wagner et al., 2008; Maruyama et al., 2010). Glu478Gly missense mutation induces the accumulation of the mutated protein in the neurons and modulates the formation of inclusion bodies, resulting in a disturbance in the cell biological functions (Maruyama et al., 2010).

Amyotrophic Lateral Sclerosis and Cyclin F

Williams et al. (2016) performed exome-sequencing for locus chromosome 16p13.3 in order to discover new leads related to ALS pathogenesis. The authors discovered a missense mutation in *CCNF* gene (nucleotide A replaced by G, resulted in amino acid substitution Ser621Gly), which encodes the cyclin F in neuronal cells. Cyclin F (786 amino acid protein) contains F-box motif that recognizes and binds to SKP1 and CUL1 in order to form SCF E3 ligase complex (SKP1-CUL1-F-box protein) (D'Angiolella et al., 2013; Williams et al., 2016). The mutated cyclin F leads to abnormal ubiquitination and aggregation of ubiquitinated proteins such as TDP-43, forming ubiquitinated inclusion (Williams et al., 2016).

Amyotrophic Lateral Sclerosis and Neural-Precursor-Cell-Expressed-Developmentally-Down-Regulated-8

Ubiquitin-like protein Neural-precursor-cell-expressed-developmentally-down-regulated-8 (NEDD8) has a structure similar to ubiquitins and called Ub-like proteins. Ub-like proteins are classified into two groups according to the manner of protein conjugation; type 1 Ub-like proteins conjugate with the target protein in a way similar but not totally identical to the known ubiquitination mechanism such as NEDD8 and small Ub-related modifier (SUMO1), while type 2 Ub-like proteins have Ub-like protein structure with broad biological functions such Elongin B (subunit of the transcription factor B), Rad23 (Radiation sensitive), and Parkin (Parkinson Protein 2 E3 Ubiquitin Protein Ligase) (Tanaka et al., 1998).

NEDD8 immuno-reactivity was detected in different neurodegenerative diseases such as Parkinson disease (in LBs and Lewy neurites), multiple system atrophy (in ubiquitinated inclusions and oligodendroglial inclusions), AD (in neurofibrillary tangles), motor neuron disease (in ubiquitinated inclusions), and in triplet repeat diseases (in intranuclear inclusions) (Mori et al., 2005). Moreover, its immune-reactivity was also detected in other diseases such as alcoholic liver disease and astrocytoma (in Rosenthal fibers) (Dil Kuazi et al., 2003).

Signaling proteins and phosphoprotein array study in muscles of 36 ALS patients revealed that there are 17 differentially expressed proteins and phosphoprteins in ALS compared to normal muscle cells. In between, heat shock protein 90 (HSP90) (chaperone), and phospho-retinonlastoma (tumor suppressor, p-Rb at Ser780) were overexpressed, while cyclin-dependent kinase 4 (CDK4) and p-p53 at Ser392 were downregulated (Yin et al., 2012). The accumulation of P53 was detected in the nucleus of lumbar spinal cord of ALS patients. Moreover, the study showed that the immuostaining for p53 was also

positive for cell cycle regulators (pRb and E2F-1) and apoptotic proteins (Bax, caspase 8 and caspase 3). P53 stabilization is regulated by MDM2 via ubiquitin-proteasome machinery. ALS is characterized by dysfunction in ubiquitin-proteasome system and this may explain the stabilization of p53 and its translocation into the nucleus of the lumbar spinal cord. P53-MDM2 interaction could be a therapeutic target for improving the survival of ALS patients.

HUNTINGTON DISEASES

HD is a progressive hereditary neurodegenerative disorder caused by an autosomal dominant defective gene on chromosome 4 that encodes the huntingtin (HTT) protein, containing a repeating sequence of CAG at the N-terminus of HTT, a protein with an abnormally long polyglutamine (polyQ) sequence. In addition, amyloidogenic mutant huntingtin (mHTT) aggregates were implicated in progression of HD (Popovic et al., 2014). Generally, these neuronal aggregates containing N-terminal fragments of polyQ HTT are located in the striatum and in the cortex of HD patients; either in nuclear or in cytoplasmic regions of affected neurons (DiFiglia et al., 1997). Cognitive decline, behavioral abnormalities and involuntary movements accounted as marked hallmarks of HD; probably caused by both neuronal dysfunction and neuronal cell death; leading to a progressive decline in functional capacity, and ultimately death. Several factors have been associated with HD including alterations in calcium level, IGF signaling, vesicle transport, endoplasmic reticulum maintenance, and autophagy (Martin et al., 2015). Yet, there is no definite cure for HD, although several medications are prescribed to treat movement difficulties (Chorea Huntington) such as monoamine depletors (i.e., Tetrabenazine); or those targeting the atypical behavioral aspects of HD such as antidepressants [i.e., serotonin reuptake inhibitor (SSRI)] and antipsychotic drugs (i.e., Olanzapine) (Chen et al., 2012).

Various *in vitro* and *in vivo* studies ratified that dysfunction in ubiquitin metabolism contributes to the pathogenesis of HD, leading to the accumulation and aggregation of insoluble ubiquitin-containing mHTT (Bennett et al., 2007). Typically, HTT is ubiquitinated at amino acids K6, K9, and K15, resulting in its degradation and decreasing the toxicity of mHTT (Kalchman et al., 1996). The accumulation of K48-, K11-, and K63-linked ubiquitin chains in HD mouse models, and the brains from HD patients was shown to be a common feature of HD (Zucchelli et al., 2011). The fact that mHTT aggregates are abnormally enriched for ubiquitin and contain ubiquitin E3 ligases, confirms that the ubiquitination is a key factor in aggregate formation. Global changes in UPS, alike the accumulation of Lys48-, Lys63-, and Lys11-linked polyubiquitin chains associated with HD pathology (Bennett et al., 2007).

Ubiquitin-Proteasome System and Tumor Necrosis Factor Receptor Associated Factor 6

Tumor necrosis factor receptor associated factor 6 (TRAF6) is an E3 ubiquitin ligase and was found to be overexpressed in postmortem brains of HD patients. *In vitro* cultured cells, TRAF6 promoted the aggregate formation through mediating atypical ubiquitination of Lys6-, Lys27-, and Lys29-linked chains related to HTT. Both mHTT and TRAF6 were localized to insoluble protein fraction.

POLYPHENOLS IN NEURODEGENERATIVE DISORDERS

Various pharmacological agents have been studied and used to find suitable therapeutic interventions in neurodegenerative diseases; however, some defects have always been associated with such treatments, since the pathophysiology of such impairments is yet to be elucidated. A wide range of phytopharmaceuticals are being explored to improve the effects of commonly used drugs in the treatment of neurogenic disorders (Farzaei et al., 2018a), both via prophylactic and disease controlling approaches (Gorji et al., 2018). Natural products can also provide templates for the development of other drug compounds and to design new effective complex molecules (Babitha et al., 2014). Phytochemicals proven to possess potential neuroprotective effects and are able to protect the central nervous system (CNS) against neuronal injury (Kumar, 2006); hence people who consumed higher amounts of natural functional foods were found to show a lower risk for diseases caused by neuronal damage (Lobo et al., 2010).

PPs are the most abundant natural phytochemicals, capable of protecting neuronal cells in different *in vivo* and *in vitro* models through diverse intracellular targets. Various epidemiological and preclinical investigations confirmed the favorable effects of PPs in neurodegenerative diseases, primarily due to their antioxidant properties and their influence on stress response, mainly through nuclear factor erythroid 2-related factor (Nrf2) signaling, triggering the antioxidant defense machinery (Pandareesh et al., 2015; Farzaei et al., 2018b). In addition, polyphenolic compounds may exert neuroprotective effects involving phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β) (PI3K/Akt/GSK3β) neuronal survival pathway, through the N-methyl-D-aspartate (NMDA) receptors and by downstream signaling in hippocampus and cognitive deficits through tyrosine receptor kinase β (Trkβ) and brain-derived neurotrophic factor (BDNF) in hippocampus (Srivastava et al., 2018). Generally, natural compounds have shown inhibitory or therapeutic effects on neurodegerative disorders via biological effects such as antioxidant, anti-inflammatory, calcium antagonization, anti-apoptosis, and neurofunctional regulation (Choudhary et al., 2013).

Beside their free radical scavenger properties, the mechanisms by which polyphenolic compounds are able to counteract and prevent neurodegenerative diseases include: (1) anti-inflammatory activity through chromatin remodeling (modulation of both DNA methyl transferase and histone deacetylase activities) and alteration in the expression of related transcription factors such as NF-kB (Rahman and Chung, 2010), and dampening of microgliosis, astrogliosis, and glia-derived pro-inflammatory cytokines (Sundaram and Gowtham, 2012; Peña-Altamira et al., 2017; Sarubbo et al., 2017); (2)

improvement of mitochondrial function through stimulating the mitochondrial membrane potential and respiratory chain complex IV, enhancing the mitochondrial complex I-IV enzymatic potential, moderating the mitochondrial free radical production, and increasing endogenous antioxidant defense (Fiorani et al., 2010; Davinelli et al., 2013; Cai et al., 2015; de Oliveira et al., 2015) as well as mitochondrial biogenesis through activation of the AMPK/Sirt1/PGC-1α axis (Ayissi et al., 2014; Cao et al., 2014; de Oliveira et al., 2016; Valenti et al., 2016); (3) antioxidant activity through activation of Nrf2/ARE signaling pathway, increasing the expression of nerve growth factor, glial cell line-derived neurotrophic factor, BDNF, TrkA/B, activation of ERK1/2-CREB-BDNF and Akt/Glycogen synthase kinase 3β signaling pathways (Lin et al., 2010; Bagli et al., 2016; Moosavi et al., 2016; Martínez-Huélamo et al., 2017; Sanadgol et al., 2017).

Anthocyanins (Orhan et al., 2015), proanthocyanidins (Strathearn et al., 2014), stilbenes (Braidy et al., 2016), isoflavons (Devi et al., 2017), and curcumin (Hu et al., 2015), are among the most studied dietary phenolic compounds demonstrating protective effects against AD and PD, while there are also studies showing the potential of S-allylcysteine as organosulfur compounds (Farooqui and Farooqui, 2018) and isothiocyanates such as 6-methylsulfinylhexyl isothiocyanate (6-HITC) and sulforaphane (Giacoppo et al., 2015) to be active as neuroprotective dietary phytochemicals.

These herbal constituents seem to create their neuroprotective effects through mechanisms involving the activation of cellular antioxidant responses including activation of the Nrf2-mediated antioxidant response, stimulation of PGC-1α-mediated mitochondrial biogenesis, and alleviating neuroinflammation evoked by the activation of glial cells (de Rus Jacquet et al., 2017a). Several flovonoids like hesperidin, kaempferol, naringin, and epigallocatechin gallate (EGCG) have also been reported to show efficacy against 3-NP-induced neurotoxicity, which is an extensively used animal model for HD (Dey and De, 2015). Onjisaponin B and trehalose enhanced autophagy as one of therapeutic approach against toxic intracytosolic aggregate-prone mHtt protein in HD (Dey and De, 2015).

A number of PPs can modulate neural toxicity or loss by means of their antioxidant properties. For example, it was shown that a synthesized mitochondria-targeted curcumin (MTC), triphenylphosphonium cation-curcumin, reduced free radicals-induced neurotoxicity and mitochondrial impairments *in vivo* and *in vitro* (Hasan et al., 2019a). Similarly, curcumin and MTC showed protective effects against oxidative damage and cerebellar toxicity induced by rotenone *in vivo*, mainly through decrease of lipid peroxidation, and nitric oxide levels, and reduction of glutathione, SOD, and catalase activities, while enhancing the acetylcholine esterase activity (Hasan et al., 2019b).

Beside flavonoids, various non-flavonoid antioxidant phytochemicals like α-mangostin, curcumin, lycopene, and melatonin, have been reported as effective natural compounds in different HD models (Choudhary et al., 2013). Considering the role of oxidative stress and chronic inflammation in the ALS pathophysiology, natural compounds targeting such stressors are supposed to be effective alone or in combination with other natural/chemical substances to find new therapeutic approach for ALS management (Nabavi et al., 2015).

EGCG, quercetin, quercitrin, and curcumin have been found to be effective in ALS models (Koh et al., 2006; Ip et al., 2017). A 12-month, double-blind, randomized, placebo-controlled study on ALS patients, demonstrated that nanocurcumin combined with riluzole improved survival rate during the trial (Ahmadi et al., 2018).

Polyphenols, Ubiquitin–Proteasome, and Neurodegenerative Diseases

PPs directly or indirectly interfere with the cellular protein degradation systems including the chaperone-mediated autophagy; the ubiquitin–proteasome degradation pathway; and the lysosome-autophagy system, by eliminating the misfolded and damaged proteins. The accumulation of insoluble protein aggregates is a common mark of neurodegenerative diseases, making PPs a great interest for therapeutic strategies (Hajieva, 2017). On one hand, proteasomal inhibition by PPs would be undesirable in neurodegenerative disorders, and in the other hand, proteasome stimulation and reduction of protein degradation by proteasome inhibitors have shown beneficial consequences and were found to be presumably neuroprotective (del Rosario Campos-Esparza and Adriana Torres-Ramos, 2010; de Rus Jacquet et al., 2017b).

PPs and their derivatives have been shown to inhibit UPS (Nabavi et al., 2018a), yet, a number of limitations have impeded their clinical applications. PPs may target various components of this system through mechanisms involving proteasome inhibition, deubiquitinase activity and/or the activities of E1, E2, or E3, thereby, physiologically affecting the essential proteins and/or by effect on the protein substrates, leading to the imbalanced coordinated intracellular protein homeostasis and the consequent off-target effects (del Rosario Campos-Esparza and Adriana Torres-Ramos, 2010; Liu et al., 2015).

Aforementioned, several PPs were reported to act as proteasome-inhibitors, mainly through chymotrypsin-like activity (on both intracellular 26S and purified 20S proteasome) (Nam et al., 2001; Smith et al., 2002; Kazi et al., 2003; Marambaud et al., 2005; Chen et al., 2007a,b; Chang et al., 2015; Ding et al., 2018). Structure activity relationship studies showed that flavonoids with a hydroxylated B ring and/or unsaturated C ring are potent proteasome inhibitors, of which the carbonyl carbon on the C ring could be the site of nucleophilic attack on the proteasome β5 subunit (Chen et al., 2007a). Anthocyanins and anthocyanidins were reported to possess proteasome inhibitory effects, contributing to their neuroprotective activities (Dreiseitel et al., 2008). Several studies demonstrated that the proteasome inhibitory activities of green tea PPs, are responsible for its anticancer and neuroprotective assets (del Rosario Campos-Esparza and Adriana Torres-Ramos, 2010), for instance, EGCG and its analogs were shown to inhibit the chymotrypsin-like activity of the purified 20S proteasome *in vitro* (Nam et al., 2001; Smith et al., 2002). As an exception, curcumin exhibits a binary

function against proteasome. Curcumin at low concentrations activates the proteasome (Jana et al., 2004), while at high doses, the compound suppresses the proteasome activity by enhancing the accumulation of ubiquitinated proteins. In neuro 2a and Hela cells, curcumin inhibited the chymotrypsin, trypsin, and post-glutamyl peptidyl-like protease activity of the proteasome, decreased the free ubiquitin levels and increased the protein polyubiquitination (Jana et al., 2004). The neuroprotective effect of curcumin could also be explained by deubiquitination enzymes that specifically regulate the protein polyubiquitination, through cleavage of ubiquitin from ubiquitin-conjugated protein substrates, preventing molecular aggregation (Reyes-Turcu et al., 2009). Curcumin treatment reduced the activities of deubiquitination enzymes in HeLa cells (Si et al., 2007), also curcumin inhibited the ubiquitin isopeptidase activity (Mullally and Fitzpatrick, 2002). In a recent *in vitro* study, myricetin modulated endogenous levels of quality control E3 ubiquitin ligase E6-AP and reduced the misfolded proteins inclusions, resulting in the maintenance of proteostasis (Joshi et al., 2019).

A polyphenol-rich extract from elderflower was shown to suppress neurotoxicity elicited by PD-related symptoms in cortical astrocytes via Nrf2 stabilization and inhibition of Nrf2 degradation mediated by the ubiquitin proteasome pathway. Another way, down-regulation of UPS by elderflower polyphenols induces the Nrf2 activation through up-regulation of macroautophagy pathway (also called the lysosome–autophagy protein degradation pathway), leading to the Nrf2 stabilization (de Rus Jacquet et al., 2017b). Nrf2 is a transcription factor involved in regulating the expression of cellular antioxidant enzymes and the genes encoding cytoprotective proteins (Tambe, 2015). Up-regulation of the Nrf2-mediated cellular antioxidant response (i.e., increase in glutathione synthesis and glutathione metabolites levels), results in alleviation of neurodegeneration in PD (de Rus Jacquet et al., 2017b). Quercetin was shown to induce the expression of proteasome subunits by a similar mechanism (Kwak et al., 2003).

In AD brains, Aβ neurotoxicity has been shown to have an inhibitory impact on the activity of UPS (Tseng et al., 2008), compelling a decrease in proteasome activity. In HEK293 and neuro 2a cells transfected with human APP695, resveratrol promoted the intracellular degradation of Aβ in a way that total activity of the proteasome did not increase. This was proved where several proteasome inhibitors such as lactacystin, Z-GPFL-CHO, and YU101 significantly prevented the resveratrol-induced inhibition of Aβ activity, and the siRNA-directed silencing of the proteasome β5 subunit (Marambaud et al., 2005). In mouse model of early PD, an extract of mulberry fruits, rich in phenolic contents (i.e., flavonoids, anthocyanins, and arotenoids), down-regulated the expression of components such as α-SNCA and ubiquitin, also reduced neuronal toxicity; representing that the neuroprotective effect of this plant might be partially mediated by inhibition of the LBs formation in the brain. LBs is thought to trigger dopaminergic neurodegeneration in PD (Gu et al., 2017) (**Figure 4**).

Polyphenols/Ubiquitin-Proteasome System Interactions, Clinical Trials

Pharmacological activities of several phytochemicals in neurodegenerative diseases are extensively supported by preclinical and epidemiological studies, which some have shown the mechanistic potential of these compounds as the UP pathway inhibitors (Murakami, 2013). Previously stated, a variety of phenolic compounds have been characterized with their proteasome-inhibitory activity such as resveratrol, EGCG, curcumin, quercetin, chrysin, genistein, kampferol, myrycetin, luteolin, apigenin, gallic acid, ellagic acid, and tannic acid. However, few clinical trials were carried out on a limited basis. Up to date, curcumin and resveratrol are the most studied compounds followed by EGCG and genistein, alone or in combination with other conventional drugs.

Curcumin

Currently, 6 clinical trials related to the effects of curcumin on AD is being conducted (http://clinicaltrials.gov/ct2/results?term=alzheimer\pmand\pmcurcumin), of which 3 have been completed (4, 5, 6) and 2 studies are still in progress, whereas 1 study has unknown status (**Table 1**). In a pilot randomized clinical trial in China, 34 patients with a diagnosis of probable or possible AD were investigated in 3 subgroups including 4, 1 (compared with 3 g placebo), or 0 g curcumin (compared with 4 g placebo) once daily. They additionally received the standardized extract of gingko leaf (120 mg/day). Compared with the placebo, there was not any significant difference in cognitive function (as secondary outcome) or plasma isoprostanes iPF2α-III and plasma Aβ40 levels (as primary outcome) between 0, 1, and 6 months. Curcumin seemed to cause no major side effects in AD patients in this study (Baum et al., 2008).

In a double-blinded, placebo-controlled trial in the United States, a mixture of curcumin derivatives (2 and 4 g/day), consisting of curcuminoids, demethoxycurcumin, and bisdemethoxycurcumin was prescribed to the patients with mild-to-moderate AD for 24 weeks and an open-label extension to 48 weeks. No significant differences in cognitive function and in plasma or cerebrospinal fluid (CSF) biomarkers of AD (including Aβ40 and Aβ42 levels, and total tau and p-tau) were observed between curcumin and the placebo groups (Ringman et al., 2012).

A double-blind, randomized, placebo-controlled trial in Iran was designed to evaluate the safety and efficacy of nanocurcumin in ALS adults. Subjects with a definite or probable ALS diagnosis were received either nanocurcumin (80 mg/day) or placebo for 12 months. The primary outcomes were considered to be survival/death and any mechanical ventilation dependency. The authors found a considerable difference between the study groups survival. No major adverse events or drug adverse effects and death were reported (Ahmadi et al., 2018).

Another pilot randomized trial was carried out in Italy to investigate the efficacy of the higher dose of oral curcumin (600 mg/day, Brainoil) on clinical parameters and biochemical markers, in 42 ALS patients. The first 3 months of the study

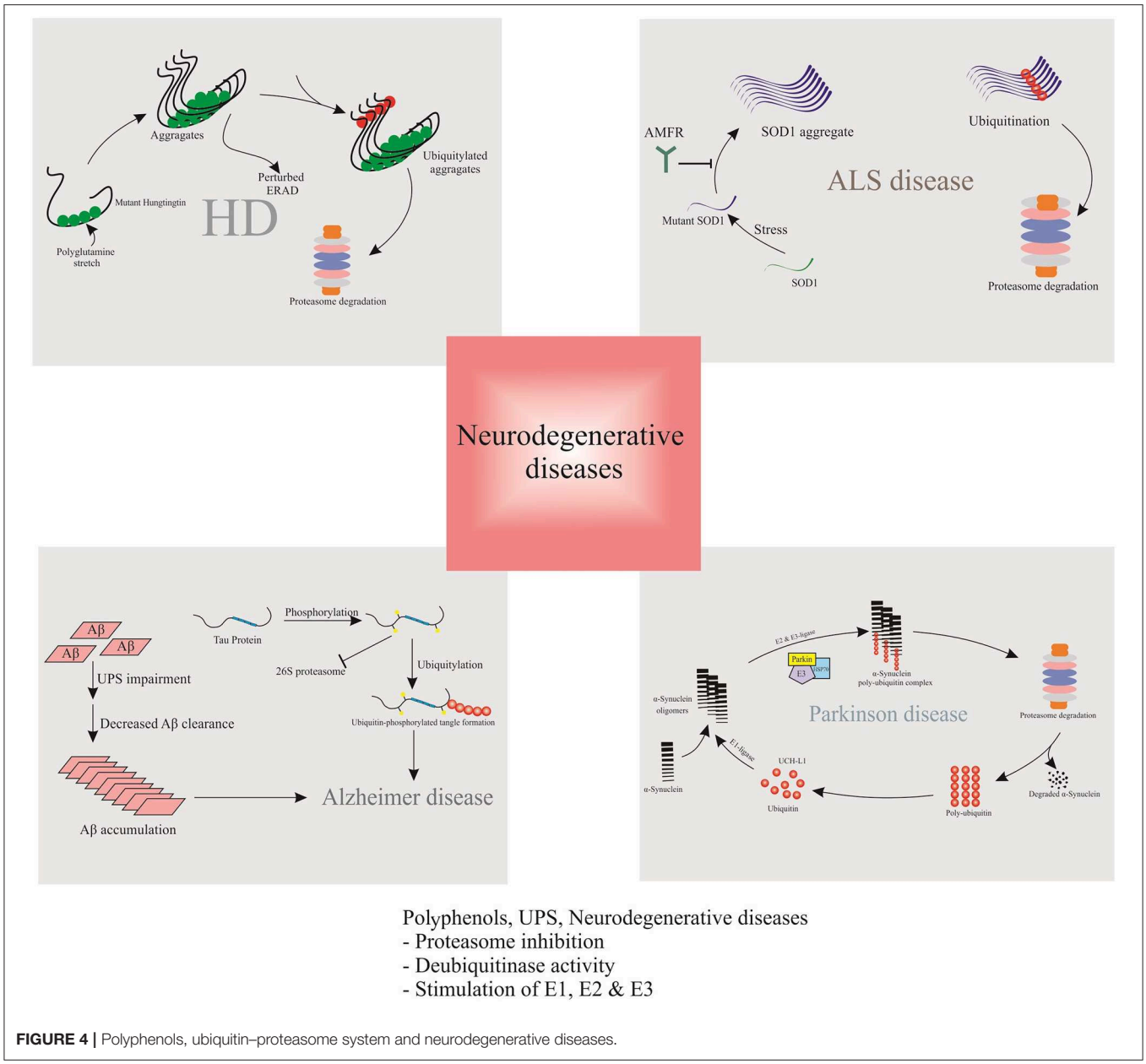


FIGURE 4 | Polyphenols, ubiquitin–proteasome system and neurodegenerative diseases.

was double blind and the last 3 months were open-label. Clinical criteria were consisted of ALS Functional Rating Scale Revised (ALS-FRS-r), BMI, Medical Research Council (MRC) scale, and Maximum Handgrip Force (MHF) scale; and the plasma metabolic biomarkers (i.e., plasma values of Advanced Oxidative Protein Products (AOPPs), ferric reducing ability (FRAP), total thiols (T-SH) groups and lactate). Data were not significant, however, the authors concluded further studies is required due to disparity of results (Chico et al., 2018).

Resveratrol

There are 5 recorded clinical trials related to the effects of resveratrol on AD (http://clinicaltrials.gov/ct2/results?term$=$alzheimer\pmand\pmresveratrol), 3 studies have been completed, 1 has been withdrawn and 1 is still active. Two clinical trials have reported that resveratrol altered several AD specific biomarkers with no major adverse effects in AD patients (Turner et al., 2015; Moussa et al., 2017). A phase 2 randomized, double-blind, placebo-controlled trial was performed in the United States, mainly to determine the safety, tolerability and pharmacokinetics of synthetic resveratrol (500 mg orally, once daily, increasing at 13 weeks intervals to a maximum of 1 g) in 119 individuals with mild to moderate AD for 52-weeks. They found that resveratrol was able to penetrate into the blood–brain barrier; likewise, the compound changed the AD biomarkers (Aβ40, Aβ42, tau, and phospho-tau) in the plasma and CSF. Plasma Aβ40 and CSF Aβ40 levels were found to be stabilized by resveratrol compared with a decrease in the

TABLE 1 | Clinical trials relevant to the phytochemicals with UPS inhibitory activity.

ID number (www.ClinicalTrials.gov)	Phase	Medication and dose	Duration	Number of subjects/Condition	Status	Country
NCT00164749	Phase 1,2	Curcumin 1 or 4 g/day (standardized ginkgo extract 120 mg/day)	6 months	34 Probable or possible AD	Published	China
NCT00099710	Phase 2	Curcumin C3 Complex® 2 or 4 g/day (1.9 or 3.8 g/day curcuminoids) [a]	24 weeks with an open-label extension to 48 weeks	36 Mild-to-moderate AD	Published	USA
NCT00595582	–	Curcumin 5.4 g/day (bioperine)	24 months	10 MCI or mild AD	Completed Results not available	USA
NCT01001637	Phase 2	Longvida® 4 or 6 g/day	2 months	26 Probable AD	Unknown	India
NCT01383161	Phase 2	Theracurmin™ 2.79 g/day (180 mg/day curcumin)	18 months	132 MCI	Active, not recruiting	USA
NCT01811381	Phase 2	Longvida Curcumin® (800 mg/day of curcumin)	12 months	80 MCI	Recruiting	USA
NCT01504854	Phase 2	Resveratrol 500 mg/day with dose excalation by 500 mg increments ending with 2 g/day	52 weeks	119 Mild-to-moderate AD	Published	USA
NCT00743743	Phase 3	Longevinex brand resveratrol supplement (resveratrol 215 mg/day)	52 weeks	50 Mild-to-moderate AD	Withdrawn	USA
NCT00678431	Phase 3	Resveratrol with glucose and malate	12 months	27 Mild-to-moderate AD	Completed Result not available	USA
NCT01126229	Phase 1	Resveratrol 300 mg/day or 1,000 mg/day	12 weeks	32 ≥65 years old	Completed Result not available	USA
NCT01219244	Phase 4	Resveratrol or omega-3 supplementation or caloric restriction	6 months	330 MCI	Recruiting	Germany
NCT01766180	-	ResVida (resveratrol 150 mg/day) alone or associated with Fruitflow [a]-II 150 mg/day	3 months	80 Subjects with memory impairment	Recruiting	USA
NCT02621554	Phase 2/Phase 3	Resveratrol (dose not reported)	12 months	60 ≥50 years Healthy or with subjective memory complaints	Recruiting	Germany
NCT02502253	Phase 1	Bioactive Dietary Polyphenol Preparation (BDPP) at low, moderate, and high dose	4 months	48 MCI	Recruiting	USA
NCT01982578	-	Genistein (60 mg BID)	180 days	50 AD	Unknown	Spain
NCT00205179	Phase 2	Soy isoflavones 100 mg/day; Novasoy®; (85% daidzin and genistin, as glycosides)	6 month	59 AD	Published	USA
NCT00951834	Phase 2, 3	Epigallocatechin-gallate (EGCG) with increasing doses (in months 1–3: 200 mg EGCG, months 4–6: 400 mg, months 7–9: 600 mg and months 10–18: 800 mg EGCG).	18 month	21 subjects in the early stages of AD	Completed Results not available	Germany
NCT01699711	Phase 2	9 mg/kg of EGCG, (in green tea extract standardized for EGCG)	12 month	84 DS	Published	Spain
NCT01662414	Phase 4	Soy protein (as placebo) vs. HMS 90® (whey protein) by the dose of 1 sachect (10 g) 2 times/day as add-on (adjuvant) therapy.	6 month	32 Idiopathic PD	Published	Thailand

(Continued)

TABLE 1 | Continued

ID number (www.ClinicalTrials.gov)	Phase	Medication and dose	Duration	Number of subjects/Condition	Status	Country
NCT02336633	–	Resveratrol (80 mg/j = 4 capsules/day)	12 month	120 HD	Recruiting	France
Not available	Pilot	Curcumin (600 mg/day, Brainoil) (for the first 3 months), followed by an open-label phase (for the last 3 months)	6 month	42 ALS	Published	Italy
Not available	–	Nanocurcumin (80 mg daily)	12 month	54 Subjects with definite or probable ALS	Published	Iran

AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington disease; DS, Down syndrome; ALS, Amyotrophic Lateral Sclerosis.

placebo group; it also stabilized CSF Aβ42 in the subset of patients with biomarker-confirmed AD (baseline Aβ42 < 600 ng/ml). Additionally, the brain volume loss was increased in resveratrol treated group (3 vs. 1%), probably due to anti-inflammatory activity of resveratrol. Totally, the results were not interpretable due to the study limitations; furthermore, no significant effects were found on clinical (secondary) outcomes. Resveratrol was found safe and well-tolerated with minor side effects such as nausea, diarrhea and weight loss (Turner et al., 2015).

Moussa et al., suggested that resveratrol treatment could preserve the integrity of the blood-brain barrier in AD patients with CSF Aβ42 < 600 ng/ml, declined the level of CSF MMP9 (matrix metallopeptidase 9), and elevated macrophage-derived chemokine (MDC), interleukin (IL)-4, and the fibroblast growth factor (FGF)-2 (Moussa et al., 2017).

Moreover, resveratrol enhanced the plasma MMP10 and decreased the pro-inflammatory makers including IL-1R4, IL-12P40, IL-12P70, TNF-α, and RANTES compared to the baseline values. It also attenuated the accumulation of Aβs in the brain with no alterations in CSF tau and p-tau. From clinical point of view, resveratrol improved cognitive and functional decline (mini-mental state examination (MMSE) and activities of daily living) during 12-months study. The authors concluded that resveratrol reduced CSF MMP9, modified neuro-inflammatory factors, and caused adaptive immunity (Moussa et al., 2017).

Another randomized, double-blind phase 3 study has been carried out on 27 participants with mild-to-moderate AD. The treatment group received resveratrol (unknown dose) with glucose and malate, delivered in grape juice. Cognitive measurements (the ADAS-Cog scale and Clinical Global Impression of Change (CGIC) scale) were used at follow up visits at months 3, 6, 9, and 12 months. Although this study was completed, results are unpublished to date (Zhu et al., 2018).

One randomized double-blind placebo controlled trial is already running to investigate the effect of resveratrol on 102 early affected HD patients in France (5≤UHDRS≤40). Subjects received either resveratrol at 80 mg or placebo for 1 year. Clinical outcomes and biological tolerance evaluated every 3 months. The primary measure is the rate of caudate atrophy after 1-year treatment.

Genistein

Despite promising pre-clinical data, there is no clinical trials on the applications of genistein for AD treatment. Up to our knowledge, only one trial was conducted. Genistein (60 mg) administration for 180 days changed the Aβ level in CSF of AD patients, besides, improved MMSE, ADAS-cog and the memory alteration test at determined intervals. Although, the study has passed its completion date, but the results are not available (Yassa et al., 2009).

Soy Isoflavones

There is a pilot randomized clinical trial examining the effect of soy isoflavones on cognitive function in old men and women over the age of 60 diagnosed with AD. Sixty-five participants were randomized to treatment for 6 months by soy isoflavone (100 mg/day; 85% daidzin and genistin as glycosides), or matching placebo capsules. No significant differences were observed between isoflavones treated group and placebo group or between the genders in terms of cognition outcomes, and self-report of mood symptoms. Besides, it was found that cognitive functions (speed dexterity and verbal fluency) were associated with equal levels (Gleason et al., 2015). A double-blind, placebo-controlled, Phase IV trial was designed in Thailand to compare HMS 90® (an immune system stimulant) vs. soy protein (as placebo), by the dose of 1 sachet (10 g) 2 times/day as adjuvant therapy in 38 individuals with idiopathic PD. No significant clinical outcomes were observed in biomarkers of oxidative stress (glutathione), plasma amino acids, and the brain function in both groups (Tosukhowong et al., 2016).

Epigallocatechin Gallate

The normalization of tyrosine phosphorylation regulated kinase 1A gene (Dyrk1A) and APP functions as therapeutic approaches for cognition improvement and slowing AD progression was investigated in a phase 2 randomized clinical study. Down syndrome (DS) patients received a daily oral dose of 9 mg/kg of EGCG, (in green tea extract standardized for EGCG) for 12 months. EGCG caused significant improvement in the adaptive behavior and brain-related changes in young adults with DS (de la Torre et al., 2016). In a phase 2 randomized placebo-controlled clinical trial, 21 patients over the age of 60 and in the early stages of AD received EGCG in an increasing manner

(in months 1–3: 200 mg EGCG, months 4–6: 400 mg, months 7–9: 600 mg, and months 10–18: 800 mg EGCG). ADAS-COG score, MMSE score, safety and tolerability, and the brain atrophy were assessed, although the study is completed but data are not available (https://clinicaltrials.gov/ct2/show/NCT00951834).

CONCLUSION AND FUTURE PROSPECTS

The unique involvement of UPS in malfunction of the nervous system encloses a broad range from drug abuse to neuroinflammation, making UPS an emerging topic in neurodegeneration and an important target for drug discovery. Since, UPS function is downregulated in vulnerable degenerating neurons in neurodegenerative diseases, thus, normal function of UPS assures a balanced regulation of misfolded protein degradation, which contributes to eliminate abnormal protein aggregates and to maintain the cellular protein homeostasis in the brain and neural network.

So far, a number of UPS regulators were developed. For example, cAMP phosphodiesterases inhibitors and UCHL1 that can modulate the brain cAMP-dependent protein kinase A (PKA)-cAMP response element binding protein (CREB) PKA-pCREB levels in AD subjects, resulting in enhanced protein degradation and synaptic functions (Cao et al., 2019). According to the collected data, a number of PPs are able to exert UPS inhibitory activity, mainly through chymotrypsin-like activity on both intracellular 26S and purified 20S proteasome. PPs interfere in many steps of degradation processes by means of proteasome inhibition, deubiquitinase activity, and stimulation of E1, E2, or E3, resulting in reduction of neurotoxicity, improvement of synaptic plasticity, and transmission, as well as enhanced neuronal survival. In this context, few clinical trials were carried out, mostly on a limited basis, however, the results are inconclusive and in most cases statistically insignificant. However, concerning PPs and their probable interaction with UPS, two hypotheses can be speculated; either their proteasomal inhibitory effects or their proteasomal stimulatory functions. PPs can induce ubiquitination which results in accelerating the elimination of damaged soluble proteins and degradation of short-lived regulatory proteins. Another way, inhibition of proteasomal activity by proteasome inhibitors i.e., PPs has been linked to synaptic plasticity.

Considering PPs and their roles in neuroprotection, curcumin, and resveratrol are the most studied polyphenolic compounds followed by EGCG and genistein, alone or in combination with other conventional drugs. Recently, it was proposed that UPS dysregulation, aberrant mRNA splicing, mitochondrial dysfunction, and excessive oxidative stress directly interplay with the process of neurodegeneration, thereby, future design of biomarkers and the drug development plans have to focus on concurrent targeting of multiple components and steps of neurodegenerative diseases (Tan et al., 2019). It is plausible that a combination of PPs and current drugs may improve the PPs therapeutic application for neuronal related destruction disorders. In this way, PPs offers considerable opportunity for development of specific therapeutics approaches via UPS, for particular groups of misfolded proteins. However, direct links and molecular mechanisms remain elusive and require to be addressed.

This is important to have a clear understanding of PPs molecular mechanism of action and their possible interplay with UPS. In addition, the upstream regulators or downstream targets of UPS have to be characterized, empowering researches to develop treatment strategies with more specificity and efficacy. Another question that has to be responded is whether the inhibition of UPS by PPs is more favorable for the neurodegenerative diseases or the stimulation of this system? Concerning neurodegeneration, further clinical interventions with greater sample size, proper duration, applicable formulations, and/or dosages should be designed to assess the potential efficacy of natural bioactive compounds as UPS inhibitors/regulators.

AUTHOR CONTRIBUTIONS

ZM, FE-S, NS, FG, MK, and AA contributed to the collection and/or assembly of data and interpretation, and the writing of the manuscript. SM, MA, and MF contributed to the provision of study material, conception and design, and final approval of manuscript. All authors have read and approved the manuscript.

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The Role of NMDA Receptors in the Effect of Purinergic P2X7 Receptor on Spontaneous Seizure Activity in WAG/Rij Rats With Genetic Absence Epilepsy

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P2X7 receptors (P2X7Rs) are ATP sensitive cation channels and have been shown to be effective in various epilepsy models. Absence epilepsy is a type of idiopathic, generalized, non-convulsive epilepsy. Limited data exist on the role of P2X7Rs and no data has been reported regarding the interaction between P2X7Rs and glutamate receptor NMDA in absence epilepsy. Thus, this study was designed to investigate the role of P2X7 and NMDA receptors and their possible interaction in WAG/Rij rats with absence epilepsy. Permanent cannula and electrodes were placed on the skulls of the animals. After the healing period of the electrode and cannula implantation, ECoG recordings were obtained during 180 min before and after drug injections. P2X7R agonist BzATP, at doses of 50 μg and 100 μg (intracerebroventricular; i.c.v.) and antagonist A-438079, at doses of 20 μg and 40 μg (i.c.v.) were administered alone or prior to memantine (5 mg/kg, intraperitoneal; i.p.) injection. The total number (in every 20 min), the mean duration, and the amplitude of spike-wave discharges (SWDs) were calculated and compared. Rats were decapitated and the right and left hemisphere, cerebellum, and brainstem were separated for the measurements of the advanced oxidation protein product (AOPP), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), glutathione peroxide (GPx), and glutathione reductase (GR). BzATP and A-438079 did not alter measured SWDs parameters, whereas memantine reduced them, which is considered anticonvulsant. BzATP did not alter the anticonvulsant effect of memantine, while A-438079 decreased the effect of memantine. Administration of BzATP increased the levels of SOD and GR in cerebrum hemispheres. A-438079 did not alter any of the biochemical parameters. Memantine reduced the levels of MDA, GSH, and GR while increased the level of CAT in the cerebrum. Administration of BzATP before memantine abolished the effect of memantine on MDA levels. The evidence from this study suggests that P2X7Rs

does not directly play a role in the formation of absence seizures. P2X7Rs agonist, reduced the antioxidant activity of memantine whereas agonist of P2X7Rs reduced the anticonvulsant action of memantine, suggesting a partial interaction between P2X7 and NMDA receptors in absence epilepsy model.

Keywords: absence, epilepsy, memantine, NMDA, oxidative, P2X7, stress, WAG/Rij

INTRODUCTION

P2X7 receptors (P2X7Rs) are purinergic cation channels. They are sensitive to high concentrations of ATP in the extracellular space, and they have essential roles in inflammation (Burnstock and Knight, 2018). P2X7Rs could be permeable to small cations when they are stimulated within milliseconds. However, prolonged stimulation (within seconds) of P2X7Rs allows permeation by molecules with a mass of up to 900 Da, leading to the release of inflammatory cytokines and apoptosis (Burnstock and Knight, 2018). Stimulation of P2X7Rs also leads to neuroinflammatory responses, including, ADAM10, and ADAM17 activation, and causes the secretion of prostaglandin E2 and proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-2, IL-4, IL-6, and tumor necrosis factor (TNF) (Engel et al., 2012b; Beamer et al., 2017). P2X7Rs mediate NLRP3 inflammasome-dependent IL-1β secretion following activation of NF-κB in the brain and immune cells (Albalawi et al., 2017; Burnstock and Knight, 2018). Therefore, P2X7Rs are a target for neurodegenerative diseases.

P2X7R expression has been widely shown in the central nervous system, including microglia, oligodendrocytes, and ependymal cells (Jimenez-Mateos et al., 2019). The P2X7R is mainly expressed on microglia, but not on the neurons (Jimenez-Pacheco et al., 2016; Kaczmarek-Hajek et al., 2018). P2X7R is expressed in the CA1 pyramidal and dentate granule neurons, as well as in microglia of epileptic mice (Jimenez-Pacheco et al., 2016) and in the neurons of rat hippocampus (Sperlagh et al., 2002). Moreover, P2X7R expression has been detected in both the astrocyte culture of brain/spinal cord slices (Illes et al., 2012, 2017). This conflicting data about functional expression of P2X7R could be attributed to both brain region-specific expression and the pathological conditions of the brain such as epilepsy (Beamer et al., 2017). P2X7R expression has increased in the hippocampus and neocortex regions of the brain in many epilepsy models (Jimenez-Pacheco et al., 2016; Huang et al., 2017; Rodriguez-Alvarez et al., 2017; Zeng et al., 2017; Jimenez-Mateos et al., 2019).

Absence epilepsy is a common neurological disease in children that affects educational success. Absence epilepsy is a loss of consciousness with a sudden pause in behavior, and if electroencephalography is recorded during seizures, bilateral synchronous 3-Hz frequency spike-wave discharges (SWDs) are observed (Russo et al., 2016; Fisher et al., 2017). Its pathophysiology is unclear, but it has been shown that absence seizures start from a glutamatergic focus located in the perioral region of the somatosensory cortex, and then this area affects the thalamus over time and creates a cortico-thalamo-cortical circuit (Meeren et al., 2005; Pinault and O'Brien, 2005). Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats are also a validated genetic model of absence epilepsy characterized by SWDs on electroencephalography with a spontaneous pause in behavior (Russo et al., 2016). IL-1β increases in the somatosensory cortex and IL-1β antagonist administration reduces SWDs in the Genetic Absence Epilepsy Rat from Strasbourg (GAERS) model, which is also a validated genetic absence epilepsy model (Akin et al., 2011).

The effects of P2X7Rs on epilepsy have been investigated in various experimental epilepsy models (Fischer et al., 2016; Huang et al., 2017; Nieoczym et al., 2017; Rodriguez-Alvarez et al., 2017; Arslan et al., 2019). In a kainic acid-induced status epilepticus (SE) model, P2X7R expression was shown to increase, and seizure severity and neuronal death decreased after pre-treatment or post-treatment with intracerebroventricular (i.c.v.) injection of A-438079 or systemic administration of JNJ-47965567 (Engel et al., 2012a; Jimenez-Pacheco et al., 2013, 2016). Systemic administration of A-438079 reduced convulsions in the kainic acid-induced SE model and reduced neuronal death more than phenobarbital in 10-day-old rats (Mesuret et al., 2014). In a penicillin-induced epilepsy model, a P2X7R agonist showed proconvulsant effects that could be reversed by A-438079 and a T-type calcium channel blocker, whereas a P2X7R antagonist, A-438079, showed an anticonvulsant effect (Arslan et al., 2019). In a pentylenetetrazol (PTZ) kindling model, P2X7R antagonists, Brilliant Blue G (BBG) and tanshinone showed a slight delay in kindling development, and JNJ-47965567 and AFC-5128 showed a strong delay (Soni et al., 2015; Fischer et al., 2016). However, P2X7R antagonists were ineffective in fully kindled rats (Fischer et al., 2016). They also did not affect the number of seizures in the kainic acid-induced kindling model, but they gave rise to less severe chronic seizures (Amhaoul et al., 2016). Systemic administration of A-438079 reduced acute seizures during hypoxia in neonatal mice but had no effect on post-hypoxia seizures (Rodriguez-Alvarez et al., 2017). P2X7R antagonists did not affect the maximal electroshock seizure threshold test or PTZ seizure threshold test, but AFC-5128 or JNJ-47965567 combination with carbamazepine increased the seizure threshold more than carbamazepine alone (Fischer et al., 2016; Nieoczym et al., 2017). BBG showed a week anticonvulsant action on the threshold of 6 Hz induced psychomotor seizures in mice (Nieoczym et al., 2017). However, P2X7R knockout mice were more susceptible to pilocarpine-induced seizures (Kim and Kang, 2011), and blockade of P2X7Rs increased the number and severity of pilocarpine-induced seizures in mice (Rozmer et al., 2017).

On the other hand, NMDA receptors are cation channels, and over-stimulation leads to an increase in intracellular calcium, which could be toxic for cells (Rosini et al., 2019). Memantine,

a non-competitive NMDA receptor antagonist, has also shown anticonvulsant effects in many experimental epilepsy models, including a penicillin-induced epilepsy model, an audiogenic seizure model, and a PTZ kindling model (Frey and Voits, 1991; Cakil et al., 2011; Kim et al., 2012; Zaitsev et al., 2015). Only one study has been conducted with WAG/Ola/Hsd rats, which are thought to be a model of genetic absence epilepsy (Frey and Voits, 1991), but there is no much information about this rat substrain. The impairment of gamma-aminobutyric acid (GABA) and glutamate leads to epilepsy. GABAergic antiepileptic drugs worsen absence seizures (Panayiotopoulos, 2001). However, the NMDA receptor NR1 subunit showed a decrease depending on age in WAG/Rij rats (van de Bovenkamp-Janssen et al., 2006). In addition, in both 2-month-old and 6-month-old WAG/Rij rats, the NR2B subunit was lower in various layers of the somatosensory cortex than in the Wistar rats of the same age (Karimzadeh et al., 2013). Stimulation of NMDA receptors increased SWDs in WAG/Rij rats, whereas NMDA receptor reduced SWDs (Peeters et al., 1994).

The P2X7R increases glutamate secretion in a vesicular and non-vesicular manner (Sperlagh et al., 2002; Cho et al., 2010). It also affects GABA and glutamate reuptake in a calcium-dependent manner (Barros-Barbosa et al., 2018). The P2X7R was found to be non-desensitizing, and it allows substantial calcium influx (Fischer et al., 2016). It activates intracellular signaling pathways (Takenouchi et al., 2010). Both P2X7R and NMDA receptor activation can activate pathways of reactive oxygen species (ROS) (Davidson and Duchen, 2006). ROS cause many changes such as aging, cardiovascular disease, cancer, and neurodegenerative diseases, including epilepsy, by damaging proteins, lipids, carbohydrates, and nucleic acids, and they can be controlled by antioxidant systems, preventing the formation of ROS, and damage (Droge, 2002; Terrone et al., 2019).

x There are limited data on the interaction between P2X7Rs and NMDA receptors in epilepsy. Thus, the effects of P2X7Rs and NMDA receptors and their relationship were investigated using a P2X7R agonist, BzATP, which is more selective for P2X7R than for other P2X receptors, a P2X7R antagonist, A-438079, which highly targets the P2X7R, and a selective antagonist of the NMDA receptor, memantine, in WAG/Rij rats with both electrophysiological and biochemical analysis methods.

MATERIALS AND METHODS

Animals

In this study, 63 male, 6–8 months old, 250–300 g weighing WAG/Rij rats were used with the permission of Ondokuz Mayis University Animal Experiments Local Ethics Committee (2015/56). Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats with spontaneous seizures were purchased from Charles River Lab (Germany). Animals were fed *ad libitum* and housed in heat-regulated rooms, on a 12 h light-dark cycle. All experimental procedures were conducted under the European Union Directive (2010/63/EU), and Turkish legislation acts concerning animal experiments.

Animals were divided into nine groups (n = 7) randomly as follows:

1. Control group (WAG/Rij rat)
2. Sham Group (WAG/Rij rat, 2 μl sterile distilled water; i.c.v.)
3. BzATP 50 μg (i.c.v.) group
4. BzATP 100 μg (i.c.v.) group
5. A-438079 20 μg (i.c.v.) group
6. A-438079 40 μg (i.c.v.) group
7. Memantine 5 mg/kg (i.p.) group
8. BzATP 100 μg (i.c.v.) + Memantine 5 mg/kg (i.p.) group
9. A-438079 20 μg; (i.c.v.) + Memantine 5 mg/kg (i.p.) group.

Surgery and Electrocorticography Recording

Animals were anesthetized and sedated with ketamine/xylazine (90/10 mg/kg; i.p.) and placed in the stereotaxic apparatus. After the skin was cut off about 3 cm and folded back, subcutaneous tissue was removed from the cranium. According to the rat brain atlas (Paxinos and Watson, 2007), four burr holes were drilled in the skull with a microdrill without damaging the dura mater. For ECoG recordings, three screw electrodes were placed into the holes as coordinates: first electrode; 2 mm anterior and 3.5 mm right lateral to bregma, second electrode; 6 mm posterior and 4 mm right lateral to bregma and earth electrode was placed on the cerebellum. For i.c.v. injections, an external cannula was advanced into the brain as coordinates: 1.1 mm posterior, 1.5 mm right lateral and 3.2 mm vertical to bregma. Afterward, the electrodes and cannula were fixed to the skull with dental cement. The cannula was covered with a dust cup until used. After the surgery, animals were housed individually for a week and habituated to the recording cage (32 cm × 30 cm in width, 50 cm high) for 3 days before the experimental procedure.

On the experiment day, animals were connected to the ECoG recording system (PowerLab, 16/SP, AD Instruments, Australia) by an isolated flexible cable. Baseline electrocorticography (ECoG) recordings were taken for 3 h from all awake animals at the same time of day (10:00 AM). After the drug injection, ECoGs recording continued for another 3 h (Aygun et al., 2019). The number of SWDs and the mean duration and amplitude of SWDs were measured and calculated for every 20 min offline with LabChart 7 Pro (AD Instruments, Australia) (Arslan et al., 2013, 2014).

Drugs and Drug Administration

Ketamine hydrochloride, xylazine hydrochloride, A-438079 hydrochloride hydrate, BzATP [2′(3′)-*O*-(4-Benzoylbenzoyl) adenosine 5′-triphosphate triethylammonium] and memantine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, United States and dissolved in sterile distilled water. After obtaining 180 min of baseline ECoG recordings, A-438079, at the doses of 20 and 40 μg, and BzATP, at the doses of 50 and 100 μg were administered into the lateral ventricle within a thin internal cannula (4.2 mm vertical to the bregma) in a volume of 2 μl. Memantine, at a dose of 5 mg/kg, was injected intraperitoneally in a volume of 0.5 ml. For the

interaction groups, memantine was administered 10 min after the chosen doses of BzATP (100 μg) or A-438079 (20 μg) (Arslan et al., 2019). The sham group was given sterile distilled water (2 μ l, i.c.v.).

Biochemical Analysis

After the end of the ECoG recordings, all rats were decapitated following ketamine/xylazine anesthesia. Right and left hemisphere, cerebellum, and brainstem were separated in oxygenated artificial cerebrospinal fluid [aCSF containing (mM): NaCl, 124; KCl, 5; KH2PO4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; HEPES, 10, at pH 7.4 when saturated with 95% O_2 and 5% CO_2]. The tissues were frozen in liquid nitrogen, homogenized immediately, and soaked in phosphate buffer solution (PBS, 10 mM, and pH 7.2). After 1 min of sonication at +4°C with 220 V (METU Electromechanical, Germany), homogenates were stored at −80°C. On the evaluation day, the homogenates were defrosted at room temperature and were centrifuged at +4°C for 5 min with 15,000 × *g* (Sigma 3K30, Germany) for biochemical analysis.

Tissue protein concentrations were determined with Lowry's method (Lowry et al., 1951). The results were expressed per mg protein. Advanced protein oxidation products (AOPP), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR) concentrations in the tissues were examined with commercial rat ELISA kits (SunRed Biotechnology Co., Shanghai, China). These kits all use a double-antibody sandwich enzyme-linked immunosorbent assay (Arslan et al., 2019).

Statistical Analysis

Spike-wave discharges parameters were calculated, by using the raw data obtained from LabChart 7-Pro, with an excel program. The total number of SWDs after drug injections was calculated for every 20 min and these data were converted to percentage values by comparing to the baseline values. The mean duration and the amplitude of SWDs during 180 min after injections were calculated as a percentage by comparing to the baseline data.

SPSS 15.0 data analysis software was used for statistical analyses. The normality of the data was tested with the Shapiro–Wilk test. After verifying that the data were normally distributed paired-samples *t*-test was performed between dependent groups, and one- or two-way ANOVA and then *post hoc* Tukey tests were used for multiple comparisons. The results are expressed as mean ± standard error (SEM). For all statistical analyses, $p < 0.05$ was considered statistically significant.

RESULTS

All rats showed SWDs in ECoG characterized by paroxysmal unresponsiveness to environmental stimuli (**Figure 1**). Paired-samples *t*-test revealed that the total numbers and the mean duration of SWDs did not significantly change after the injection of solvent compared to baseline values. The total numbers and mean durations of SWDs were 108.4 ± 4.8, 111.0 ± 3.8 and 8.67 ± 0.18, 8.76 ± 0.21 s during 180 min before and after the administration of sterile distilled water, respectively. The mean amplitudes of SWDs were 0.635 ± 0.039 and 0.648 ± 0.048 mV before and after the administration of sterile distilled water, respectively. There was no statistical difference regarding the parameters of SWDs between the control and sham groups ($p > 0.05$).

Table 1 shows the biochemical analysis for the left and right hemispheres, cerebellum, and brainstem of the groups. There was no significant difference between the sham group and the control group in any of the biochemical parameters.

The Role of P2X7Rs in WAG/Rij Rats

Administration of P2X7Rs agonist BzATP, at the doses of 50 and 100 μg, did not significantly change any of the total numbers [$F_{(8,162)} = 0.12$] and durations [$F_{(2,18)} = 1.54$] of SWDs compared to the control group (**Figure 2**). Total numbers, mean durations and mean amplitudes of SWDs were 12.2 ± 0.9, 11.1 ± 0.9; 9 ± 0.4, 8.9 ± 0.4 s;0.6490 ± 0.013, 0.6148 ± 0.016 mV in the 80th minute in the BzATP 50 μg and BzATP 100 μg groups, respectively. After the injections of BzATP, at doses of 50 and 100 μg, the total numbers and mean durations of SWDs were 107.3 ± 4.2, 102.7 ± 5.4; 8.82 ± 0.38, 8.52 ± 0.29 s during 180 min, respectively. Injection of A-438079, at the doses of 20 and 40 μg, did not alter the total numbers [$F_{(8,162)} = 0.54$] and durations [$F_{(2,18)} = 6.54$] of SWDs compared to the control group (**Figure 3**). The total numbers and mean durations of SWDs were 107.8 ± 7.7, 102.7 ± 9.4; 8.38 ± 0.28, 8.2 6 ± 0.34 s during 180 min after the injections of 20 and 40 μg A-438079, respectively.

BzATP, at a dose of 100 μg, injection significantly increased SOD and GR levels in the left and right hemispheres ($p < 0.05$). Other biochemical parameters were not different in the BzATP group compared to the control group ($p > 0.05$). A-438079, at a dose of 20 μg, did not alter any of the biochemical parameters (**Table 1**).

The Effect of Memantine on WAG/Rij Rats

Intraperitoneal injection of memantine (5 mg/kg) significantly decreased the total number [$F_{(8,108)} = 0.66$, $p < 0.001$] and mean duration of SWDs [$F_{(1,12)} = 6.34$, $p < 0.05$] 20 min after injection compared to control group (**Figure 4**). The total number, mean duration and amplitude of SWDs were 32.5 ± 3.6; 7.74 ± 0.19 s and 0.635 ± 0.03 mV during 180 min after the administration of memantine, respectively.

Memantine significantly decreased MDA levels in all brain regions compared to the control group ($p < 0.05$). In the cerebrum, injection of memantine decreased GSH and GR levels, and increased CAT levels ($p < 0.05$). Other biochemical parameters were not different in the memantine group compared to the control group (**Table 1**).

Interaction Between P2X7R and Memantine in WAG/Rij Rats

Injection of BzATP (100 μg) 10 min before memantine decreased the total number of SWDs after 20 min [$F_{(8,216)} = 1.10$, $p < 0.001$] and the mean duration of SWDs [$F_{(3,24)} = 10.60$,

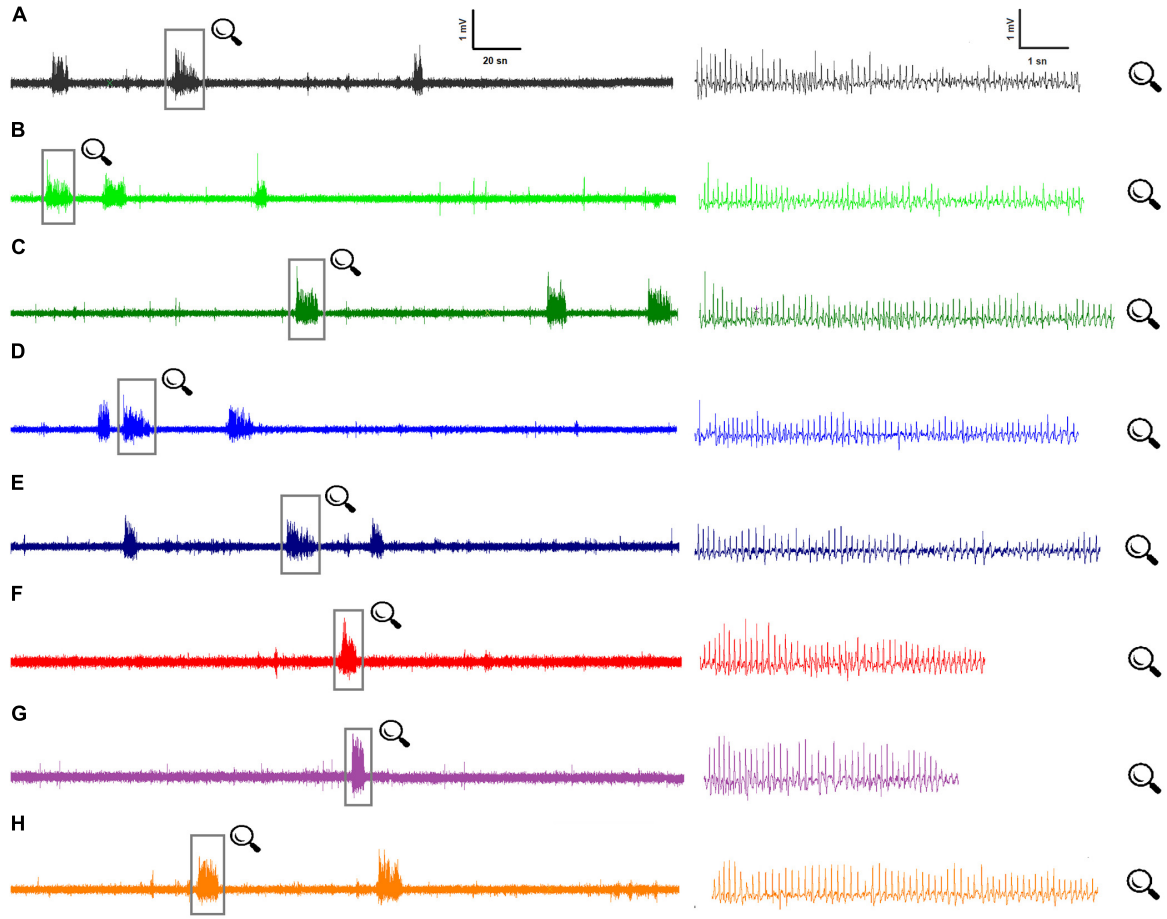

FIGURE 1 | Representative ECoG recordings for all groups at 80th minute: **(A)** Control ECoG activity in WAG/Rij rats (n = 7); **(B)** BzATP, at the dose of 50 μg, i.c.v. (n = 7); **(C)** BzATP, at dose of 100 μg, i.c.v. (n = 7); **(D)** A-438079, at dose of 20 μg, i.c.v. (n = 7); **(E)** A-438079, at dose of 40 μg, i.c.v. (n = 7); **(F)** Memantine administration, at dose of 5 mg/kg, i.p. (n = 7); **(G)** BzATP (100 μg) administration 10 min before memantine (n = 7); **(H)** A-438079 (20 μg) administration 10 min before memantine (n = 7).

$p < 0.05$] compared to the control group (**Figure 4**). However, the total number and the mean duration of SWDs were not found to be different after the co-administration of BzATP with memantine compared to the alone memantine injection. The total number and the mean duration of SWDs were 26.3 ± 2.8 and 7.56 ± 0.42 s for 180 min after the co-administration of BzATP with memantine, respectively.

Co-administration of BzATP with memantine decreased the levels of GSH and GR, and increased CAT levels in the cerebrum compared to the control group ($p < 0.05$). Other biochemical parameters did not alter with the co-administration of BzATP with memantine compared to the control group.

Although administration of A-438079 10 min prior to memantine decreased the total number of SWDs after 40 min compared to the control group ($p < 0.05$), but it appears that A-438079 partially reversed the anticonvulsant activity of memantine after 20th minute compared to the memantine group alone [$F_{(8,216)} = 1.97, p < 0.001$]. The mean duration of SWDs did not alter compared to both the control and memantine groups [$F_{(3,24)} = 6.74$, **Figure 5**]. The total number and mean duration of SWDs were 59.7 ± 6.6 and 8.05 ± 0.21 s for 180 min after the co-administration of A-438079 with memantine, respectively.

Malondialdehyde levels significantly decreased in all regions after the co-administration of A-438079 with memantine ($p < 0.05$). In the cerebrum, the combination of A-438079 + memantine decreased GSH and GR levels, and increased CAT levels compared to the control group ($p < 0.05$). Interestingly, CAT levels were decreased in the brainstem with the injections of A-438079 and memantine ($p < 0.05$). The rest of the measured biochemical parameters were not changed in the A-438079 + memantine group compared to the control group (**Table 1**).

DISCUSSION

In the present study, the electrophysiological results revealed that the administration of memantine showed an anticonvulsant effect without changing the mean amplitude of SWDs. Neither BzATP nor A-438079 affected the parameters of SWDs in

TABLE 1 | The levels of advanced oxidation protein products (AOPP), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), glutathione peroxide (GPx), and glutathione reductase (GR) in the left and right hemispheres, cerebellum and brainstem of all experimental groups.

		AOPP (nmol/mL)	MDA (nmol/mL)	SOD (ng/mL)	GSH (ng/mL)	CAT (ng/mL)	GPx (ng/mL)	GR (ng/mL)
LEFT HEMISPHERE	Control	4.7 ± 0.7	5.7 ± 0.8	5.1 ± 0.5	52.8 ± 5.6	10.8 ± 0.8	8.8 ± 0.7	5.9 ± 0.5
	BzATP (100 μg)	5.65 ± 0.9	6.7 ± 0.8	7.1 ± 0.6*	60.6 ± 5.5	10.1 ± 0.7	9.7 ± 1.04	8.7 ± 1.1*
	A-438079 (20 μg)	4.4 ± 0.5	6.04 ± 0.7	4.6 ± 0.2	48.4 ± 4.3	11.8 ± 1.3	6.4 ± 0.9	4.7 ± 0.7
	Memantine (5 mg/kg)	3.6 ± 0.5	2.6 ± 0.4*	4.6 ± 0.4	35.02 ± 3.5*	17.3 ± 0.6**	7.6 ± 0.6	2.6 ± 0.3**
	BzATP + memantine	4.9 ± 0.7	4.7 ± 0.6	4.5 ± 0.7	37.4 ± 2.9*	15.06 ± 0.3*	9.5 ± 1.1	3.7 ± 0.3*
	A-438079 + memantine	3.4 ± 0.6	3.1 ± 0.3*	4.3 ± 0.6	32.2 ± 4.1*	16.1 ± 0.5*	6.9 ± 0.4	3.07 ± 0.4*
RIGHT HEMISPHERE	Control	5.02 ± 1.03	5.8 ± 0.7	5.06 ± 0.4	47.5 ± 4.7	11.3 ± 1.8	7.6 ± 0.5	5.7 ± 0.3
	BzATP (100 μg)	6.1 ± 1.07	7.1 ± 1.1	8.03 ± 0.8*	56.4 ± 53	10.5 ± 1.3	8.8 ± 0.8	8.1 ± 0.5*
	A-438079 (20 μg)	4.6 ± 0.7	5.06 ± 1.01	4.5 ± 0.3	50.6 ± 5.1	9.9 ± 2.01	7.04 ± 0.33	5.07 ± 0.4
	Memantine (5 mg/kg)	4.5 ± 0.6	3.1 ± 0.4*	5.2 ± 0.6	27.9 ± 3.1*	18.2 ± 0.7*	6.04 ± 0.7	2.5 ± 0.4**
	BzATP + memantine	6.09 ± 0.9	4.7 ± 0.6	5.2 ± 0.7	25.5 ± 3.09*	19.7 ± 1.3*	6.4 ± 0.8	3.4 ± 0.5*
	A-438079 + memantine	4.2 ± 1.02	3.3 ± 0.3*	4.6 ± 0.4	29.06 ± 4.1*	17.8 ± 0.6*	5.7 ± 0.4	2.4 ± 0.6**
CEREBELLUM	Control	11.2 ± 1.3	7.4 ± 1.05	9.07 ± 1.06	73.8 ± 8.3	15.7 ± 1.9	10.5 ± 1.3	9.1 ± 0.5
	BzATP (100 μg)	12.1 ± 1.2	7.6 ± 1.07	10.3 ± 0.8	78.5 ± 2.06	15.6 ± 2.4	12.5 ± 0.9	9.02 ± 0.4
	A-438079 (20 μg)	10.3 ± 1.01	6.6 ± 0.9	8.4 ± 0.9	62.8 ± 8.4	13.7 ± 2.1	10.7 ± 1.05	8.4 ± 0.8
	Memantine (5 mg/kg)	8.9 ± 0.7	4.4 ± 0.6*	7.7 ± 1.2	60.02 ± 7.1	20.7 ± 2.4	9.7 ± 0.7	7.9 ± 0.8
	BzATP + memantine	9.1 ± 1.1	7.08 ± 0.8	7.2 ± 1.1	66.4 ± 6.5	17.6 ± 3.4	8.7 ± 1.05	10.03 ± 1.2
	A-438079 + memantine	7.7 ± 1.4	3.6 ± 0.5*	8.03 ± 0.8	59.7 ± 5.9	19.9 ± 3.6	8.6 ± 0.8	8.9 ± 0.7
BRAINSTEM	Control	9.3 ± 0.9	11.8 ± 1.2	7.1 ± 0.5	100.4 ± 9.9	18.1 ± 1.9	17.8 ± 2.6	8.05 ± 1.1
	BzATP (100 μg)	10.1 ± 1.3	13.3 ± 0.9	9.3 ± 0.7	103.7 ± 11.7	17.9 ± 1.7	19.7 ± 2.1	8.4 ± 0.6
	A-438079 (20 μg)	8.6 ± 0.9	10.7 ± 0.6	6.8 ± 0.6	108.4 ± 11.05	19.5 ± 3.08	17.45 ± 1.3	7.1 ± 0.7
	Memantine (5 mg/kg)	7.6 ± 0.7	6.4 ± 1.1*	6.3 ± 0.8	92.3 ± 10.5	17.8 ± 2.1	16.9 ± 1.4	7.3 ± 0.8
	BzATP + memantine	8.3 ± 0.8	9.1 ± 0.8	5.9 ± 0.4	89.7 ± 8.8	21.8 ± 3.4	14.3 ± 1.2	10.1 ± 1.2
	A-438079 + memantine	7.5 ± 1.05	7.1 ± 0.7**	6.09 ± 0.6	82.3 ± 7.1	14.6 ± 1.7*	16.5 ± 1.07	7.6 ± 0.7

*$p < 0.05$, **$p < 0.01$ compared to control group.*

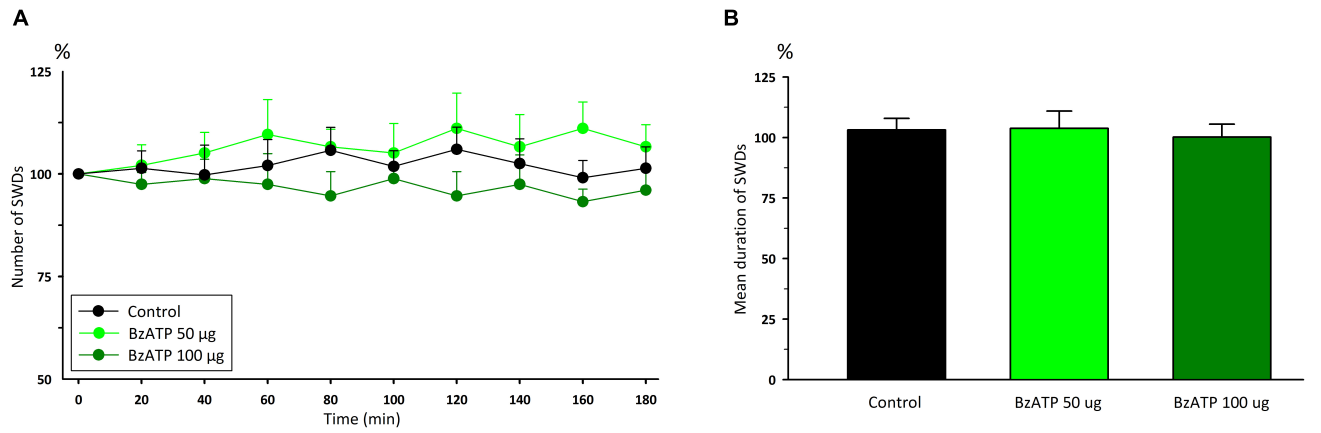

FIGURE 2 | The effects of P2X7R agonist BzATP on **(A)** the total number of SWDs in every 20 min and **(B)** the mean duration of SWDs for 180 min.

WAG/Rij rats. BzATP did not reverse the anticonvulsant activity of memantine. However, A-438079 reduced the anticonvulsant activity of memantine.

BzATP increased only the SOD and GSH levels in the cerebrum, whereas A-438079 did not significantly affect any of the biochemical parameters. Memantine showed antioxidant effects by decreasing MDA levels in all tissue samples. Memantine also reduced GSH and GR and increased CAT levels in the cerebrum. BzATP reversed the antioxidant effects of memantine on MDA, while A-438079 enhanced the anticonvulsant effects of memantine in the brainstem.

The Role of P2X7R in WAG/Rij Rats

The P2X7R is the newest member of the purinergic receptor family and has been the subject of epilepsy research with its widespread presence in the nervous system (Jimenez-Mateos

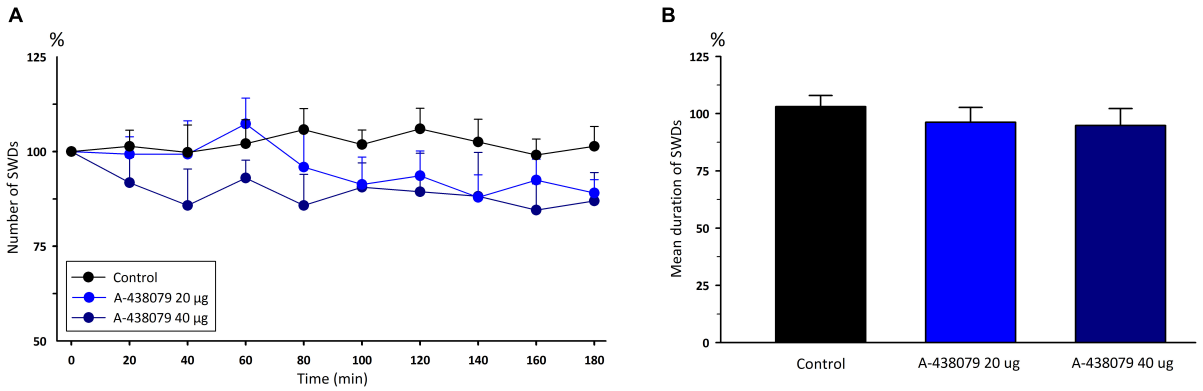

FIGURE 3 | The effects of P2X7R antagonist A-438079 on **(A)** the total number of SWDs in every 20 min and **(B)** the mean duration of SWDs for 180 min.

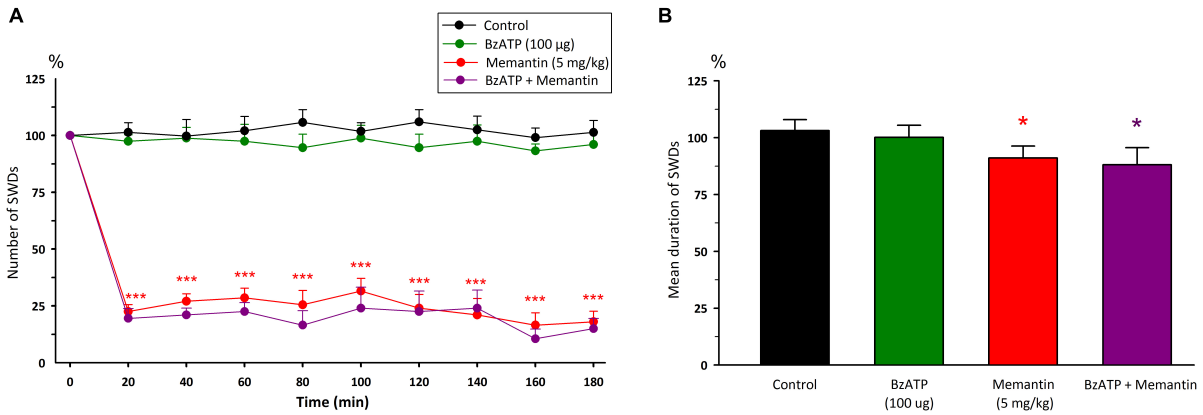

FIGURE 4 | The effects of memantine at a dose of 5mg/kg and its interaction with BzATP on **(A)** the total number of SWDs in every 20 min and **(B)** the mean duration of SWDs for 180 min. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control group.

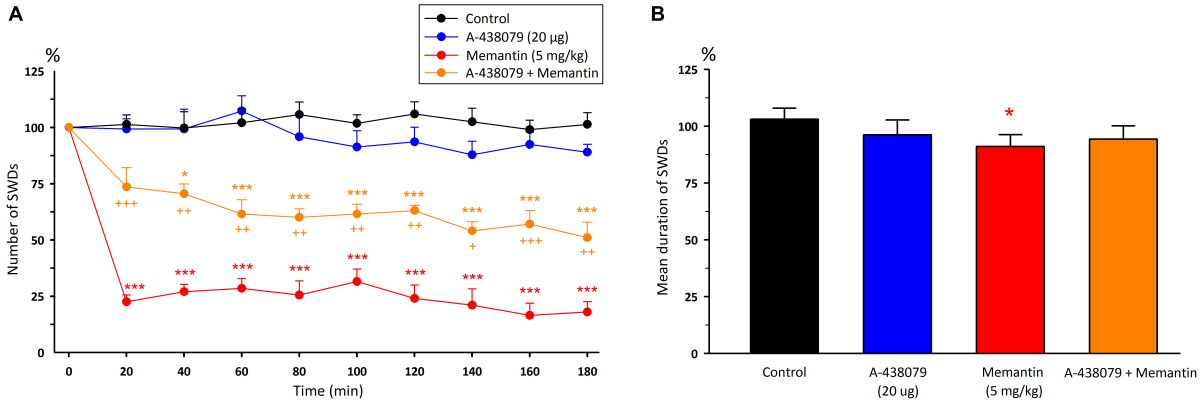

FIGURE 5 | The effects of memantine at a dose of 5mg/kg and its interaction with A-438079 on **(A)** the total number of SWDs in every 20 min and **(B)** the mean duration of SWDs for 180 min. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to the control group. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to the – memantine group.

et al., 2019). The involvement of the P2X7R in epilepsy has been demonstrated by several researchers with either anticonvulsant, proconvulsant, or no effects in various models of epilepsy (Jimenez-Pacheco et al., 2016; Rodriguez-Alvarez et al., 2017; Arslan et al., 2019). These controversial results depend on experimental differences, such as the selection of the epilepsy model, the dosage of the P2X7R agonist and antagonist used, and the species of the experimental animals. We used an absence

epilepsy model in this study, which was a chronic non-convulsive epilepsy model, and there are some unique mechanisms known to create absence seizures (Meeren et al., 2005; D'Antuono et al., 2006; Luttjohann and van Luijtelaar, 2012; Scicchitano et al., 2015). The P2X7R is a non-selective cation channel, and it allows calcium influx, which is essential for the absence epilepsy pathogenesis.

BzATP and A-438079 have high potency for the P2X7R. BzATP is the most potent for the P2X7R, but it is not a selective P2X7R agonist (Donnelly-Roberts and Jarvis, 2007). Thus, BzATP may also be effective in other P2X receptors that may also have an important role in epilepsy (Michel et al., 2001; Henshall et al., 2013). The potent inhibition of the P2X7R with A-438079 confirms that the P2X7R accounts for ATP-triggered Ca^{2+} entry (Fischer et al., 2016). The moderate bioavailability and moderate plasma elimination half-life were 19% and 1.02 h, respectively, for intraperitoneal A-438079 in rats (McGaraughty et al., 2007). Brain levels of A-438079 rapidly declined after parenteral injection, and Mesuret et al. (2014) suggested that the rapid elimination of the compound restricts the therapeutic window of this compound. However, in our previous study, i.c.v. injection of BzATP and A-438079 showed their effects on the penicillin-induced epileptiform activity within 20 and 60 min after their injections, respectively, and lasted for 120 min (Arslan et al., 2019). BzATP is the most potent agent for the P2X7R, and A-438079 is specific and can almost abolish the effects of BzATP (Anderson and Nedergaard, 2006; Fischer et al., 2016). The multi-targeting of different P2X receptors and their stability in the brain may be considered possible limitations for such studies in epilepsy. In addition, P2X7Rs release cytokines during normal brain function (Sperlagh and Illes, 2014). In particular, P2X7R activation might be linked to the regulation of various aspects of immunocompetent cells through the expression and secretion of many inflammatory mediators, including IL-1b, IL-2, IL-4, IL-6, IL-8, and TNFα (Engel et al., 2012b). However, inflammatory mediators were not measured, which might be another limitation of the present study. Therefore, further studies are required to determine both receptor-specific localization and the level of inflammatory mediators in an absence epilepsy model.

In an Alzheimer's disease model, P2X7R activity affected nicotinamide adenine dinucleotide phosphate (NADPH) activity and increased the formation of the $O^{\cdot -}$ radical by acting on p38 MAPK (Parvathenani et al., 2003). The antioxidant enzyme that converts the $O^{\cdot -}$ radical into H_2O_2 is SOD, and an increase in the SOD level promotes the increase of $O^{\cdot -}$ radicals (Younus, 2018). Activation of the P2X7R due to BzATP promotes ROS production through NADPH oxidase in macrophages, microglia, and neurons that can be blocked by P2X7R inhibitors (Hewinson and Mackenzie, 2007; Mead et al., 2012; Munoz et al., 2017). The P2X7R has also been shown to directly affect SOD in amyotrophic lateral sclerosis models and cell culture studies (Gandelman et al., 2013; Fabbrizio et al., 2017). GR is used in the reduction reaction of NADPH to GSH disulfide, which is a way to decrease NADPH. BzATP increases the production and release of ROS in the substantia gelatinosa of the spinal cord by stimulating P2X7Rs in astroglia (Ficker et al., 2014). However, Safiulina et al. (2006) showed ATP-induced ROS generation in CA3 pyramidal neurons due to the stimulation of P2Y1 receptors, not P2X7Rs. ATP treatment increased the expression of Cu/Zn SOD in the RBA-2 astrocyte cell line of cell culture (Chen et al., 2006). In our previous study, BzATP increased lipid peroxidation and the levels of protein oxidation and antioxidant proteins in the brain of penicillin-induced epileptic rats (Arslan et al., 2019). In the present study, BzATP and A-438079 did not affect protein or lipid oxidation, but BzATP increased SOD and GR in the cerebrum. Accordingly, the P2X7R seems to be more effective in the cerebral cortex in WAG/Rij rats with absence epilepsy. Since it has been suggested that oxidative mechanisms and inflammatory mechanisms do not cause the formation of absence epilepsy (Grosso et al., 2011) and there are no inflammatory processes in WAG/Rij rats, it is logical to expect that the low number of P2X7Rs and efficacy may be the reason for the ineffectiveness of BzATP and A-438079 observed in the present study in absence epileptic rats. However, children with absence epilepsy have shown no oxidant markers, while increased lipid peroxidation and protein oxidation levels have been observed in epileptic encephalopathic patients (Grosso et al., 2011).

The Role of NMDA Receptors in WAG/Rij Rats

Although memantine is mostly used in Alzheimer's disease to improve cognitive function, the anticonvulsant activity of memantine has been demonstrated in various models of experimental epilepsy (Frey and Voits, 1991; Cakil et al., 2011; Kim et al., 2012; Zaitsev et al., 2015). Memantine showed an anticonvulsant effect both in a penicillin-induced epilepsy model and in Krushinsky–Molodkina rats with audiogenic seizures (Cakil et al., 2011; Kim et al., 2012). Memantine was also effective on the tonic component of seizures in a PTZ kindling model, and it prevented neuronal death (Zaitsev et al., 2015). Moreover, memantine showed anticonvulsant effects in WAG/Ola/Hsd rats, which are thought to be a model of genetic absence epilepsy (Frey and Voits, 1991), but there is no sufficient information about this rat substrain. In agreement with these studies, memantine has an anticonvulsant effect on absence epilepsy in WAG/Rij rats, which was used in the present study. Recent studies have shown that the initial focal point of absence epilepsy is in the perioral area of the somatosensory cortex and that the cortex is present in NMDA receptor-mediated glutamatergic pyramidal neurons and is present in layers V and VI (Miras-Portugal et al., 2003; Scicchitano et al., 2015; Russo et al., 2016). Thus, it can be concluded that seizures can be prevented without initially entering the thalamo-cortical circuit by decreasing the activity in this region in the presence of memantine.

As an NMDA receptor blocker, memantine has an antioxidant effect by blocking calcium entry, affecting intracellular signaling pathways (Flores et al., 2011; Annweiler and Beauchet, 2012; Gubandru et al., 2013; Lee et al., 2018). Memantine decreased the level of MDA, which is the final product of polyunsaturated fatty acid peroxidation, in all brain tissue samples and decreased GSH and GR levels but increased CAT levels in the cerebrum in this study.

Functional Interaction Between P2X7Rs and NMDA Receptors

Pre-treatment of organotypic hippocampal slice cultures with ATP reduced NMDA-induced neuronal death in microglia, suggesting microglia-mediated neuroprotection depends on P2X7Rs (Masuch et al., 2016). The glutamatergic agonists NMDA and AMPA increased the BzATP-induced current amplitudes in organotypic hippocampal slice cultures (Khan et al., 2018). Intravitreal injection of A-438079 and BBG significantly reduced NMDA-induced cell loss in the retinae of male Wistar rats, suggesting a strong link between P2X7R and NMDA (Sakamoto et al., 2015). Activation of the P2X7R has been shown to trigger the release of glutamate from neurons and astrocytic cells by vesicular and non-vesicular pathways (Sperlagh et al., 2002; Cho et al., 2010). Stimulation of NMDA receptors also increases ATP release (Cho et al., 2010; Engel et al., 2012a,b). In addition, P2X7Rs have been observed on presynaptic in glutamatergic pyramidal neurons (Sperlagh et al., 2002; Miras-Portugal et al., 2003), suggesting a P2X7-NMDA receptors interaction in pyramidal cells located in the perioral region of the somatosensory cortex, which is considered to be the initial focal point of absence epilepsy. In addition, both receptors are known to increase the intracellular calcium level. Thus, the interaction between P2X7 and NMDA receptors may increase excitability in the brain and may cause neurotoxicity by increasing ROS (Carrasco et al., 2018). In a phencyclidine-induced schizophrenia model, prefrontocortical postsynaptic NMDA currents slightly decreased due to both genetic deletion (P2X7R -/-) and pharmacological blockade with JNJ-47965567 (Kovanyi et al., 2016). However, NMDA currents were not affected in either wild type or P2X7R-deficient mice in *in situ* cortical astroglia (Oliveira et al., 2011). In contrast, electrophysiological recordings revealed that stimulation of the P2X7R with BzATP did not reverse memantine's anticonvulsant effect; the anticonvulsant activity of memantine was maintained in the presence of BzATP, but it neutralized the level of MDA. The administration of A-438079 reduced the anticonvulsant activity of memantine, but the net effect remained anticonvulsive in this study. This finding might have been due to increased glutamate through BzATP activity, leading to cytotoxic effects through other glutamate receptors, such as AMPA and kainate (Coyle and Puttfarcken, 1993; Quincozes-Santos et al., 2014). There is evidence that some intracellular pathways affect the interaction of the two receptors. They both affect phosphokinase C (Ortega et al., 2010), pannexin-1 (Bravo et al., 2015), and the MAP kinase pathway (Gandelman et al., 2013; Fabbrizio et al., 2017; Lee et al., 2018). Therefore, it appears that this effect is due to the possible interaction of P2X7 and NMDA receptors in intracellular pathways. In addition, A-438079 and memantine decreased MDA levels in all tissues and changed antioxidant parameters similar to the memantine group in the present study, suggesting a strong interaction between the P2X7R and NMDA receptors in the brainstem.

CONCLUSION

Electrophysiological data from the present study suggest that P2X7Rs are ineffective for absence epilepsy whereas a biochemical analysis revealed a partial interaction between P2X7 and NMDA receptors in WAG/Rij rats with absence epilepsy. It seems logical to expect this interaction since P2X7R and the NMDA receptor both allow calcium influx. P2X7 and NMDA receptors use common intracellular signal pathways, but this interaction cannot be limited to calcium influx in epilepsy. Many other systems and receptors are involved in calcium influx without P2X7 or NMDA receptors and contribute to epileptogenesis. Besides, the P2X7R has been linked to inflammatory mediators in neurological diseases. Therefore, further studies are required to determine the level of inflammatory mediators and their localization in absence epilepsy.

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 Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2015/56).

AUTHOR CONTRIBUTIONS

EA planned and supervised all experiments. ED, HA, GA, and MA conducted the experiments. BA and ER provided biochemical measurements. ED, HA, GA, and BA analyzed the data. EA wrote the manuscript.

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MINI REVIEW
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Evidence of a Role for the TRPC Subfamily in Mediating Oxidative Stress in Parkinson's Disease

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Parkinson's disease (PD) represents one of the most common multifactorial neurodegenerative disorders affecting the elderly population. It is associated with the aggregation of α-synuclein protein and the loss of dopaminergic neurons in the substantia nigra pars compacta of the brain. The disease is mainly represented by motor symptoms, such as resting tremors, postural instability, rigidity, and bradykinesia, that develop slowly over time. Parkinson's disease can also manifest as disturbances in non-motor functions. Although the pathology of PD has not yet been fully understood, it has been suggested that the disruption of the cellular redox status may contribute to cellular oxidative stress and, thus, to cell death. The generation of reactive oxygen species and reactive nitrogen intermediates, as well as the dysfunction of dopamine metabolism, play important roles in the degeneration of dopaminergic neurons. In this context, the transient receptor potential channel canonical (TRPC) sub-family plays an important role in neuronal degeneration. Additionally, PD gene products, including DJ-1, SNCA, UCH-L1, PINK-1, and Parkin, also interfere with mitochondrial function leading to reactive oxygen species production and dopaminergic neuronal vulnerability to oxidative stress. Herein, we discuss the interplay between these various biochemical and molecular events that ultimately lead to dopaminergic signaling disruption, highlighting the recently identified roles of TRPC in PD.

Keywords: TRPC channels, Parkinson's disease, oxidative stress, dopamine release, neuronal apoptosis

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INTRODUCTION

Neurological disorders continue to increase in tandem with longer lifespans in populations, with aging remaining the biggest risk factor for developing neurodegenerative diseases. Parkinson's disease (PD) is one of the most common multifactorial neurodegenerative disorders. Indeed, it affects approximately 2% of the elderly population and 4% of individuals aged over 80 years (Berman and Nichols, 2019).

Disease onset usually occurs at the age of 65–70 years (Marino et al., 2019). However, its pathological changes can be observed as early as 20 years prior to the appearance of motor symptoms and include unspecific signs such as fatigue, hyposmia, and constipation (Hawkes et al., 2010). Motor symptoms develop slowly over time and are the main clinical characteristics of PD. These include dysfunctions of the somatomotor system such as resting tremors, rigidity,

bradykinesia, and postural instability (Schapira et al., 2017). In turn, there is a progressive physical limitation, in addition to impairments in non-motor functions such as neuropsychiatric (sleep disorders, depression, and dementia) and autonomic symptoms (bladder and gastrointestinal alterations) (Sakakibara et al., 2012; Fasano et al., 2015).

The pathogenesis of PD is not completely understood. However, different studies have contributed to the dissection and determination of some of the mechanisms involved in its establishment and progression. Classically, the progressive neurodegeneration in PD is associated with the aggregation of α-synuclein, a small lipid-binding protein, into structures called Lewy bodies in the substantia nigra pars compacta (SNpc).

Accumulation of dopamine (DA) and DA products has also been pointed as a potential mechanism involved in neuronal death (Mullin and Schapira, 2015). Indeed, the neurotoxic effects of the endogenous DA derivative *N*-methyl-(*R*)-salsolinol (NMSAL) (Naoia et al., 2002) was shown to induce oxidative stress and decrease the levels of reduced glutathione (GSH) in dopaminergic SH-SY5Y cells (Wanpen et al., 2004). The progressive loss of DA neurons leads to a subsequent reduction of DA levels. All these alterations contribute to an abnormal neuronal functioning, and thus, to motor deficiency and worsening of the quality of life of patients at advanced stages of PD (Magrinelli et al., 2016).

For instance, many studies have provided substantial evidence of the role of neuroinflammation (Tansey and Goldberg, 2010), mitochondrial dysfunction (Park et al., 2018, 2019), and oxidative and nitrosative stresses in PD (Puspita et al., 2017). In this context, disruption of neuronal calcium ion (Ca^{2+}) homeostasis in the central nervous system plays a critical role in the cascade of events that culminates in the degeneration of dopaminergic neurons (Zaichick et al., 2017). Also, a correlation between reactive oxygen species (ROS) production and Ca^{2+} channel activation has already been explored (Görlach et al., 2015).

Recent studies have focused in the identification of a link between Ca^{2+}-mediated signaling and neuroinflammation (Sama and Norris, 2013). It observed an association between neurodegeneration, mitochondrial dysfunction, and, oxidative and nitrosative stresses (Celsia et al., 2009). This evidence points to a role for transient receptor potential channels (TRP) in PD (Takahashi and Mori, 2011).

First discovered in *Drosophila melanogaster* as key molecules in phototransduction, the TRP channels comprise a family of non-selective cation channels that are widely expressed on mammalian cells, including neurons and different types of non-neuronal cells. They are distributed in six different subfamilies: ankyrin (TRPA1), canonical (TRPC1-7), melastatin (TRPM1-8), mucolipin (TRPML1-3), polycystin (TRPP1-3), and vanilloid (TRPV1-6). Their broad tissue expression confers them the ability to influence different pathologies and physiological states. In this context, it is now known that these channels participate in the development and maintenance of inflammation and pain, are important sensors of molecules such as lipids and ROS, and are involved in thermoregulation, tissue remodeling, and neuronal plasticity, among other responses.

OXIDATIVE AND NITROSATIVE STRESSES IN PARKINSON'S DISEASE

Reactive oxygen species and reactive nitrogen intermediates (RNIs) are natural byproducts necessary for cellular homeostasis (Liguori et al., 2018) (**Figure 1**). ROS are formed during metabolic redox reactions and include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl (•OH), and superoxide (O_2•−) radicals (Sies et al., 2017). RNIs are produced in neuronal cells from arginine by the neuronal nitric oxide synthase (nNOS) and include nitric oxide (NO•), nitrite (NO_2), and *S*-nitrosothiols and peroxynitrite (OONO−) (Adams et al., 2015).

Excessive ROS and RNI formation during oxidative and nitrosative stresses results in a variety of detrimental effects in the cell, thus, contributing to organelle and membrane structural damages and cellular apoptosis (Guo et al., 2018). This cytotoxic environment has been recognized as a common underlying phenomenon in the dopaminergic neurodegenerative process (Dias et al., 2013). Indeed, an irregular oxidation of macromolecules, such as lipids, proteins, and nucleic acids, was observed in the brain tissues of PD patients (Bosco et al., 2006; Nakabeppu et al., 2007). Also, higher levels of the oxidative stress markers 8-OhdG (8-Oxo-2′-deoxyguanosine) and malondialdehyde, in addition to NO_2, were detected in the peripheral blood of PD patients in comparison with healthy individuals (Wei et al., 2018). The same patients presented systemic down-regulation of the antioxidant proteins glutathione and catalase (CAT).

In addition, major genetic insights indicate that specific mutations in a series of primary genes that are responsible for PD-related synucleopathy and the regulation of mitochondrial and ROS equilibrium can disrupt cellular homeostasis (Cacabelos, 2017). For instance, an elevated expression of the wα-synuclein protein and oxidative stress genes [*HSPB1*, Heat Shock Protein Family B (Small) Member 1; *NOX1*, NADPH oxidase 1; and *MAOB*, Monoamine oxidase B] was observed in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons (Nguyen et al., 2011). Similarly, *iPSC* midbrain dopaminergic neurons from patients with PTEN-induced putative kinase 1 (*PINK1*) or *Parkin* mutations presented abnormal mitochondria (Chung et al., 2016) (**Figure 1**).

Accordingly, evidence suggests that in PD, the mitochondrion represents the primary source of ROS, contributing to intracellular oxidative stress and therefore, to the vulnerability of dopaminergic neurons to apoptosis (Beal, 2005). Moreover, knockout mice for Dynamin-1-like protein (*Drp1*), a guanosine triphosphate (GTP)ase that regulates mitochondrial fission, exhibited degeneration of nigrostriatal dopaminergic neurons (Berthet et al., 2014). This response was associated with a reduced mitochondrial mass in axons, which was associated with impaired mitochondrial dynamics denoted by the loss of coordination of mitochondrial movements.

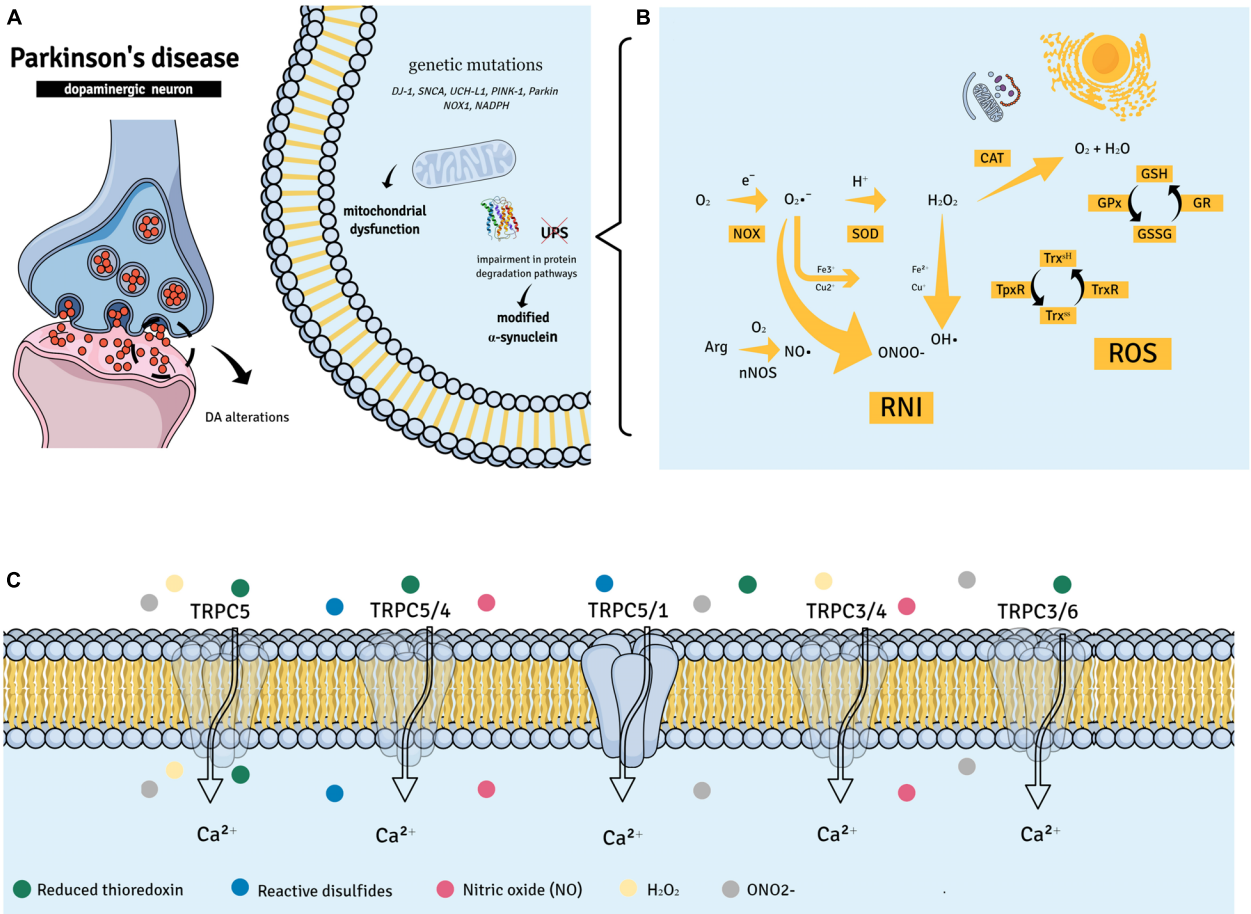

FIGURE 1 | Parkinson's disease (PD) suggested pathways. **(A,B)** PD has been associated with the aggregation of α-synuclein into *Lewy* bodies in dopaminergic neurons of the substantia nigra pars compacta. Other factors such as gene mutations (DJ-1, SNCA, UCH-L1, PINK-1, and Parkin) may contribute to mitochondrial dysfunction and neuronal death in PD. The accumulation of dopamine (DA) and its products in DA neurons may also be a causative factor of neuronal death. This may lead to mitochondrial dysfunction, changes in protein degradation [by impairing the ubiquitin-proteasome system (UPS) function], and increased generation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNIs). **(C)** Members of the transient receptor potential canonical (TRPC) subfamily of non-selective Ca^{2+} channels are able to recognize ROS and RNIs and have been implicated in neuronal survival; in fact, different oxidative/nitrosative stress products can directly activate TRPC complexes.

Additionally, disruption of respiratory chain complexes, especially the mitochondrial complex I (NADH-quinone oxidoreductase), was implicated in the enhanced production of ROS in PD (Ryan et al., 2015). Human studies also indicated that the dysfunction of this specific complex occurs in the SNpc of PD patients (Schapira et al., 1990). Of note, mitochondrial integrity in SNpc neurons was found to be dependent on Parkin expression (Park et al., 2006; Stichel et al., 2007).

In regard to RNIs, the excessive or inappropriate generation of NO and O_2•−-derived reactive species, plays a critical role in mediating the neurotoxicity associated with mitochondrial damage (Kaludercic and Giorgio, 2016). The reaction between NO and O_2•− represents an important source of OONO−, a highly reactive molecule for a broad range of chemical targets that potently inhibits mitochondrial proteins. OONO− overproduction was found to enhance the levels of oxidized lipids and DNA in the dopaminergic neurons of PD patients (Ebadi and Sharma, 2003). Depletion of antioxidant defenses, including GSH, was also observed in the same samples (Franco and Cidlowski, 2009). Interestingly, nNOS- and inducible NO synthase (iNOS)-dependent NO levels were increased in the SNpc of PD patients (Hancock et al., 2008). Also, high levels of NO and OONO− correlated with a worse prognosis in PD (Kouti et al., 2013), corroborating the hypothesis that both RNI and ROS generation may strongly contribute to neurodegeneration in PD.

Antioxidant proteins such as superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx), and GSH counteract excessive ROS production. Therefore, reductions in their activities and/or expression may favor lipid peroxidation or promote neuronal excitotoxicity with subsequent protein modifications and eventual neuronal death (Deponte, 2013; Patlevič et al., 2016). Interestingly, evident differences were

found in the levels of GSH of post-mortem brain samples of PD patients in comparison with other brain regions (Perry et al., 1982; Sian et al., 1994). Also, animal studies revealed that down-regulation of GSH synthesis results in a progressive degeneration of nigrostriatal dopaminergic neurons (Garrido et al., 2011).

By using agonists and antagonists, knockout mice and cells, and a diverse range of molecular biology techniques, several roles have been suggested for the TRPC subfamily. These include their importance as sensors of molecules involved in oxidative and nitrosative stresses (**Figure 1**) known to influence neuronal survival and function (Chen et al., 2009; Delgado-Camprubi et al., 2017).

TRANSIENT RECEPTOR POTENTIAL CHANNELS AND THE CANONICAL SUBFAMILY

In humans, the TRPC subfamily is formed by six channels (TRPC1 and TRPC3-7), which are considered the mammalian TRPs most closely related to those of *D. melanogaster*. TRPC channels are formed by four subunits and each subunit has six transmembrane domains and a pore region between the fifth and the sixth transmembrane domain (Feng, 2017).

TRPCs assemble into tetramers to form functional channels. Each monomer consists of a transmembrane domain and a cytosolic domain (Li et al., 2019). The cytosolic domain contains the N- and C-terminal subdomains. The N-terminal is composed of four ankyrin repeats and linker helices, whilst the C-terminal is formed by a connecting helix and a coiled-coil domain (Li et al., 2019). All TRPC channels contain the calmodulin and inositol trisphosphate (IP_3) receptor-binding motif, which is able to interact with phosphoinositides, inositol polyphosphates, Gαi/o proteins, and SEC14 domain and spectrin repeat-containing protein 1 (SESTD1), a Ca^{2+}-dependent phospholipid/cytoskeleton-binding protein (Wang et al., 2020). These different interacting pathways may influence TRPC functions.

Distributed in two subgroups, diacylglycerol (DAG)-activated (TRPC3/6/7) and non-DAG-activated receptors (TRPC1/4/5), TRPC channels can form homo- and heterotetramers (Strübing et al., 2001; Zagranichnaya et al., 2005; Poteser et al., 2006; Woo et al., 2014; Myeong et al., 2016; Bröker-Lai et al., 2017; Sunggip et al., 2018; Ko et al., 2019). Their assembly in these complexes may vary with their expression sites and functions. Additionally, members of the TRPC subfamily, such as TRPC1, can also form heterotetramers with channels of other TRP subfamilies, including TRPV4 and TRPP2 (Kobori et al., 2009; Greenberg et al., 2017). Despite the advances in elucidating the structure and assembly of TRPCs, the definite functions of their homo- and heterotetramers remain unclear and represent a whole new avenue of knowledge to be pursued.

So far, different roles have been identified for TRPC channels including in cardiovascular, lung, kidney and neurological diseases, inflammation, and cancer, among others. Of importance to our review, TRPCs are involved in neurotransmission, neural development, excitotoxicity, and neurodegeneration (Wang et al., 2020). Interestingly, TRPC channels, especially TRPC1, have topped the list of molecules involved in store-operated Ca^{2+} entry. However, it is now well-established that their importance goes beyond the endoplasmic reticulum Ca^{2+} store (Wang et al., 2020). Herein, we will focus on the importance of TRPC channels as oxidative and nitrosative sensors in PD.

In regard to oxidative stress, TRPC5 is perhaps the most well investigated member of the TRPC subfamily. It can be activated by both oxidant and antioxidant molecules such as H_2O_2 and reduced thioredoxin, respectively (Yoshida et al., 2006; Xu et al., 2008; Naylor et al., 2011). TRPC5 can be also activated by NO and reactive disulfides (Yoshida et al., 2006; Maddox et al., 2018). However, TRPC5 sensitivity to NO has been argued by other studies (Xu et al., 2008; Wong et al., 2010), indicating this response may vary with cell type, generated NO concentrations, and other experimental conditions. Interestingly, TRPC5/TRPC4 complexes were found to be involved in the regulation of Ca^{2+}-dependent production of NO by endothelial cells (Yoshida et al., 2006). TRPC5-dependent NO generation via endothelial NOS (eNOS) activation was later confirmed (Sunggip et al., 2018).

Another interesting finding is the ability of oxidant products such as OONO− to up-regulate both the mRNA and protein expressions of TRPC6 and TRPC3 in monocytes. Of note, OONO−-induced Ca^{2+} influx in these cells is reversed by the TRPC channel blocker 2-APB (Wuensch et al., 2010). Additionally, TRPC3/TRPC4 assembly forms redox-sensitive complexes on endothelial cells (Poteser et al., 2006). Adding another layer of complexity to TRPC roles in oxidative/nitrosative stresses, it is important to highlight that these channels do not only form complexes but are also able to down-regulate each other's' responses. Indeed, TRPC3/TRPC6-mediated Ca^{2+} influx can be down-regulated by activation of the TRPC5-NO axis (Sunggip et al., 2018).

Evidence also indicates that TRPC1 negatively regulates TRPC5-mediated Ca^{2+} influx in striatal neurons undergoing oxidative stress (Hong et al., 2015). Interestingly, TRPC1/TRPC5 complexes have been shown to mediate the protective effects of reduced thioredoxin in inflammation, therefore acting as a target for this antioxidant molecule (Xu et al., 2008).

Importantly, TRPCs are highly expressed in various regions of the brain in which they play different roles (**Table 1**). Thus, due to their ability to sense and modulate oxidative/nitrosative stress responses, they should be considered as potential mediators of neuroinflammation. Therefore, the importance of TRPC channels in PD will now be discussed.

TRPC CANONICAL CHANNELS IN PARKINSON'S DISEASE

Reports of the contribution of TRPC channels in PD are relatively new and we have not yet uncovered their definite roles in disease progression and maintenance. Also, few studies have attempted to link their expression and/or activation with the ongoing oxidative and nitrosative stresses that occur in PD.

TABLE 1 | Neuronal expression and functions of TRPC channels.

Receptor	Animal species/strains/cell lines	Expression site	Possible roles/effects following activation	References
TRPC1	Sprague-Dawley rats	Telencephalon	Renewal of neural stem cells	Fiorio Pla et al., 2005;
	Wistar rats	cerebellum, and midbrain cortical pyramidal and SNpc neurons	Modulation of neuronal firing somato-dendritic release of dopamine following activation of mGluR and synaptic plasticity	Martorana et al., 2006; Valero et al., 2015; Martinez-Galan et al., 2018
	C57BL/6J mice	Hippocampal neural progenitor cells and neurons	Mediation of store-operated Ca^{2+} entry and neuronal cell differentiation and mediation of glutamate-induced cell death	Narayanan et al., 2008; Li et al., 2012
	SH-SY5Y cells and TRPC1 wild type and knockout mice (C57BL/6J background)	Neuroblastoma cells and mouse DA neurons from SNpc	Increased cell survival	Selvaraj et al., 2012
	Human	Brain cortical lesions from epilepsy patients and healthy tissues,	Mediation of astrocyte-induced epilepsy	Zang et al., 2015
	Cell line	D54 human glioma cells, H19-7 hippocampal neurons, PC12 cells	Store-operated Ca^{2+} entry and activation of Cl^{-} channels, differentiation of hippocampal neuronal cells, stimulation of neurite outgrowth and down-regulation of TRPC5-mediated responses	Wu et al., 2004; Heo et al., 2012; Cuddapah et al., 2013
TRPC3	Sprague-Dawley rats	Cerebellum, striatal cholinergic interneurons, striatal cholinergic interneurons, cortical neurons	Increased neuronal survival, modulation of the tonic activity of striatal cholinergic interneurons following activation of mGluR1/5, neuronal depolarization via interaction with dopamine receptors, mediation of low calcium and magnesium-induced depolarization, epileptiform activity, and redox-signaling	Berg et al., 2007; Jia et al., 2007; Roedding et al., 2013; Xie and Zhou, 2014; Zhou and Roper, 2014
	Wistar rats	Hippocampus	Integrity of the neuronal morphology, synaptic plasticity and cognition	Qin et al., 2015
	Balb/c	Prefrontal cortex	Depression-like behavior	Buran et al., 2017
	Wild type and *Mwk* mice	Cerebellum	Regulation of Purkinje cell development and survival, and synaptic plasticity	Becker et al., 2009; Dulneva et al., 2015
	C57Bl6J/SJL, and TRPC3 wild type and knockout (Sv129 background)	Hippocampus	Decrease in neuronal excitability, and early-onset memory deficits	Neuner et al., 2015
	Human	Cerebellar Purkinje neurons	Downstream signaling to mGluR activation; contribution of the TRPC3c isoform to focal ischaemic brain injury	Cederholm et al., 2019
	Cell line	H19-7 hippocampal neurons	Differentiation of hippocampal neuronal cells via store-operated calcium entry	Wu et al., 2004
TRPC4	TRPC4 wild type and knockout rats	Dopamine neurons	Dopaminergic activity and cocaine addition	Klipec et al., 2016
	C57BL/6 mice	Hippocampus, cortex, olfactory bulb, lateral septum, coronal brain slices, and prefrontal cortex	Neuronal development, anxiety, and depression	Zechel et al., 2007; Yang et al., 2015; Just et al., 2018

(Continued)

TABLE 1 | Continued

Receptor	Animal species/strains/cell lines	Expression site	Possible roles/effects following activation	References
	Gonadotropin-releasing hormone (GnRH) transgenic mice	GnRH neurons from the pre-optic area	Sustained excitation of GnRH neurons and gonadotropin release	Zhang et al., 2013
	TRPC4 wild type and knockout mice (mixed background)	Amygdala, hippocampus, lateral septum, and hippocampus	Innate fear responses, downstream signaling to mGluR activation, seizure-induced excitotoxicity and neurodegeneration	Phelan et al., 2012; Riccio et al., 2014
	BL/6 P0 mice	Hippocampal neurons	Inhibition of neurite outgrowth	Jeon et al., 2013
	Human	Brain cortical lesions from epilepsy patients and healthy tissues	Seizure events	Wang et al., 2017
	Cell line	PC12 cells	Exocytosis in neuroendocrine cells	Obukhov and Nowycky, 2002
TRPC5	Sprague-Dawley rats	Pyramidal and hippocampal neurons	Seizure events, inhibition of dendritic development	Tai et al., 2011; He et al., 2012
	C57BL/6 mice	Coronal brain slices, cerebellar granular neurons, hippocampus, prefrontal cortex and retinal ganglion cells	Anxiety and depression, neuronal regeneration, retinal ganglion cell death	Yang et al., 2015; Wu et al., 2016; Just et al., 2018; Oda et al., 2019
	TRPC5 wild type and knockout mice (129/SvImJ background)	Cortical neurons	Oxidative stress-induced neuronal cell death	Park et al., 2019
	YAC128 mutant Huntington's disease transgenic mice	Striatal cells	Oxidative stress-induced neuronal damage	Hong et al., 2015
	TRPC5 wild type and knockout mice (C57BL/6 and 129/SvImJ mixed background)	Hippocampus and amygdala	Fear-related responses	Riccio et al., 2009
	Human	Brain cortical lesions from epilepsy patients and healthy tissues	Seizure events	Xu et al., 2015
	Cell line	E18 hippocampal neurons, PC12 cells, NG108-15 neuroblastoma/glioma hybrid cells	Axon formation, neuronal development and plasticity, growth cone morphology and motility, neuronal regeneration	Greka et al., 2003; Wu et al., 2007; Davare et al., 2009; Wu et al., 2016
TRPC6	Sprague-Dawley rats	Cerebellum and substantia nigra	Neuronal survival, downstream signaling to mGluR activation	Giampà et al., 2007; Jia et al., 2007
	C57BL/6J mice TRPC6 wild type and over-expressing mice Cell line	Hippocampus E18 hippocampal neurons	Neuronal survival Synaptic and behavioral plasticity Dendritic growth	Kunert-Keil et al., 2006; Tai et al., 2008; Zhou et al., 2008; Boisseau et al., 2009; Du et al., 2010; Lin et al., 2013; Yao et al., 2013
TRPC7	Sprague-Dawley rats	Cholinergic interneurons, substantia nigra, subthalamic nucleus neurons	Downstream signaling to striatal mGluR1/5 receptors and NMDA-induced depolarization-activated inward current and firing	Zhu et al., 2005; Berg et al., 2007

TRPC1 is the most well investigated member of the canonical subfamily in PD. A study in SH-SY5Y cells demonstrated that TRPC1 protein expression becomes down-regulated in these cells following incubation with salsolinol (Bollimuntha et al., 2006), a neurotoxin endogenously found in the nigrostriatal cells and cerebrospinal fluid samples of patients with PD (Moser et al., 1995; Maruyama et al., 1996). Despite its low expression on the cell membrane, the TRPC1 protein was detected in the cytosol (Bollimuntha et al., 2006). This result suggests that salsolinol may cause TRPC1 translocation from the neuronal cell membrane to the cytoplasm.

Interestingly, the endogenous salsolinol derivative NMSAL was detected in the nigrostriatum and intraventricular fluid samples of patients with PD (Maruyama et al., 1996). NMSAL induces neuronal apoptosis via mitochondrial and caspase-3-dependent pathways (Akao et al., 1999; Maruyama et al., 2001; Arshad et al., 2014) and it is considered to be far more toxic to neurons than salsolinol (Maruyama et al., 1996). NMSAL exhibited similar effects to those of salsolinol in neuronal TRPC1 expression and localization (Arshad et al., 2014). All this evidence indicates a protective role for TRPC1 in PD.

Ca^{2+}-induced ROS generation in cultured rat DA neurons treated with the neurotoxin 1-methyl-4-phenylpyridinium ion (MPP^+) was also linked to TRPC1 (Chen et al., 2013). Another study by Selvaraj et al. (2009) showed that 1-methyl-4-phenyl-1, 2,3,6-tetrahyrdro-pyridine (MPTP), a compound known to cause PD in mice by inducing mitochondrial dysfunction and neuronal apoptosis, reduces the expression of TRPC1 in the SNpc. A similar result was observed in PC12 cells incubated with MPP^+. The same study also found that TRPC1 over-expression increases the survival of PC12 cells incubated with MPP^+ by preserving mitochondrial membrane potential and regulating the expression of the anti-apoptotic genes Bcl2 and Bcl-xl (Selvaraj et al., 2009). Of note, the authors highlighted in their study that TRPC1 over-expression only partly restores mitochondrial membrane potential and neuronal survival.

The contribution of other TRPCs to PD has also been investigated. Analysis of TRPC3 expression patterns revealed that the TRPC3 protein is increased in the SNpc following exposure to MPTP (Selvaraj et al., 2009). On the other hand, no alterations in TRPC3 levels were noted in DA neurons from PD patients (Sun et al., 2017). Of note, these controversial data on TRPC3 expression have been obtained in different experimental settings. Therefore, TRPC3's role in PD cannot be overruled. Also, it is possible that other TRPC channels and their complexes may contribute to changes in neuronal survival in PD.

In this context, it is important to highlight the complexes formed by TRPC1 with TRPC5. Although no studies have yet investigated these complexes in PD, they have been pointed as mediators of other neurodegenerative diseases such as Huntington's. In a recent report, it was demonstrated that intracellular oxidized glutathione activates TRPC5 in striatal cells of Huntington's disease (Q111 cells). The same study showed that upon oxidative stress, TRPC5-mediated Ca^{2+} influx leads to increased cytosolic Ca^{2+} levels and activation of the calpain-caspase pathway, leading to apoptosis of striatal neurons (Hong et al., 2015). In parallel, as observed for PD, TRPC1 protein and mRNA expression is down-regulated in Huntington's striatal cells favoring the formation of TRPC5 heterotetramers in these cells (Hong et al., 2015). These results reinforce the protective role of TRPC1 in neurodegenerative diseases and shed light on the deleterious importance of TRPC5 in neuronal survival.

From the best of our knowledge, no studies have yet investigated the association between TRPC channels and RNI in PD, highlighting the need for further studies to fill this gap of information.

FUTURE PERSPECTIVES

Herein, we presented evidence and discussed the importance of TRPC channels in the recognition and regulation of oxidative and nitrosative stress responses, as well as their contributions to PD. The recent advances in the field of TRPC channels, in particular the protective functions of TRPC1 and the deleterious role of TRPC5 in PD, highlight their importance as pharmacological targets in treating neurodegenerative diseases. Considering the ability of TRPC channels to assemble as homo- and heterotetramers with channels of the same subfamily and also as members of other subfamilies of TRPs, and the lack of antagonists and agonists capable of selectively differentiating the individual actions of each one of these channels, their targeting of PD may become a difficult task. Therefore, efforts need to be made in order to develop effective and more selective pharmacological tools to investigate TRPC channels. This will be an essential step to achieve a broader knowledge of the pathophysiological roles of their different assembly modes and establish their definite importance in PD.

AUTHOR CONTRIBUTIONS

DM-F, NO, LS, and EF contributed to the conception and design and drafted and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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TRPV1-Estradiol Stereospecific Relationship Underlies Cell Survival in Oxidative Cell Death

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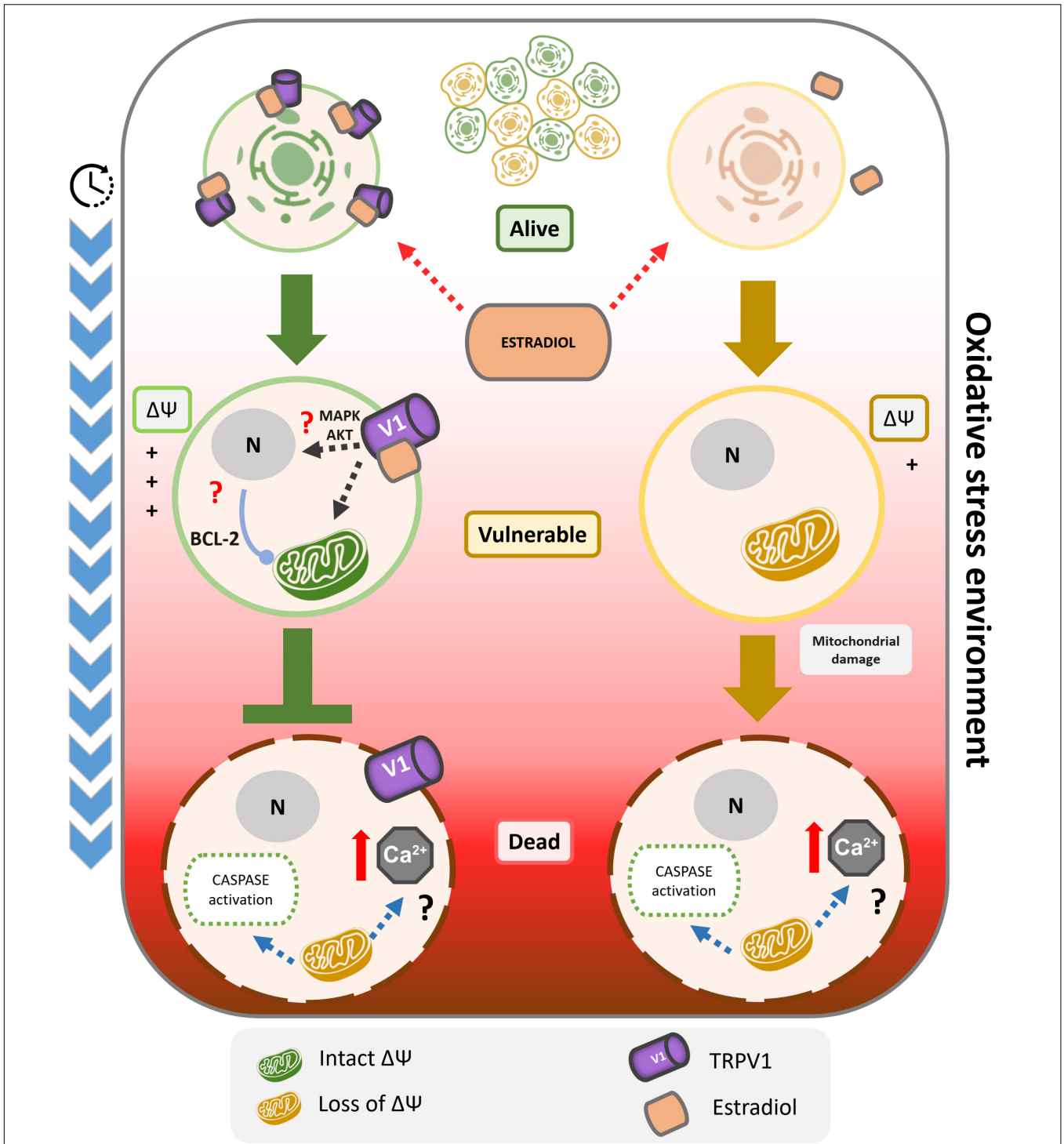
17β-estradiol is a neuronal survival factor against oxidative stress that triggers its protective effect even in the absence of classical estrogen receptors. The polymodal transient receptor potential vanilloid subtype 1 (TRPV1) channel has been proposed as a steroid receptor implied in tissue protection against oxidative damage. We show here that TRPV1 is sufficient condition for 17β-estradiol to enhance metabolic performance in injured cells. Specifically, in TRPV1 expressing cells, the application of 17β-estradiol within the first 3 h avoided H_2O_2-dependent mitochondrial depolarization and the activation of caspase 3/7 protecting against the irreversible damage triggered by H_2O_2. Furthermore, 17β-estradiol potentiates TRPV1 single channel activity associated with an increased open probability. This effect was not observed after the application of 17α-estradiol. We explored the TRPV1-Estrogen relationship also in primary culture of hippocampal-derived neurons and observed that 17β-estradiol cell protection against H_2O_2-induced damage was independent of estrogen receptors pathway activation, membrane started and stereospecific. These results support the role of TRPV1 as a 17β-estradiol-activated ionotropic membrane receptor coupling with mitochondrial function and cell survival.

Keywords: TRPV1, 17β-estradiol, cell death, membrane receptor, neuroprotection

Abbreviations: 17α-E_2/αE_2, 17α-Estradiol, 17β-E_2/βE_2, 17β-Estradiol, 17β-E_2-BSA/βE_2-BSA, 17β-Estradiol conjugated with Bovine Serum Albumin, AVD, Alive-Vulnerable-Dead, CAP, Capsaicin, CPZ, Capsazepine, DMEM, Dulbecco modified Eagle medium, ERα, Estrogen receptor α, ERβ, Estrogen receptor β, FACS, Fluorescence activated cell sorting, FBS, Fetal bovine serum, H_2O_2, Hydrogen Peroxide, HP/HeLa-p, Parental HeLa cells, mRNA, Messenger RNA, NT, Non-treated, PI, Propidium Iodide, Q-PCR, Quantitative PCR, Rhod123, Rhodamine 123, ROS, Reactive oxygen species, RT-PCR, Reverse transcription PCR, shRNA, Short RNA hairpin, shRNA-SS, shRNA-Scrambled Sequence, st-TRPV1, Stable expression of TRV1, TRPV, Vanilloid transient receptor potential cannel, TMX, Tamoxifen, TUB-III, Beta Tubulin III.



GRAPHICAL ABSTRACT | TRPV1 is sufficient condition for 17β-estradiol resistance against oxidative stress-induced cell death. The cell death induced by oxidative stress could be characterized by a kinetic model of three state: alive, vulnerable and dead. In particular, the transition from alive to vulnerable state involves the depolarization of mitochondrial membrane potential, and the evolution to cell death the activation of caspases. Only in TRPV1 expressing cells, the initial application of 17β-estradiol protects against the irreversible damage triggered by oxidative stress, by inhibition of the transition from alive to vulnerable state. This mechanism includes blocking oxidative stress-dependent mitochondrial depolarization and the activation of caspases 3/7. Furthermore, 17β-estradiol potentiates TRPV1 activity associated with an increased open probability. Despite the relationship between the activation of TRPV1 and the maintenance of the mitochondrial membrane potential should be clarified, it could include the increase of the calcium buffer capacity of the mitochondria and/or the overexpression of anti-apoptotic proteins such as the Bcl-2 family as previously reported. Finally, the 17β-estradiol cell protection was independent of estrogen receptors, and was membrane started and stereospecific. These results support the role of TRPV1 as a 17β-estradiol ionotropic receptor being critical to cell survival.

INTRODUCTION

Oxidative dynamics is involved in several physiological processes, and disruption of redox control is a general pathological condition that induces cell dysfunction and death (Ghezzi et al., 2017). Oxidative stress is involved in several chronic conditions such as Parkinson's disease and Alzheimer's disease (Islam, 2017) and also in acute injuries as stroke and ischemia/reperfusion, damaging damaging several organs (Rodrigo et al., 2013). In this sense, aromatization of testosterone to estradiol is not restricted to classical endocrine tissues and has been associated with neurogenesis and tissue response to injury (Tenkorang et al., 2018; Duncan and Saldanha, 2019). Apart from classical steroid mechanism of action, a large body of evidence nowadays shows a new mechanism which appear to involve membrane-associated signaling complexes (Wu et al., 2005, 2011). Such responses could be independent or in conjunction with estrogen receptors α and β, suggesting that estrogens such as 17β-estradiol modulate neural function by direct effects on membrane receptors (Balthazart and Ball, 2006; Vega-Vela et al., 2017). These "membrane actions" of estrogen involve mainly ionic channels conduction and permeation regulation, kinase activation and transient increase of intracellular Ca^{2+}. These effects can trigger different signaling pathways that are critical for regulation of plasticity, cognition, neuroprotection and maintenance of homeostasis. Moreover, this extra-nuclear action has been shown to be critical for protection against oxidative stress-induced cell death. For instance, it has been demonstrated that estrogen-induced rapid Ca^{2+} influx, via the voltage-gated L-type Ca^{2+} channel is key to initiate the downstream Src/ERK signaling pathways leading to estrogen neuroprotection through activation of the transcription factor CREB and subsequent increase of Bcl-2 expression in hippocampal neurons (Wu et al., 2005). Also, this Ca^{2+} himself triggered by estrogen could induce an increase in mitochondrial Ca^{2+} sequestration and promote mitochondrial tolerance against glutamate excitotoxicity in hippocampal neurons.

In this context, the polymodal TRP ion channels have emerged as potential targets of membrane signaling of steroids, in particular, those of the melastatin (M) and vanilloid (V) families (Kumar et al., 2015). TRPM3 was the first described steroid-sensitive ionotropic receptor shown to be rapidly and reversibly activated by pregnenolone sulfate, inducing transient calcium influx (Wagner et al., 2008; Thiel et al., 2013). Additionally, 17β-estradiol triggers a physiological rapid intracellular calcium response, via Ca^{2+} influx through TRPV5 and TRPV6, during transepithelial Ca^{2+} transport (Irnaten et al., 2008, 2009; Kumar et al., 2017). In particular, 17β-estradiol modulates TRPV1 expression and activity in cervical afferent neurons, in dorsal root ganglion cells and hippocampus (Tong et al., 2006; Wu et al., 2010; Cho and Chaban, 2012; Pohóczky et al., 2016; Yamagata et al., 2016) controlling pain sensation and tissue viability, through fine regulation of calcium homeostasis. It has been recently demonstrated that the TRPV family located at the central nervous system (hypothalamus, hippocampus, cortex, brainstem) has estrogen receptor binding sites that are inducible by gene promoters, whose expression may be regulated by the estrous cycle (Kumar et al., 2018).

The modulation of TRP family by estrogens could be relevant due to the variety of roles that TRPs play in excitable and non-excitable cells, accounting for sensory physiology, proliferation, growth, male fertility, neuronal plasticity and regulation of oxidative stress-induced cell death. The function of TRPV family and particularly TRPV1 member in cell death merits special consideration. With a still unclear role in cell viability, TRPV1 non-selective cation channel is able to integrate physicochemical stimuli such as temperature, voltage, proton gradients and osmolarity (Caterina et al., 1997; Nishihara et al., 2011; Canul-Sánchez et al., 2018). Micromolar concentrations of the classical TRPV1-agonist capsaicin (CAP) and acid solution (pH 5.5) induce cytosolic calcium increase, ROS production, mitochondrial membrane depolarization and cell death (Hu et al., 2008). In rat cortical neurons TRPV1 activation by CAP induces apoptotic cell death via L-Type Ca^{2+} channels, generating Ca^{2+} influx, ERK phosphorylation, ROS production and caspase-3 activation (Shirakawa et al., 2008). However, similar results have been reported for CAP without TRPV1 participation, suggesting both dependent and independent effects of this vanilloid (MacHo et al., 2000). Our explanation is that the amount of CAP is able to modify the amount of calcium entry and release from inner cell stores (Ramírez-Barrantes et al., 2018).

The regulation of the channel might be critical for maintaining cellular homeostasis in oxidative environment. TRPV1 *knock-out* (*KO*) mice have estrogen sensitive tissues like testis, much more sensitive to cell death by oxidative stress stimuli (Mizrak and van Dissel-Emiliani, 2008). Moreover, in hippocampus subjected to 10 min ischemia, CA1 neurons pre-treated with CAP are less sensitive to cell death and the effect is inhibited by the TRPV1 antagonist capsazepine (CPZ). The mechanism suggested involves a moderate increase in Ca^{2+} via TRPV1. This transient Ca^{2+} influx may induce tolerance to the subsequent calcium overload, preconditioning the response and inducing neuroprotection (Pegorini et al., 2005; Huang et al., 2017). Also, in rats, the activation of TRPV1 by CAP in substancia nigra pars compacta is able to diminish cell death triggered by MPP, via reduced activation of microglia and decrease of ROS levels (Park et al., 2012).

This paradoxical effect of TRPV1 points the importance of the channel in cell survival, choosing the activation of different responses depending on the cell context, the moment of activation, and transience of the signal.

The ability of estrogen to modulate the expression and function of TRPV1 channel may imply a specific mechanism to control cellular homeostasis. Cholesterol, pregnenolone and testosterone can inhibit TRPV1-mediated currents by different ways. However, 17β-estradiol is the only steroid able to modulate the activation of the channel during enhanced CAP-evoked current in dorsal root ganglion neurons (Chen et al., 2004) and in CAP-induced nociception (Lu et al., 2009). Although the differential role of 17β-estradiol, an aromatic steroid, in allosteric modulation of TRPV1 is unclear, aromatization seems to convert an inhibitory steroid to an excitatory one. Then, can steroids differentially modulate cell viability through TRPV1? and can be the aromatic capacity relevant for paracrine and autocrine cellular protection against oxidative cell death? Here, we show that 17β-estradiol and not testosterone or 17α-estradiol, induced

cell protection via modulation of TRPV1 activity during oxidative injury independently of estrogen receptor expression.

METHODS

Cell Culture

HeLa cells were obtained from ATCC (Manassas, VA, United States). We used culture medium DMEM (Dulbecco's Modified Eagle medium) supplemented with 10% fetal bovine serum (FBS) and 50 U/mL of penicillin-streptomycin. We incubated cells in conventional incubator at 37°C, in steam saturated 95% air, 5% CO_2 atmosphere.

TRPV1 Stable Line Construction

Transfections were performed using DNA: Transit IT-LT1 (Mirus Bio LLC, Madison, United States) at a ratio of 1:3 according to the manufacturer's protocol. Cell dishes were transfected with the pCDNA3.1-TRPV1. We selected the HeLa cells 48 h after transfection using Geneticin (Sigma-Aldrich, St. Louis, MO, United States, 800 mg/mL). Cells were maintained at this Geneticin concentration in all experiments. Time course of stable TRPV1 expression was followed by PCR once a week.

Reverse Transcription PCR (RT-PCR)

Total RNA from both parental and transfected TRPV1 cell lines was extracted with Trizol (Invitrogen, Carlsbad, CA, United States). cDNA libraries were generated by RT-PCR using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, United States). Equal amounts of RNA were used as templates in each reaction. The RT-PCR product for TRPV1 generated a 167 bp amplicon. The other targets had the following sizes: estrogen receptor α, 153 bp; estrogen receptor β, 139 bp; aromatase, 134 bp; and S16, 102 bp. All of the samples were simultaneously amplified with appropriate primers and annealing temperature. The PCR reaction was performed using the Go Taq master mix (Promega Corp., Madison, WI, United States) containing all of the reagents for the amplification reaction except for the cDNA template. The protocol consisted in denaturation at 94°C for 5 min, followed by 40 cycles of: 30 s denaturation at 94°C, 30 s annealing at 55–58°C and 30 s extension at 72°C. The final elongation was performed at 72°C for 10 min, and the samples were held at 4°C once the final PCR step was completed. The PCR products and ladder (New England Biolabs, United Kingdom) were loaded onto a 2% agarose gel (Lonza Rockland, ME, United States), electrophoresed and stained with ethidium bromide (Merck KGaA, Darmstadt, Germany).

Calcium Signal Recordings

Cell cultures were loaded with Fura-2AM (Molecular Probes, Eugene, OR, United States) for 30 min at room temperature in extracellular solution containing 130 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4 (adjusted with Tris base). The cells were then rinsed and allowed to equilibrate for 5–10 min. CAP-induced Ca^{2+} activity was recorded by epifluorescence microscopy using an Olympus IX81 microscope (Olympus, Japan) equipped with dual-excitation wavelength with a minimum recording time of 2 s for Fura 2. The maximum resolution was obtained using objective lens Olympus Plan Apo X40 oil 1.3 NA. We calculated the concentration of cytosolic calcium from the recorded fluorescence intensity using the following equation:

$$[Ca^{2+}] = Kd \times \left[\frac{R - R_{\min}}{R_{\max} - R}\right] \times \frac{Sf}{Sb}$$

where Kd is the Fura 2 dissociation constant at 37°C (224 nM), R is the ratio of fluorescence measured at 340 and 380 nm, respectively, and Sf/Sb is the 380 nm ratio of fluorescence in low-calcium buffer referred to high-calcium buffer.

Animal Experimentation

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the National Institute of Health (USA) and performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ethics Committee for Animal Experimentation Committee as well as the Biosecurity Committee of the University of Valparaíso. All of the animals were handled according to approved institutional animal care and used committee protocols (BEA125-18) of the University of Valparaiso. All surgery was performed under tricaine anesthesia, and every effort was made to minimize suffering.

Heterologous Expression System

Xenopus laevis oocytes were used to measure TRPV1 currents. mMESSAGE mMACHINE from Ambion (Waltham, MA, United States) was used for *in vitro* transcription of the cRNA of wild type TRPV1 rats (GenBank™ accession no. NM031982). The oocytes were injected with 3 ng of cRNA and then incubated in ND96 solution (in mM: 96 NaCl, 2 KCl, 1.8 CaCl2, 1 $MgCl_2$, 5 HEPES, pH 7.4) at 18°C for 3–5 days before electrophysiological recordings.

Electrophysiological Recordings

Macroscopic and single channel current recordings were made employing the patch-clamp technique with the cell-attached and inside-out configurations, respectively. Symmetrical recording solutions contained: 150 mM NaCl, 10 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4. 17β-estradiol (E2) and other hormones were prepared in recording solutions at the final concentrations indicated, and perfused into the recording chamber, exchanging at least 10-times the chamber volume. Data were acquired with an Axopatch 200B amplifier (Molecular Devices), and the Clampex 10.7 acquisition software (Molecular Devices). Both the voltage command and current output were recorded at 100 kHz and filtered at 20 kHz using an 8-pole Bessel low-pass filter (Frequency Devices) and sampled with a 16-bit A/D converter (Digidata 1550B; Molecular Devices). Borosilicate capillary glasses (1B150F-4, World Precision Instruments, Sarasota, FL, United States) were pulled in a horizontal pipette puller (Sutter Instrument, Novato, CA, United States) and fire-polished with a microforge (MF-830, Narishige, Tokyo, Japan). All experiments

were performed at room temperature (20–22°C). Macroscopic current recordings were evoked by pulses of −100 to +350 mV in 20 mV increments, with pulses of decreasing duration as potential increases, followed by a step at 190 mV, to obtain the tail currents.

Cell Death Protocols

Cells were exposed to the experimental conditions in DMEM supplemented with 1% bovine serum albumin instead of FBS. H_2O_2 (Merck KGaA, Darmstadt, Germany) was added to cell cultures for 24 h in the absence or presence of the following drugs: CAP, CPZ, 17β-estradiol, 17α-estradiol (Tocris Bioscience, Bristol, United Kingdom), 17β-estradiol-BSA and testosterone (Sigma-Aldrich, St. Louis, MO, United States). After 24 h, the cultures were stained with Rhodamine 123 (Rhod 123; Invitrogen, Carlsbad, CA, United States; 100 nM) and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, United States; 10 μg/mL), JC-1 (Invitrogen, Carlsbad, CA, United States, 2 μM), or cell event caspase 3/7 (Invitrogen, Carlsbad, CA, United States).

Three State Model Evaluation by Flow Cytometry

We interpreted our results using a three-state, alive (A)-vulnerable (V)-dead (D) model. To quantify the three cellular states, mitochondrial function and plasma membrane integrity were recorded over time using Rhod 123 and PI fluorescence intensity. Mitochondrial membrane potential ($\Delta\psi$) was monitored using Rhod 123 in non-quenching mode. Rhod 123 is a fluorescent membrane-permeant cation, which passively distributes across membranes according to the membrane potential. In non-quenching mode, mitochondrial depolarization causes Rhod 123 efflux from the mitochondrial matrix into the cytosol resulting in a decrease of fluorescence intensity. Depolarized mitochondria will have lower cationic dye concentrations and lower fluorescence, while hyperpolarized mitochondria will have higher dye concentrations and fluorescence. To calibrate Rhod 123, we performed a temporal course of mitochondrial depolarization using the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 10 μM) by flow cytometry. We determined that a 15 min exposition to Rhod 123 at 0.5 μg/mL was sufficient to measure the mitochondrial depolarization in non/quenching mode and applied this strategy in each experiment. To measure the integrity of plasma membrane, we used propidium iodide (PI, 10 μg/mL), a DNA intercalating that binds to cellular DNA when plasma membrane integrity is lost. We recorded the time course of cell death in ethanol 10% to determine the maximum signal of PI. Each experiment was accompanied with an ethanol control.

The A-V-D model distinguishes three states from the fluorescence intensity of Rhod 123 and PI. The alive state corresponds to cells with high fluorescence intensity for Rhod 123 and low PI fluorescence intensity, which indicate, respectively, optimal function of mitochondrial membrane potential and impermeability of plasma membrane. Conversely, the dead state identifies cells with low Rhod 123 fluorescence intensity and high PI fluorescence intensity, indicating a fall in the mitochondrial membrane potential and the permeabilization of the plasma membrane. Finally, the vulnerable state corresponds to cells with one of these two parameters altered. Cells were measured by flow cytometry (FACScalibur, BD, Biosciences, CA, United States). We acquired a minimum of 10,000 cells in each experiment and excluded from the analysis debris and duplets. The analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, United States). To calculate the cell fraction or probability of each state, the data were normalized using the following formula:

$$X_{(A-V-D)} = \frac{n^{\circ}x}{n^{\circ}(A+V+D)}$$

Where, X is cell state fraction or probability, n∘x the number of cells in state x, and n∘(A + V + D) total number of cells.

Measurement of Mitochondrial Membrane Potential (ΔΨm)

JC-1, a sensitive fluorescent probe for ΔΨm, was used (Invitrogen, Carlsbad, CA, United States) after specific experimental procedure. Parental HeLa cells and st-TRPV1 were rinsed twice with PBS and stained with 2 μM JC-1 for 30 min at 37°C. Cells were rinsed twice with PBS and immediately analyzed by FACScalibur flow cytometer (BD, Biosciences, CA, United States). We used a 488 nm excitation filter, a 530 nm emission filter (FL1) and a 585 nm emission filter (the fluorescence 2: FL2). A logarithmic transformation was applied to the values of photomultiplier. Green fluorescence (FL1) represents the monomeric form of JC-1 corresponding to the mitochondrial mass. Red-orange fluorescence (FL2) corresponds to the J-aggregate form of JC-1. The analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, United States). Mitochondrial depolarization was indicated by an increase in the red/green fluorescence intensity ratio.

Caspases-3/7 Activity Measurement

Cell Event™ assay (Invitrogen, Carlsbad, CA, United States) was used to measure the activity of caspase-3/7 enzymes. After specific experimental procedure Parental HeLa cells and st-TRPV1 cells were collected, and the reagents were incubated for 30 min. The fluorescent intensity (at 485 nm excitation and 535 nm emission) was monitored with FACScalibur cytometer, BD, Biosciences, CA, United States) using 488 nm laser of excitation and FL1 emission filter (530/30 bp). The analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, United States).

Primary Hippocampal Cultures

Pregnant *Sprague Dawley* rats were handled under standard conditions of temperature (12 h light/dark cycle) and *ad libitum* feeding, according to the guidelines of the Animal Care Committee of the University of Valparaíso (CICUAL-UV). Hippocampi were isolated at embryonic day 18 and washed with Hanks saline solution containing in mM (135 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 0.33 Na_2HPO_4, and 5.5 D-glucose) balanced at pH 7.4 at 4°C. They were trypsinized and mechanically

disaggregated into MEM 10 (MEM, 19.4 mM D-glucose, 26 mM $NaHCO_3$, supplemented with 10% horse serum, 10 U/mL penicillin, 10 μg/mL streptomycin). The non-disintegrated tissue was centrifuged at 800 rpm for 10 s. The cell suspension was seeded in MEM 10 at a density of 40,000 cells per 12 mm diameter glass cover previously treated with poly-lysine (50 μg/mL) and kept in a humid atmosphere, saturated with 5% CO_2. After 1 h, MEM 10 was replaced by serum-free neurobasal medium supplemented with B27 and 2 mM GlutamaxTM (Invitrogen, Carlsbad, CA, United States). Cells were used at 11–14 DIV (Muñoz et al., 2011).

Immunofluorescence

Adult rats (1 month) were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffer. The brain was equilibrated in 30% sucrose solution, embedded in cryopreservant (OCT) and sectioned at 20 μm using a cryostat (Leica CM1900). Floating cuts were incubated in permeabilization/blocking buffer (0.7% Triton X-100, 0.1% sodium borohydride and 10% goat serum in PBS) overnight at 4°C. Sections were washed and incubated with primary rabbit polyclonal antibody against TRPV1 (dilution 1: 200, abcam, Cambridge, MA, United States) and with primary mouse monoclonal antibody against anti-β-Tubulin III (1: 500, Millipore) overnight at 4°C in PBS-TX (0.7% Triton X-100 and 10% goat serum in PBS). The slices were then washed and incubated for 2 h with donkey-antirabbit Alexa Fluor 546 and donkey-antimouse AlexaFluor 488 antibodies (1: 500), obtained from Molecular Probes. Hoechst® 33342 was used as nuclei marker according to the manufacturer's instructions (Molecular Probes). Images were obtained using a confocal microscope (Nikon Eclipse C180i).

For immunofluorescence in cultured neurons, we used a similar protocol to that previously described, with the exception that the cells were fixed directly by incubation with 4% PFA and 4% sucrose for 40 min and subsequently blocked in solution without borohydride.

Neuronal Viability Determined Using 4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyl Tetrazolium (MTT)

MTT assay was used to evaluate the reduction-oxidation status of living cells and mitochondrial activity, reflecting cell survival due to the formation of formazan. A density of 1×10^4 cells/well in 96-well plates was used for the MTT assay. Briefly, after treatment neurons were incubated in medium containing 500 μg/mL MTT for 3 h at 37°C. MTT medium was removed by plate inversion and 100 μL DMSO was added to each well to dissolve the formazan crystals. The plates were read using an Anthos2020 microplate reader at a wavelength of 570 nm and a reference of 690 nm.

Data Analysis

All the results are presented as mean ± S.D. from at least three independent assays for each experimental condition. Data were analyzed with Origin Pro (OriginLab Corporation Northampton, United States). We compared multiple groups with the Fisher's least significant difference procedure and ANOVA followed by the Bonferroni *post hoc* test in Statgraphics Plus 5.0 (GraphPad Software, Inc., San Diego, CA, United States). The results were considered statistically significant with $P < 0.05$. All electrophysiology data analyses were performed with Clampfit 10.7 (Molecular Devices), GraphPad Prism 6, and Excel 2013 (Microsoft, Redmont, WA, United States). Tail currents were used to build the G-V relationships, fitted with a Boltzmann function: $G = Gmax/(1\text{-}\exp^{(-zF(V-V0.5)/RT)})$, where Gmax is the maximum conductance, z is the voltage dependence of activation, $V_{0.5}$ is the half-activation voltage, T is the absolute temperature, F is the Faraday's constant and R is the universal gas constant. Gmax, $V_{0.5}$, and z were determined by using the solver complement of Microsoft Excel. Data were aligned by shifting them along the voltage axis by the mean $\Delta V_{0.5} = (-V_{0.5})$, then binning them in a range of 25 mV, between −100 mV and up to 350 mV. Statistical analysis used a two-tailed Student's *t*-test with a non-parametric *t*-test.

RESULTS

17β-Estradiol Enhanced TRPV1–Dependent Calcium Influx

In order to study the effect of 17β-estradiol in TRPV1 activity, parental cell cultures were stably transfected with pCDNA3.1-TRPV1 (st-TRPV1) and examined for TRPV1 functional expression. TRPV1 mRNA expression in st-TRPV1 was confirmed by RT-PCR that yielded a 104 bp amplicon in st-TRPV1 but not in parental cells. Immunofluorescence microscopy and flow cytometry also confirmed the expression of the protein, which was present in more than 80% of the cells (**Supplementary Figure S1**). To demonstrate that st-TRPV1 cells express a functional TRPV1, we performed functional analysis by means of the calcium imaging. st-TRPV1 showed a transient calcium influx after the exposition to 250 nM of the TRPV1 agonist CAP, followed by a decay to basal levels, even under sustained stimulation. Only st-TRPV1 cells were able to respond to several concentrations of CAP. TRPV1 activation saturated at 1 μM CAP. The dose-response CAP data were fitted using a Hill function finding an EC_{50} of 78 nM for st-TRPV1. Next, we studied the effect of 17β-estradiol in the TRPV1-dependent changes in intracellular Ca^{2+} concentration. The increase in calcium influx triggered by CAP was enhanced by 17β-estradiol (Fig. 1A) shifting the EC_{50} to 18 nM. The 17β-estradiol effect was completely inhibited by 10 μM CPZ. Additionally, st-TRPV1 incubated with 17α-estradiol failed to induce the increase of Ca^{2+} entry mediated by CAP (Fig. 1B), suggesting that estradiol enhances TRPV1-dependent intracellular Ca^{2+} concentration in a stereospecific manner.

Specificity of 17β-Estradiol-Induced Cell Death Protection

To evaluate the role of TRPV1 in estrogen protection against oxidative stress-induced cell death, we utilized as inductor

hydrogen peroxide (H_2O_2) and recorded simultaneously the mitochondrial function and plasma membrane integrity by flow cytometry applying the AVD model analysis (Ramírez-Barrantes et al., 2018). We performed a dose-response curve of H_2O_2 in both cell types and measured cell viability (**Figure 1C**). The mere expression of TRPV1 induced resistance to H_2O_2 injury at three different concentrations, but, at 1 mM H_2O_2 the cell dies independent of TRPV1 expression. The concomitant application of CAP (250 nM), however, favored the protective effect at 1 mM H_2O_2; this effect being abolished by CPZ (**Supplementary Figures S2A,B**).

In turn, 17β-estradiol exposure induced resistance to H_2O_2-induced cell death only in st-TRPV1 cells (**Figure 1D**). 17β-estradiol oxidative cell death protection was concentration-dependent and occurred through TRPV1 modulation (**Figure 1D**). In parental cells, 17β-estradiol had no protective effect, except for saturated concentration (100 μM) possibly via triggering a non-specific effect (**Figure 1D**). The protection elicited by 17β-estradiol was abolished using 10 μM CPZ reaching levels similar to controls (**Figure 1E**). None of the compounds used except for H_2O_2 modified the viability of HeLa cells (**Supplementary Figure S3**). Moreover, 17β-estradiol-mediated H_2O_2-induced cell death protection was significantly decreased by knocking down the expression of TRPV1 in st-TRPV1 cells with a shRNA-TRPV1 (**Figure 1E**). In addition, st-TRPV1 cells transfected with a scramble shRNA-SS did not show any difference compared with non-transfected cells (**Figure 1E**). This evidence strongly suggests that cell protection against oxidative stress by 17β-estradiol is mediated by TRPV1.

The Membrane-impermeable 17β-estradiol conjugated with albumin (17β-estradiol-BSA) was employed in order to test whether estrogen could exert its effects through membrane receptor. Interestingly, this probe preserved the cellular protection against H_2O_2 in st-TRPV1 cells, which was abolished by the TRPV1 antagonist CPZ (**Figure 1F**), supporting that a membrane receptor mediates the estrogen effect. To study whether the protective effect of 17β-estradiol was stereospecific, we carried out an experiment using 17α-estradiol, the 17β-estradiol stereoisomer. Parental and st-TRPV1 cells were exposed to 1 mM H_2O_2 in the absence or presence of 17β-estradiol, 17α-estradiol or testosterone and cell viability was tested by flow cytometry. 17α-estradiol (1 μM) was unable to protect against H_2O_2 (**Figure 1G**). Similarly, cells treated with testosterone did not show any protection against H_2O_2-induced cell death (**Figure 1G**). These results indicate that the TRPV1-dependent protection against H_2O_2-induced cell death is specifically mediated by 17β -estradiol.

17β-Estradiol Increased TRPV1 Activity

In order to explore if 17β-estradiol can modulate TRPV1 activity, we measured TRPV1 currents in *Xenopus laevis* oocytes using the patch-clamp technique. The presence of 1 μM 17β-estradiol promoted a remarkable leftward shift in the conductance versus potential (G/V) relationships (**Figure 2**). The half voltage, $V_{0.5}$, for activation shifted from 131.6 ± 9.4 mV to 46.2 ± 8.3 mV, revealing that TRPV1 can be directly activated by this steroid hormone. However, the stereoisomer 17α-estradiol did not increase TRPV1 activity. On the contrary, 1 μM 17α-estradiol rightward shifted the G/V curve to 206.7 ± 18 mV (**Figures 2A,B**). Moreover, an increase in TRPV1 activity induced by 17β-estradiol was also observed in single-channel recordings where the probability to find the channel open (P_o) was significantly increased compared to controls when −100 mV was imposed to the patch membrane. 17β-estradiol NPo was: 0.052 ± 0.012 (n = 3; Control) and 0.355 ± 0.066 (0.5 μM 17β-estradiol; n = 3). This effect, however, was not reproduced by 17α-estradiol; 1 μM 17α-estradiol produced a NPo = 0.058 ± 0.017 (n = 5) a value similar to the control (**Figures 2C–F**).

An Early Pulse of 17β-Estradiol Is Sufficient to Trigger TRPV1-Dependent Cell Protection

We have studied the characteristics of the time-course of H_2O_2-induced cell death by performing a viability bioassay and kinetic recordings of the progression of the three-state cell model in parental and st-TRPV1 cells. Independently of TRPV1 expression, H_2O_2 exposure elicited almost complete cell death (**Figures 3A,B**). The analysis of vulnerable state indicated that H_2O_2 induced a vulnerable state via the loss of mitochondrial function in both cell lines, followed by plasma membrane disruption and cell death (**Supplementary Figure S2**). However, in st-TRPV1 we observed a delayed decrease in the alive state under H_2O_2 challenge compared to parental cells, and the peak of vulnerable state changed from 1 h for parental cells to 3 h for st-TRPV1 cells (**Figures 3A,B**), suggesting that TRPV1 expression is sufficient condition to improve cell viability under oxidative stress. Also, parental and st-TRPV1 cells were treated during the first 3 h of H_2O_2 challenge with a pulse of 1 μM 17β-estradiol. Parental cells showed similar results to those obtained without 17β-estradiol incubation (**Figures 3A,C**). Conversely, the exposure to 3 h 17β-estradiol was sufficient to induce cell protection against H_2O_2 cytotoxicity in TRPV1 expressing cell lines (**Figures 3B,D**). It is noteworthy that the preservation of the healthy condition was accompanied by the abolition of the vulnerable state peak. These data suggest that cells subjected to an initial pulse of 17β-estradiol became protected against oxidative cell death via a TRPV1 activated pathway.

TRPV1 Mediated 17β-Estradiol-Improved Mitochondrial Function and Avoided Caspase Activation

To investigate the link between the TRPV1 activation by 17β-estradiol and mitochondrial function, we measured mitochondrial membrane potential (ΔΨ) using JC-1 probe. As shown in **Figure 4E**, exposure to 1 mM H_2O_2 reduced the mitochondrial membrane potential in both cell lines exhibiting the same delay in the decay as previously shown during the alive to vulnerable state transition (**Figures 3A,B**). However, a pulse of 1 μM 17β-estradiol preserved the mitochondrial function in st-TRPV1 parental cells (**Figure 3E**). The loss of ΔΨ could be related to the opening of MMP and the activation of caspases generally associated with apoptotic-like cell death by an intrinsic pathway. To determine the role of the activation of caspases

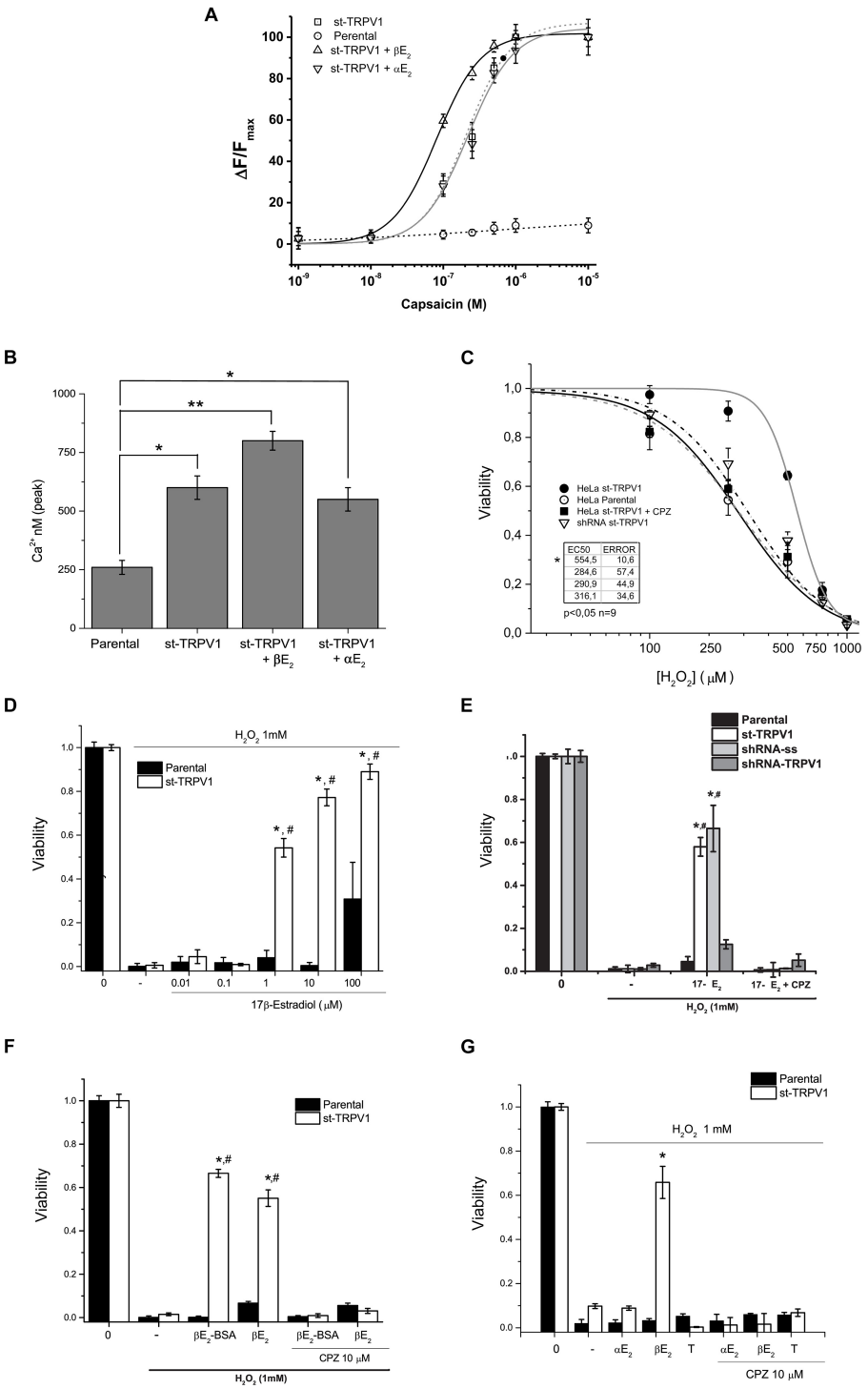


FIGURE 1 | TRPV1 expression is necessary and sufficient condition to deploy specific 17β-estradiol protection against oxidative stress. **(A)** Fura2-AM calcium imaging of HeLa parental and HeLa st-TRPV1 in the presence or absence of 17β-/17α-estradiol exposed to different concentrations of capsaicin (M). **(B)** Calculated concentration of cytosolic calcium (nM) from the peak signal of previous recorded fluorescence. **(C)** Cell viability as a function of H_2O_2 (10^{-5}–10^{-3} M) in parental and st-TRPV1 cells. (N = 9). **(D)** Dose-response bars graph of 17β-Estradiol (10^{-8}–10^{-4} M) in parental (filled bars) and st-TRPV1 (empty bars) cells in presence of H_2O_2 (N = 9). **(E)** Bar graph of the effect of knocking-down TRPV1 (sh-RNA-TRPV1) on H_2O_2-induced cell death in presence of 17β-estradiol (N = 9, scramble shRNA, shRNA-ss). **(F)** H_2O_2-induced cell death (1 mM) in cells treated with 17β-estradiol (10^{-6} M) or 17β-estradiol-BSA (10^{-6} M). **(G)** Effect of H_2O_2 co-administered with steroids 17β-estradiol, 17α-estradiol (10^{-6} M) and testosterone (10^{-6} M). Graph bars show means ± SD. All the viability experiments were registered at 24 h and the results expressed as data normalized to untreated condition. Statistical differences were assessed by one-way analysis of variance followed by Bonferroni's *post hoc* test. $^{\#}P < 0.05$ vs. NT st-TRPV1, $^{*}P < 0.01$ vs. parental cells.

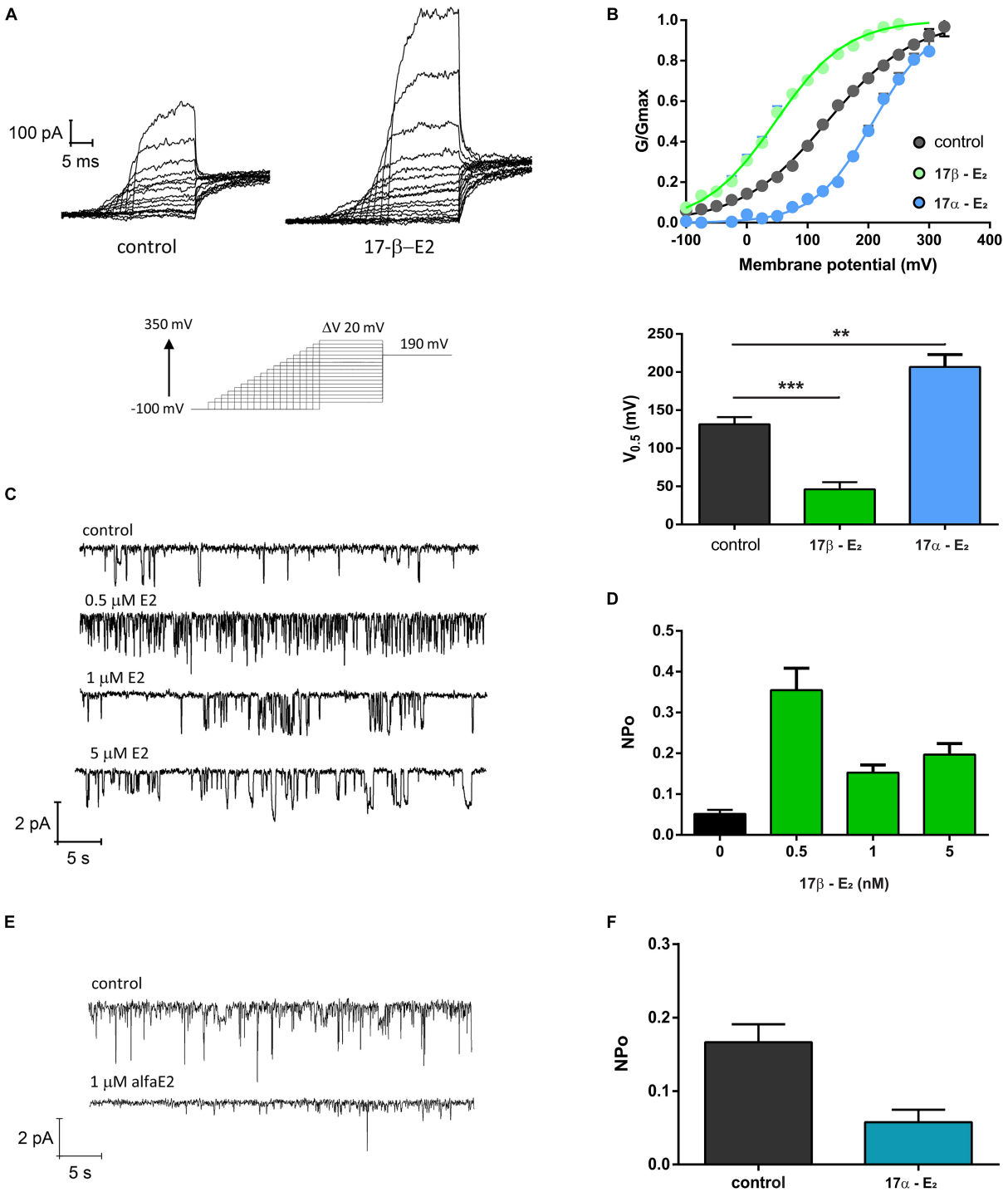


FIGURE 2 | TRPV1 activity is enhanced by 17β-estradiol. **(A)** Macroscopic current recordings of TRPV1 channel from *X. laevis* membrane patches evoked by pulses of −100 to + 350 mV in 20 mV increments, with decreasing duration as potential increases, followed by a step at 190 mV to obtain the tail currents (*bottom*). 17-βE2: 17β-estradiol; 17-αE2: 17α-estradiol. **(B)** G/Gmax versus Voltage relationships generated from the tail currents and adjusted to a Boltzmann fit as follows: 1/(1 + exp(z(Vh-V)/RT)). 17β-estradiol produced a left shift of the G/V curve decreasing the $V_{0.5}$ for activation from 131.6 ± 9.4 mV (n = 25, gray) to 46.2 ± 8.3 mV (n = 6, green). In contrast, 17α-estradiol produced a right shift of the G/V curve to 206.7 ± 18 mV (n = 7, blue). A bar plot showing $V_{0.5}$ for each experimental condition is provided (*lower* panel). ***P < 0.0001 and **P < 0.001, non-parametric t test followed by Mann Whitney test. **(C)** Single channel recordings of TRPV1 exposed to different concentrations of 17β-E2. **(D)** Quantification of NPo for the experiment showed in **(C)**. NPo for control was 0.052 ± 0.009 (n = 3), 0.5 μM 17β-E2 increases NPo to 0.35 ± 0.05 (n = 3), NPo for 1 μM was 0.15 ± 0.018 (n = 2) and NPo for 5 μM was 0.21 ± 0.027 (n = 3). **(E)** Single channel recordings of TRPV1 exposed to 1 μM 17β-E2. **(F)** Quantification of NPo from experiment showed in **(E)**. Estimated NPo for control was 0.16 ± 0.023 (n = 5). When membrane patches were exposed to 1 μM 17α-E2 on NPo was 0.58 ± 0.017 (n = 4). Single channels were recorded at −100 mV.

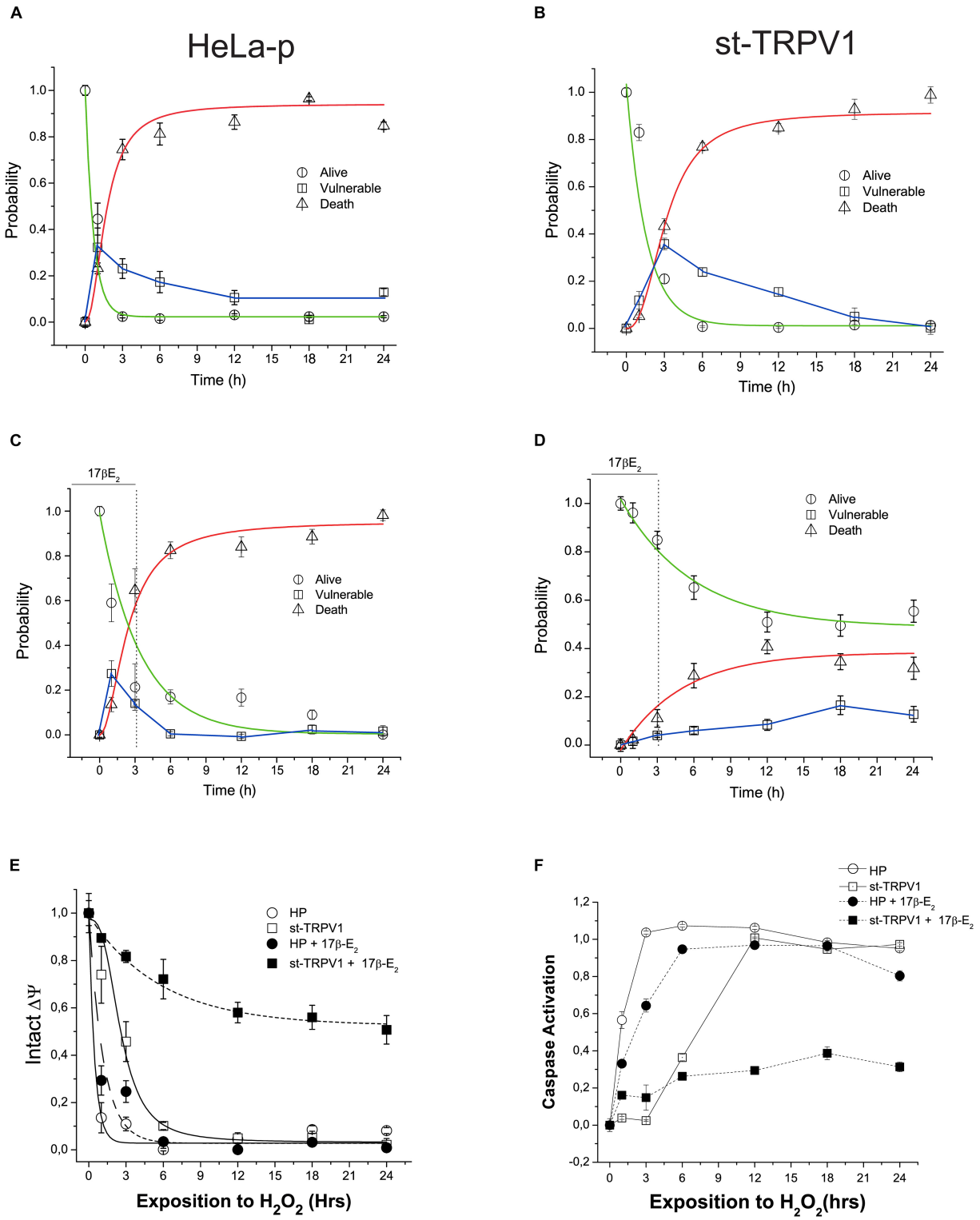


FIGURE 3 | TRPV1 mediated 17β-estradiol improving of mitochondrial stability during oxidative stress. Time course of H_2O_2-induced cell death (1 mM) following a kinetic model of cell death in HeLa parental (HeLa-P) **(A)** and st-TRPV1 cells **(B)** ($N = 9$). **(C,D)** Effect of initial pulse of 17β-estradiol on time course of H_2O_2-induced cell death in HeLa Parental **(C)** and st-TRPV1 **(D)** cell line ($N = 9$). **(E)** Temporal course of $\Delta\Psi$ in parental and st-TRPV1 cell lines measured by the rationometric probe JC-1. The data shows the first 3 h effect of 1 mM H_2O_2 and 17β-estradiol ($N = 9$). **(F)** Activation of caspase 3/7 by H_2O_2 in parental and st-TRPV1 cell lines in presence or absence of 10^{-6} M of 17β-estradiol at the first 3 h ($N = 6$).

in the H_2O_2 induced-cell death, we measured the time course of activation of total caspase (3–7) by flow cytometry in both cell lines compared with those obtained after 3 h exposure to 1 μM 17β-estradiol. We found that H_2O_2 induced the activation of caspases in both cell lines at different temporal windows. In parental cells, H_2O_2 exposure elicited the activation of caspase in the first hour (**Figure 3F**). However, at 3 h on 17β-estradiol we observed a reduction of caspase activity only in st-TRPV1. Overall, this evidence suggests that the activation of caspases induced by loss of mitochondrial membrane potential can be decreased by 17β-estradiol through an increase in TRPV1 activity. Altogether, our data suggest that TRPV1 is a membrane receptor of 17β-estradiol and that the protection against oxidative stress is related to the maintenance of mitochondrial function to preventing caspase activation.

TRPV1 Mediated Membrane 17β-Estradiol-Induced Protection Against Neuronal Oxidative Stress

To corroborate our results, we tested the participation of TRPV1 in 17β-E_2 cell protection in a primary cell culture derived from hippocampal neurons. Hippocampal neurons express functional TRPV1 and show intrinsic capacity to produce steroids and particularly estrogens (Mukai et al., 2006; Chávez et al., 2011). We confirmed the expression of TRPV1 in neurons only, both in hippocampus tissue and hippocampus-derived neurons by immunofluorescence, with highest expression in the cellular layer of CA1 region, mainly in cell bodies and dendrites (**Figures 4A,B**), confirming previous reports. Using cultured hippocampal neurons, we measured the effect of 17β-estradiol against oxidative stress produced by H_2O_2. We evaluated the cell viability of neuronal cells after 1 h of treatment with increasing concentrations of H_2O_2 in presence and absence of 17β-estradiol added 3 h before the oxidative insult (**Figure 4C**). Under all conditions, we observed a decrease in cell viability compared to controls and cell protection against H_2O_2 in those exposed to 17β-estradiol. Besides, the protective effect showed a biphasic component, with a peak of effect at 10^{-7} M and a decrease at micromolar concentration (**Figure 4D**). The half maximal effective concentration (EC_{50}), was $52 \pm 5\ 10^{-6}$ M. Using the EC_{50} for H_2O_2, we compared the effect of 17β-estradiol added 3 h before the oxidative insult. We found that the incubation of 10^{-7} M 17β-estradiol was sufficient to induce neuroprotection against H_2O_2 (**Figure 4E**). To evaluate the possible role of TRPV1 in cell protection, we blocked the channel activity with CPZ. We observed that, whereas CPZ significantly prevented the neuroprotective effect mediated by 17β-estradiol, tamoxifen and ICI 182780 (10^{-6} M) antagonists of estrogen receptors α and β, did not alter 17β-estradiol- induced neuroprotection (**Figure 4E**). Furthermore, 17β-estradiol-BSA produced the same protective effect as 17β-estradiol alone, suggesting that 17β-estradiol does not require diffusing through the membrane to exert its effect (**Figure 4F**). Altogether these data suggest that the expression of TRPV1 can mediate neuroprotection against oxidative stress, acting as a membrane receptor of the steroidal hormone.

DISCUSSION

This study demonstrates that 17β-estradiol is able to induce cell protection against oxidative stress through a mechanism dependent on TRPV1 activity. In addition, 17β-estradiol exposure prior to the oxidative injury only is sufficient to prevent H_2O_2-induced cell death. This level of control may be relevant in tissues that have the ability to aromatize androgens (Carreau et al., 2008; Zhang et al., 2014). For instance, mammalian glial cells do not produce 17β-estradiol under basal conditions. However, following brain injury and ischemia (Yague et al., 2008; Zhang et al., 2017) brain tissue expresses aromatase.

In general, estrogens are able to induce differential physiological effects through several mechanisms, some of them depend on the interaction with nuclear estrogen receptors whereas others may result from the activation of alternative estrogen-dependent routes with differential timelines. Here, TRPV1 expression was sufficient condition to confer 17β-estradiol-mediated protection against H_2O_2 challenge, independent of any intracellular classic receptor or plasma membrane diffusion. It has been reported that 17β-estradiol allows brain tissue protection, possibly by activating a voltage-dependent calcium channel (VDCC) (Wu et al., 2005; Feng et al., 2013). However, TRPV1-expressing HeLa cell lines exhibit 17β-estradiol-induced cell protection disregarding the expression of VDCC (Negulyaev et al., 1993).

In particular, estrogen can regulate TRPV1 activity and expression, playing a role in the sensitization of nociception (Cho and Chaban, 2012; Ho, 2013; Kumar et al., 2018). TRPV1 is differentially regulated by sexual steroids estrogen and testosterone, acting as positive and negative modulators, respectively (Chen et al., 2004). Our results show that 17β-estradiol-induced TRPV1 activity was not mimicked by its 17α-estradiol stereoisomer, suggesting that TRPV1 is able to discriminate between optical isomers with differential consequences. Moreover, the high lipophilicity of steroids raises the possibility that action mechanisms may occur through specific interaction with a protein inserted in the plasma membrane or by unspecific perturbation of lipid rafts surrounding the TRPV1 channel. 17β-estradiol may enhance TRPV1-mediated transient calcium influx in a stereospecific manner. In addition, the protection against oxidative stress obtained using 17β-estradiol-BSA, an impermeable probe which prevents plasma membrane diffusion, indicated that estrogen protection was initiated at the plasma membrane. This modulatory effect of 17β-estradiol has been previously reported with endogenous TRPV1 agonists in other tissues. For instance, 17β-estradiol enhances TRPV1-mediated vasorelaxation induced by CAP and anandamide (Ho, 2013). This role is particularly important considering that TRPV1 can integrate environmental physicochemical signals that are critical in controlling excitability and cell survival. Thus, integration of several signals may converge to improve cellular ability to deal with injury.

Similar to TRPV1, TRPM8 channel has been recently described as a testosterone receptor (Asuthkar et al., 2015). Both TRPV1 and TRPM8 are involved in pain nociception, inflammation and cell death, highlighting the therapeutic

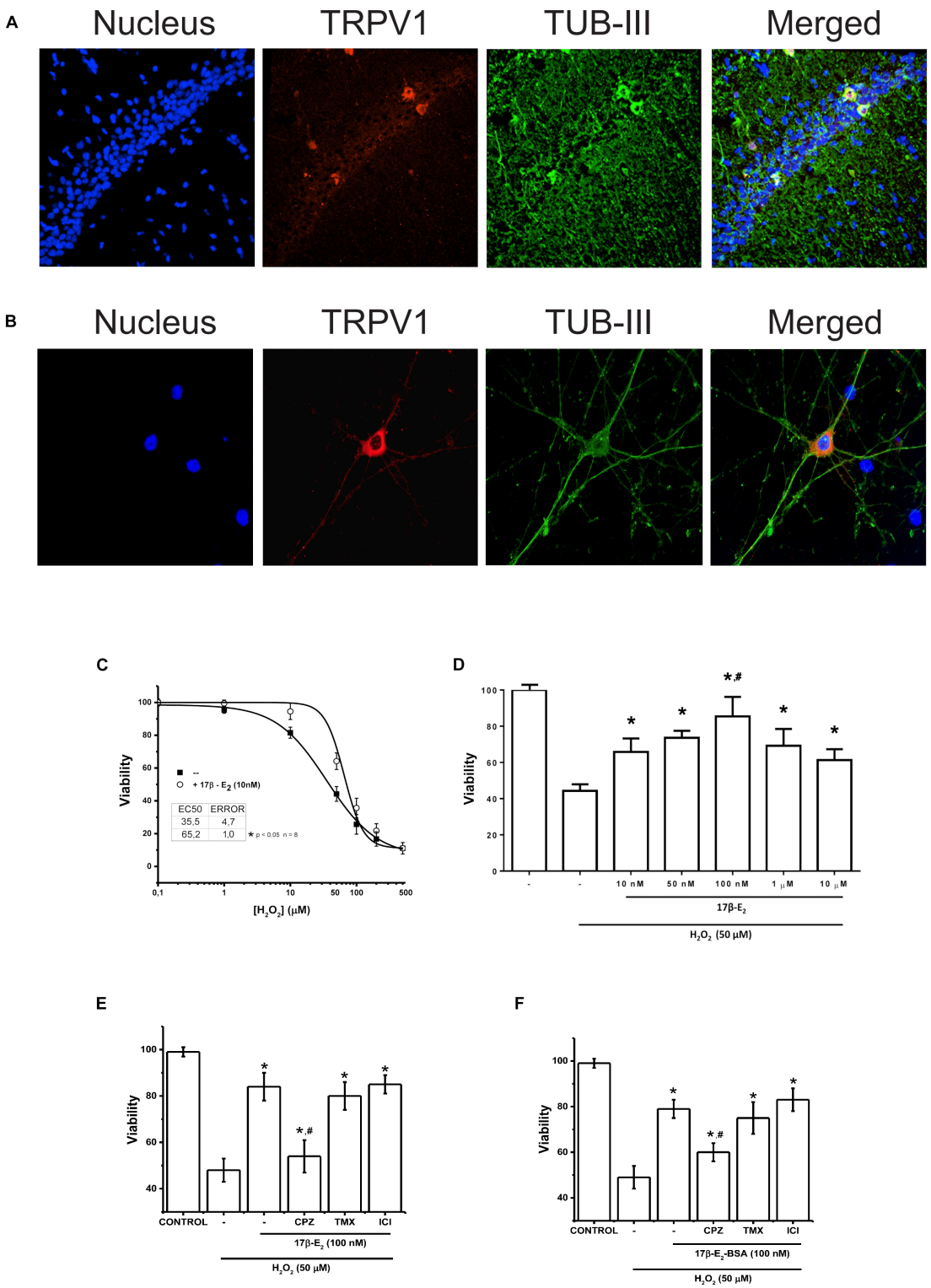


FIGURE 4 | Membrane activity of TRPV1 is sufficient condition to run 17β-estradiol protection against oxidative stress. **(A)** Immunostaining for TRPV1 expression in rat hippocampus. The technique selectively detected the CA3 region of hippocampus. **(B)** TRPV1 detection in 7-day cultured hippocampal neurons. **(C)** Changes in cell viability after 24 h incubation with H_2O_2 at increasing concentrations in presence and absence of 17β-estradiol in primary culture of hippocampal neurons. **(D)** Bar graph summarizes the effect of increasing doses of 17β-estradiol over H_2O_2 50 nM ($N = 3$) **(E)** Bar graph shows the effect of 10^{-7} M of 17β-estradiol on

(Continued)

FIGURE 4 | Continued
5×10^{-5} M of H_2O_2 in hippocampus-derived neurons (N = 5). **(F)** The graph summarizes the effect of the impermeable adduct 17β-estradiol-BSA, 10^{-7} (17β-estradiol-BSA) on cell death induced by 5×10^{-5} M of H_2O_2 ($N = 3$). CPZ: capsazepine (10 μM); TMX, inhibitor of estrogen receptor α tamoxifen (10^{-6} M); ICI, inhibitor of estrogen receptor β ICI 182780 (10^{-6} M). Results are expressed as data normalized to untreated condition (UT) (without H_2O_2 or 17β-estradiol). Bars indicate means ± SD. Statistical differences correspond to one-way analysis of variance and Bonferroni's *post hoc* test. $^{\#}P < 0.05$ vs. 17β-estradiol CPZ; $^{*}P < 0.01$ vs. 17β-estradiol H_2O_2.

potential of determining the role of these channels in hormone related activities and cell stress injuries. Besides the wide distribution of TRPV1 channels, their ability to activate different response pathways according with the nature of the stimuli, their intensity or time pattern, support the idea that they are far more complex structures than sensory transmitters. We believe that TRPV1 has to be considered as a stress response protein. By integrating multiple signaling pathways, TRPV1 can modulate intracellular calcium levels to run the cellular response to stress and injury, a strategy that may underlie the implication of TRPV1 in glial, neuronal and cardiomyocyte death.

On the other hand, we propose that 17β-estradiol is also an inductor of stress response, because it is able to directly activate classical proteins involved in stress response such as heat shock protein (Stice et al., 2011) and also, because it exerts control of mitochondrial function by several means in oxidative environment. It is likely that both 17β-estradiol and TRPV1 are involved in oxidative stress survival response.

17β-estradiol may act as an acute or allosteric modulator of TRPV1 activity (Chen et al., 2004). It is well known that transient calcium influx is necessary to induce cell protection (Bickler and Fahlman, 2004; Feng et al., 2013). It is thus possible that TRPV1 mediates extra-nuclear action of steroids, similar to other TRP channels (Thiel et al., 2013; Wagner et al., 2008). This process must be rapidly activated in order to prevent calcium overload and loss of chemical potential energy (Feng et al., 2013). It can be regulated by transitory calcium signaling to establish direct or indirect coupling between the mitochondria and calcium waves (Malli et al., 2003; Zhao and Brinton, 2007). TRPV1 could act as a critical sensor that stimulates mitochondrial function in oxidative environment.

Actually, TRPV1 channels control the mitochondrial integrity through regulation of mitochondrial membrane depolarization in neurons (Medvedeva et al., 2008; Ramírez-Barrantes et al., 2018). For example, low doses of capsaicin in dorsal root ganglion trigger a transitory calcium signaling by TRPV1, which, in turn, activates calcium uptake by mitochondria and slow release (10–20 min later) to prolong glutamate release from these sensory neurons (Medvedeva et al., 2008). This particular ability to integrate environmental signals as steroid hormones and coupling with such vital organelles as mitochondria, could explain the opposite effects of TRPV1 described in the literature. We hypothesize that, at low concentrations, TRPV1 activators can induce beneficial effects on cell viability, i.e., the agonist CAP, preventing oxidative stress-induced cell death. High concentrations of activators or sustained activation of the channel might induce toxicity by deregulation of mitochondrial function. If TRPV1 is overactivated, a loss of transience of the signal is accompanied by dysfunction of mitochondria, calcium overload and cell death (Ramírez-Barrantes et al., 2018). It is likely that the physiological mechanism of action of TRPV1 (Pegorini et al., 2005; Huang et al., 2017) consists in, sequentially: activation, transient raise in intracellular calcium and consequent calcium-mediated desensitization. These regulated actions would allow preserving the integrity of mitochondrial function and cell viability. Estrogen, as suggested by the effect of a 3 h pulse, would produce a high-intensity initial signal activating TRPV1 with a large calcium influx, followed by an increase in mitochondrial calcium buffer capacity.

Further experiments are needed to clarify the specific steroid interaction with the TRPV1 channel and coupling of mitochondrial function. Considering that 17β-estradiol has been related to controlling the production of ATP, it is possible that cell protection is due to modulation of mitochondrial function. Estradiol can potentiate a cell protective pathway associated to functional coupling between TRPV1 activity and mitochondrial function in addition to other extra-nuclear estrogen actions described (Bao et al., 2011). The TRPV1 contribution to mitochondrial function needs to be further studied with focus on the coupling between TRPV1 activity and mitochondrial membrane potential.

Evidence that an initial pulse of 17β-estradiol is sufficient for cell protection is noteworthy. It is likely that H_2O_2 induces cell vulnerability through early mitochondrial failure, determining progression to cell death. Here, TRPV1 mediated 17β-estradiol ability to inhibit mitochondria depolarization thus preserving its function, diminishing the size of vulnerable population and decreasing cell death.

Furthermore, previous experiments of calcium imaging suggest that mitochondrial function is coupled to TRPV1-dependent intracellular calcium increase (Ramírez-Barrantes et al., 2018). We hypothesized that in the context of oxidative environment the possible mechanism of TRPV1-dependent protection could be mediated by a transient calcium increase leading to expand the mitochondrial calcium buffer capacity and cellular survival potential as has been previously suggested (Ge et al., 2016). Additionally, this transient calcium increase has been associated with a primary signal for expressing the anti-apoptotic protein Bcl-2 (Wu et al., 2005, 2011).

The role of mitochondria in cytosolic Ca^{2+} signaling has been related to calcium uptake and calcium buffering. Usually, when mitochondria are depolarized, the transient raise in cytosolic Ca^{2+} induced by different stimuli is larger (Vay et al., 2007) than in basal optimal mitochondrial conditions and inhibits the production of regenerative oscillations (Dedov et al., 2001; Vay et al., 2007; Medvedeva et al., 2008). This evidence indicates that mitochondria take up significant amounts of Ca^{2+} during cell stimulation and shows their vital role in regulating the calcium influx as a specific signal rather than a basic response to unspecific calcium overload. This function seems to be

very important for many models of oxidative cell death, both mitochondria depolarization and calcium overload are present in cell death in glutamate cytotoxicity and ischemia reperfusion models (Nilsen and Diaz Brinton, 2003; Bickler and Fahlman, 2004; Wu et al., 2005). However, it has been reported that when calcium influx is triggered concomitantly with or just before the cytotoxic event, an increase in the mitochondrial calcium buffer capacity prevented cell death. This coupling activity between calcium influx and mitochondria is associated with a long-lasting conservation of the mitochondrial membrane potential due to the expression of anti-apoptotic protein Bcl-2 (Nilsen and Diaz Brinton, 2003). In fact, the calcium influx elicited by 17β-estradiol through VDCC, or even by ionomicin, is able to induce the active calcium uptake by mitochondria directly, but it relates also to the activation of MAPK pathway and AKT pathway to induce the expression of Bcl-2, inhibiting the mitochondria outer membrane permeabilization, and preventing the release of cytochrome c and the activation of caspases (Nilsen and Diaz Brinton, 2003; Bickler and Fahlman, 2004; Wu et al., 2005). In the same line, we suggest that the maintenance of mitochondrial function was able to diminish the activity of caspase enzymes necessary to induce apoptotic-like cell death via intrinsic pathways. It is possible that the membrane-associated action of 17β-estradiol through TRPV1 was able to induce cell protection not only by increasing the calcium buffering capacity but also by avoiding the activation of intrinsic apoptotic pathway. Nevertheless, the mechanisms of coupling between intracellular calcium influx, calcium buffer capacity and apoptosis are still to be clarified (**Graphical Abstract**).

We employed primary cultures of hippocampal neurons because they are responsive to estrogen and express TRPV1 endogenously (Chávez et al., 2011; Mukai et al., 2006). These neurons are very sensitive to oxidative environment. In these cells we demonstrated that 17β-estradiol and estradiol-BSA are capable of exerting protective effects, depending on a membrane receptor different from estrogen receptors α and β. In addition to classical TRPV1-expressing tissues, the channel appears at diverse locations in the context of oxidative injury. Our evidence that 17β-estradiol potentiates the activity of the channel by extra-nuclear mechanisms introduces important perspectives regarding the function of polymodal channels and of steroidal hormones (Ramírez-Barrantes et al., 2016). The study of the properties of TRPV1 may have several implications in cell physiology and therapeutic development. TRPV1 control of cellular response against oxidative environments to improve cell survival may lead to progress in stem cells graft, organ transplant, ischemia reperfusion disorders and neurodegenerative disease. TRPV1 have been used to control specific functions in neuronal and non-neuronal context, *in vitro* and in behaving animals (Huang et al., 2010; Stanley et al., 2012). In pancreas and neurons, the controlled activation of TRPV1 has been used to modulate responses as insulin secretion or producing fast activity onset. The interaction proposed in this paper points in the same line, being especially relevant in tissues that express TRPV1 and have the ability to produce relevant quantities of 17β-estradiol such as the gonads and brain. This could reveal new interesting routes for development of multidrug strategies on the basis of molecular discoveries conducted by clinical questions.

INTEREST

The novel mechanism of 17β-estradiol may directly activate TRPV1-driven plasma membrane signaling coupled with mitochondrial function in a stereospecific manner, and it role to regulation of oxidative stress-induced cell death. This mechanism described in this study could generate molecular strategies for preventing oxidative stress-induced cell death, which occurs during neural-degeneration.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RR-B and PO conceived the experiments and drafted the work. RR-B, BR, CC, PD, KC, and KC-Z performed the experiments. RR-B, CC, CL, FS, PM, IM, KC, and PO analyzed the data. CL, KC, PO, and RL contributed reagents, materials, and analysis tools. RR-B, IM, PO, RL, and KC wrote the manuscript. All authors critically revised the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00444/full#supplementary-material

FIGURE S1 | TRPV1 overexpression in heterologous expression system. **(A)** Representative RT-PCR experiment showing TRPV1 mRNA expression (167 pb) for HeLa Parental (HeLa), HeLa st-TRPV1, Hippocampal tissue and transfection vector pCDNA3.1-TRPV1 plasmid. Housekeeping gene was s16 (102 pb). **(B)** Flow cytometry histogram showing TRPV1 protein expression by Immuno-FACS for HeLa Parental and HeLa st-TRPV1 and in presence of Anti-TRPV1. Negative control corresponds to the same technique in the absence of the primary antibody for TRPV1 (n = 4). **(C)** Immunofluorescence for TRPV1 by epifluorescence microscopy show distribution of TRPV1 protein in st-TRPV1 and parental HeLa cells.

FIGURE S2 | Capsaicin and 17β-Estradiol protects against H_2O_2-induced cell death by preserving mitochondrial function in TRPV1 expressing cells. **(A)** Representative plots of the effect of CAP a TRPV1 agonist on H_2O_2-iduced cell death. **(B)** Bar graph summarizes the effect of CAP in cell death induced by H_2O_2. Bars show the mean ± SD (N = 9). **(C,D)** Representative dot-plot of temporal course of cell death from the kinetic model of cell death (**Figure 3**). **(C,D)** The data shows an initial phase of cell damage induced by H_2O_2 (1 mM) represented by the transition from alive (A) to vulnerable (V) state due to the collapse of mitochondrial function in both st-TRPV1 and HeLa-P, which eventually end in cell death for both cell lines. **(E,F)** However, after 3 h of 17β-Estradiol treatment only st-TRPV1 cells show a decrease in the number of vulnerable cells due to loss of mitochondrial function which in turns decrease the total number of dead cells (N = 9).

FIGURE S3 | Effect on cell viability of pharmacological activators and inhibitors of estrogen receptor and TRPV1. Effect of 17β-estradiol E_2, capzasepine (CPZ), tamoxifen (TMX), ICI-182 and hydrogen peroxide (H_2O_2) in st-TRPV1 cell viability measured by flow cytometry.

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REVIEW
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TRP Channels Role in Pain Associated With Neurodegenerative Diseases

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Transient receptor potential (TRP) are cation channels expressed in both non-excitable and excitable cells from diverse tissues, including heart, lung, and brain. The TRP channel family includes 28 isoforms activated by physical and chemical stimuli, such as temperature, pH, osmotic pressure, and noxious stimuli. Recently, it has been shown that TRP channels are also directly or indirectly activated by reactive oxygen species. Oxidative stress plays an essential role in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, and TRP channels are involved in the progression of those diseases by mechanisms involving changes in the crosstalk between Ca^{2+} regulation, oxidative stress, and production of inflammatory mediators. TRP channels involved in nociception include members of the TRPV, TRPM, TRPA, and TRPC subfamilies that transduce physical and chemical noxious stimuli. It has also been reported that pain is a complex issue in patients with Alzheimer's and Parkinson's diseases, and adequate management of pain in those conditions is still in discussion. TRPV1 has a role in neuroinflammation, a critical mechanism involved in neurodegeneration. Therefore, some studies have considered TRPV1 as a target for both pain treatment and neurodegenerative disorders. Thus, this review aimed to describe the TRP-dependent mechanism that can mediate pain sensation in neurodegenerative diseases and the therapeutic approach available to palliate pain and neurodegenerative symptoms throughout the regulation of these channels.

Keywords: pain, Alzheimer's disease, Parkinson's disease, TRP channels, neurodegeneration

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Transient receptor potential (TRP) proteins constitute a group of non-selective cation channels (Gees et al., 2010) found in most cell membranes, except in the nuclear and mitochondrial membranes. TRPs are expressed in plasma membrane and help to modulate the driving force for the influx of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} ions, and trace metal ions (Nilius and Owsianik, 2011), while in specific organelles, such as the cilium and lysosomes, they regulate organelle and cellular activity (Moran, 2018). Numerous excitable and non-excitable tissues express TRPs, where they are involved in sensory signal transduction (nociception, taste, pressure, temperature, vision, and pheromone signaling), as well as homeostatic regulation (muscle contraction, vessel relaxation, and cell proliferation) (Gees et al., 2010). In the central nervous system (CNS), several TRP channels are expressed in both neurons and glia, fulfilling critical roles in neurogenesis, structural/functional plasticity, and cell homeostasis (Nilius, 2012; Vennekens et al., 2012; Katz et al., 2017).

It has been described that diverse ion channels expressed in the brain's cells, including TRPs, are involved in the progression of neurodegenerative diseases such as Parkinson's and Alzheimer's. Also, several members of TRPs subfamilies are highly expressed in neurons and microglia mediating neuropathic pain (Haraguchi et al., 2012). TRP channels are part of cellular pathways related to the synthesis of many inflammatory mediators associated with neuroprotection/neurotoxicity, where they contribute to intracellular calcium regulation and signaling and painful stimuli transduction (Ji and Suter, 2007; Miyake et al., 2014; Lee and Kim, 2017). Therefore, TRP channels became of interest as promising targets for the treatment of both neurodegenerative diseases and pain.

In this review, we summarize the evidence of the role of TRP channels in the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Also, we discussed the possible involvement of TRP channels in pain associated with these neurodegenerative diseases and the use of TRP channels as possible pharmacological targets for pain treatment in patients with neurodegenerative diseases. A better understanding of the molecular mechanisms involved in neurodegeneration and pain is necessary to prevent and treat neurodegeneration and chronic pain.

TRPs STRUCTURE AND EXPRESSION

TRP multigene superfamily is formed by 28 members that encode integral membrane proteins that function as cation channels (Vennekens et al., 2012). TRP channels have some structural similarity, sharing as common a three-dimensional structure with six transmembrane segments (S1 through S6), two variable cytoplasmic domains (N- and C terminal), and small loop forming the channel pore between S5 and S6 segments (Catterall and Swanson, 2015). The distinguishing features between TRP channel subfamilies have been reported in the N- and C-terminal cytosolic domains, which contain residues and regulatory motifs unique for each family (Gaudet, 2008).

Unlike other cation-selective channel families, TRPs are classified by primary amino acid sequence rather than selectivity, ligand function, mechanisms of regulation, or sequence homology (Moran et al., 2004; Wu et al., 2010). TRP channels are divided into seven subfamilies: TRPC (Canonical), TRPV (Vanilloid), TRPA (Ankyrin), and TRPM (Melastatin), TRPP (Polycystic), and TRPML (Mucolipin). The seventh family, the no mechanoreceptor potential C channels (NOMPC or TRPN), is not found in mammals (Skryma et al., 2011). Alternatively, based on their sequence and topological features, TRP genes superfamilies are divided into Group 1 (TRPC, TRPV, TRPM, TRPA, and TRPN), and Group 2 (TRPP and TRPML). TRP subunits, in the same or different subfamilies, form functional homomeric or heteromeric ion channels with distinct biophysical and regulatory properties (Hellwig et al., 2005; Cheng, 2018). Heteromultimerization among mammalian TRP subunits have been observed for the TRPC, TRPV, TRPM, and TRPP families, displaying distinctive features (Hellwig et al., 2005; Cheng, 2018). For instance, formation of heteromeric complexes TRPC1/3, TRPC1/4, TRPC1/5, TRPC3/4, TRPC4/5 showed novel non-selective cationic channels with a voltage dependence or dynamic gating (Cheng et al., 2010; Kim et al., 2014; Woo et al., 2014). Also, TRPV1/3, TRPV5/6, TRPM6/TRPM7 or TRPML1/2 channels form heteromeric channels with intermediate conductance levels and gating kinetic properties (Cheng et al., 2007; Ma et al., 2011; Zhang et al., 2014; Goldenberg et al., 2015; Kim et al., 2016). Heteromerization within the mammalian TRP channel superfamily has also been observed. For instance, heteromeric TRPP2/TRPC1 and TRPP2/TRPV4 channels exhibit new receptor-operated property implicated in mechanosensation or thermosensitive roles (Du et al., 2014), and TRPC1/TRPC6/TRPV4 may mediate mechanical hyperalgesia and primary afferent nociceptor sensitization (Cheng, 2018).

The first TRP subfamily characterized was the canonical TRPC. The seven members of this subfamily are divided into four groups according to their sequence homology into Group I (TRPC1), group II (TRPC2), group III (TRPC3, TRPC6, TRPC7), and group IV (TRPC4 and TRPC5) (Nilius and Flockerzi, 2014). TRPC channels at the N-terminal domain show ankyrin repeats (3–4), a coiled-coil region, and a caveolin binding region. Meanwhile, the cytoplasmic C-terminal domain contains the TRP motif EWKFAR, a highly conserved proline-rich motif, and a region to interact with the IP_3 receptor as well with calmodulin (calmodulin/IP_3 receptor-binding region) (Putney et al., 2004). All TRPC are non-selective cation channels permeable to Ca^{2+} (Bon and Beech, 2013) linked to cellular processes such as cell division, differentiation, apoptosis, transduction of external stimuli, and refill of intracellular Ca^{2+} stores. In addition, they act amplifying receptor-activated Ca^{2+} signaling *via* interaction with second messengers (Numaga-Tomita et al., 2019). TRPC channels are widely distributed in cells of different tissues, including brain, heart, smooth muscle, liver, testis, ovaries, salivary glands (Beech et al., 2003), endothelium, kidneys (Freichel et al., 2005), and adrenal glands (Philipp et al., 2000). For instance, TRPC4/5 mRNA has been found in cortico-limbic brain regions, like the hippocampus and prefrontal cortex of adult rats (Fowler et al., 2007). TRPC channels are involved in diverse neuronal functions *via* receptor-mediated regulation by neurotrophic factors or neuropeptides, and cation influx through TRPCs control cellular functions and neuronal activity by regulating the membrane potential (Katz et al., 2017).

The TRPV subfamily is made up of six members, which are classified into four groups according to their homology: TRPV1/TRPV2, TRPV3, TRPV4, and TRPV5/TRPV6 (Smith et al., 2002; Xu et al., 2002; Nilius and Owsianik, 2011). TRPV channels were named after the discovery that its founding member (TRPV1) was activated by the vanilloid capsaicin, the compound responsible for a hot spicy taste (Szallasi and Blumberg, 1999). TRPV channels form homo- or hetero-tetramers, highly calcium selective, and mostly located on the plasma membrane. Each monomeric subunit typically contains three to five N-terminal ankyrin repeats and a TRP box at their C terminal. To this date, the most studied member of the

TRP family is the TRPV1 receptor. TRPV1, TRPV2, TRPV3, and TRPV4 are moderately Ca^{2+} permeable, while TRPV5 and TRPV6 are highly selective Ca^{2+} channels and strictly regulated by $[Ca^{2+}]i$ (Gees et al., 2010). It is known that TRPV members have different gating properties, as studies using wild type and knockout mice models revealed that although TRPV2–6 channels share high sequence similarities with TRPV1, and they do not respond to temperature stimuli (Samanta et al., 2018). Furthermore, TRPV2 and TRPV4, unlike to other members of the family, are not sensitive to capsaicin (Caterina et al., 1999). TRPV1 channel is a homo-tetramer in which each monomer contains six ankyrin repeats in the N-terminal domain. The ion-conducting pore is formed by the transmembrane segments S5 and S6 and the pore-forming P loop and is similar to voltage-gated Na^+ and K^+ channels (Samanta et al., 2018). TRPV1 channels were first described in pain-sensitive neurons in dorsal root ganglia (DRG) and trigeminal ganglion neurons (Gees et al., 2010). Specifically, they are localized in peripheral small unmyelinated C- fibers, where they act as polymodal integrators of noxious stimuli in skin, muscles, joints, and internal organs (Samanta et al., 2018); also, TRPV2-4 channels are expressed in DRG neurons. TRPV3 is found in the brain, tongue, testis (Xu et al., 2002), skin, keratinocytes, and in cells surrounding hair follicles (Mandadi et al., 2009), while TRPV4 is expressed in non-neuronal cells like insulin-secreting β-cells, keratinocytes, smooth muscle cells, and different epithelial and bone cell types (Nilius et al., 2008).

The TRPA subfamily is constituted exclusively by the mammalian TRPA1 channel, first identified as an ankyrin-like transmembrane protein sharing similarities with other TRP channel subfamilies (Jaquemar et al., 1999). TRPA1 is a non-selective cation channel formed by homo- or hetero-tetramer subunits. The structure of human TRPA1 (hTRPA1) was determined by cryo-electron microscopy and shares a common structure with other TRP channels (Paulsen et al., 2015). TRPA1 has calcium-binding domains located in the C-terminal (Meents et al., 2019), 16 ankyrin repeat sequences in the N-terminal domain (Meents et al., 2017; Samanta et al., 2018), a putative selectivity filter located at the entrance of the pore, and a voltage sensor in the C-terminal (Meents et al., 2019). These domains allow TRPA1 channels to interact with other proteins, form molecular springs, and have better elasticity. This channel is expressed throughout the body, including the brain, heart, small intestine, lung, bladder, joints, and skeletal muscles (Kono et al., 2013). TRPA1 is highly expressed in DRG and trigeminal ganglia neurons (Takahashi et al., 2008) and acts as a mechanosensor in peripheral sensory pathways and the inner ear (Brierley et al., 2011).

TRPM channel subfamily consists of eight members grouped in four pairs: TRPM1 and TRPM3; TRPM2 and TRPM8; TRPM4 and TRPM5; and TRPM6 and TRPM7 (Fleig and Penner, 2004). All TRPM family members share common structural characteristics with other TRP channels (Fujiwara and Minor, 2008); however, they have a large cytosolic domain of between 732 and 1,611 amino acids for each subunit, which makes them the largest members of the TRP superfamily (Huang et al., 2020). Furthermore, unlike the TRPC, TRPV, and TRPA subfamilies, TRPM have a unique N-terminal (TRPM homology domain) without ankyrin repeats implicated in the channel assembly and trafficking (Kraft and Harteneck, 2005). Within subfamily members, the C-terminal section of TRPM channels is particularly variable, with TRPM2, TRPM6, and TRPM7, including active enzymatic domains (Samanta et al., 2018). TRPM2 has a nucleoside diphosphate pyrophosphatase domain (Chubanov et al., 2004) that specifically binds and hydrolyzes to ADP-ribose, while TRPM6 and TRPM7 contain α-kinase domains (Nadler et al., 2001; Drennan and Ryazanov, 2004). TRPMs are widely expressed in different tissues and organs; for instance, TRPM 2, 3, 4, 5, 6, and 7 are expressed in the CNS and periphery nervous system (PNS) (Mickle et al., 2015). Also, TRPM4, TRPM5, and TRPM8 are preferentially expressed in the prostate, while TRPM4, TRPM5, and TRPM6 are expressed in the intestine, and TRPM7 in heart, pituitary, bone, and adipose tissue (Bernardini et al., 2015). By contrast, TRPM1 is expressed by melanocytes and in malignant melanoma cells (Mickle et al., 2015).

As mentioned before, several members of the TRPC, TRPV, TRPM, and TRPA families are expressed in neurons and glial cells in the CNS and PNS (**Figure 1**; Riccio et al., 2002; Moran et al., 2004; Abel and Zukin, 2008; Harteneck and Leuner, 2014; Zhang and Liao, 2015; Echeverry et al., 2016; Belrose and Jackson, 2018). Evidence has shown that TRPs in the CNS have critical roles in modulating growth cone guidance, synaptogenesis, synaptic plasticity, and in the development of several neurodegenerative diseases (Nilius, 2012; Vennekens et al., 2012; Katz et al., 2017). Notably, even when the role of TRPs in nociception in the PNS has been extensively described, their role in the CNS is almost unknown, and it has only recently gained attention.

ACTIVATION MECHANISMS OF TRPs

TRP channels display a wide variety of activation mechanisms, which include physical stimuli, ligand binding, second messengers, and reactive oxygen and nitrogen species (Vennekens et al., 2012).

TRPC channels are modulated by a diverse group of second-messengers lipids that either regulate the channel activity or its insertion into the plasma membrane (Nilius and Szallasi, 2014). TRPCs activation mechanism converges various types of intracellular stimuli, including phospholipase C (PLC), protein kinase C (PKC) activity, diacylglycerol (DAG), intracellular calcium, and phosphatidylinositol 4,5-bisphosphate (PIP_2) levels to modulate membrane potential and calcium input (Ramsey et al., 2006). Due to the flexible role of TRPC3 channel in calcium signaling and functional coupling with metabotropic receptors involving the PLC pathway in DRGs, as well as its regulation by pro-inflammatory molecules inducing channel sensitization (Séguéla et al., 2014), it has been of interest as a potential target for the management of chronic pain. Although most TRPCs are activated through PLC, which is a downstream effector of growth factors and neurotrophins, For instance, TRPC3, 6, and 7 are activated primarily by Gq/11 proteins, which are

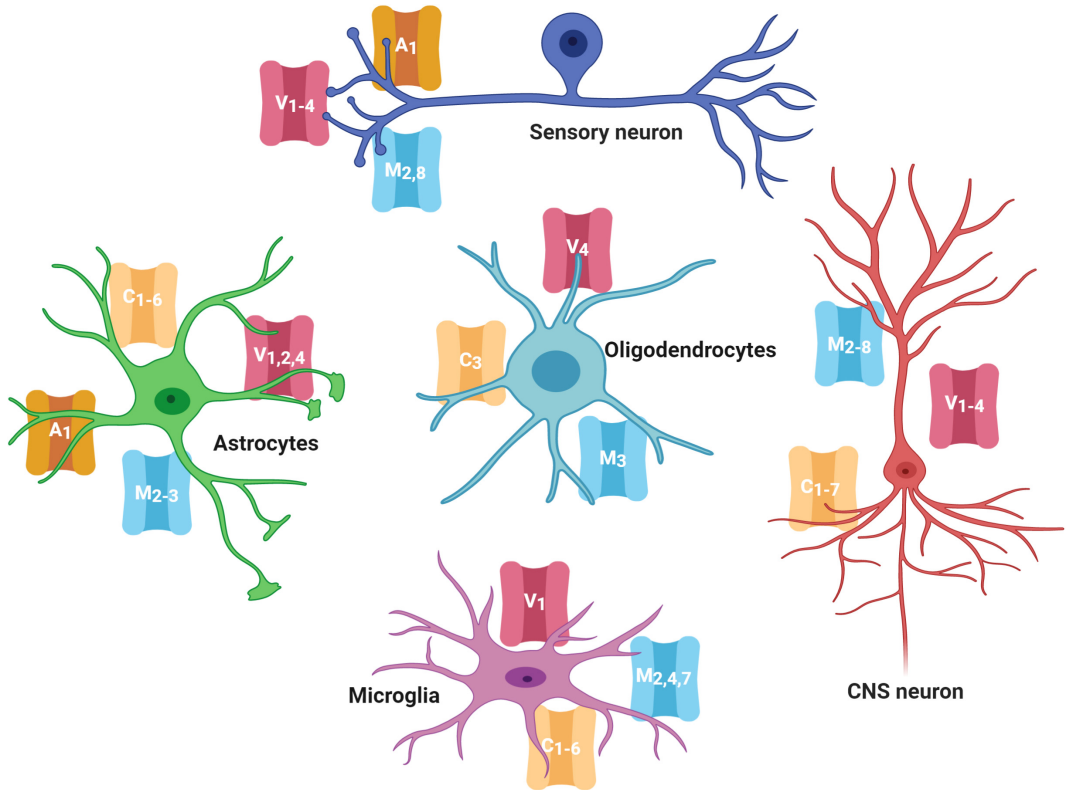

FIGURE 1 | TRP channels expressed in the nervous system cells. Several members of the TRPC, TRPV, TRPM, and TRPA families are highly expressed in cells of the central and peripheral nervous system (neurons, astrocytes, oligodendrocytes, and microglia). TRP families are represented by capital letters as follow, C, TRPC; M, TRPM; V, TRPV; A, TRPA. Numbers indicates specific members of each family.

coupled downstream PLC-β; nevertheless, the $G\alpha_{i/o}$ family are the dominant activators for TRPC4 and 5 (Nilius and Szallasi, 2014), which effectors of the PLC pathway are critical for the activation of TRPC channels remains a matter of debate, however, it is thought that specific TRPC channels may use different signaling effectors of this pathway (Putney, 2005). In this vein, it has been described that TRPC activation is dependent on recognition and lipid signals, and for instance, TRPC1, 2, 4, and 5 are activated by several DAGs (Lucas et al., 2003).

Besides lipid signaling, oxidative metabolism has a pivotal role in regulating TRPC channels activity (Kitajima et al., 2011) since they can be modulated by the production of reactive oxidative species (ROS) and reactive nitrogen species (RNS). TRPC channels can be considered redox-sensitive proteins that are targeted by ROS (Kim et al., 2013), and specifically, it has been reported that TRPC3 and TRPC4 are directly activated in response to oxidative stress (Aarts and Tymianski, 2005; Miller, 2006). It has been described that redox sensed by TRPC channels let the system indirectly to transduce lipid accumulation produced by the PIP_2/DAG pathway (Malczyk et al., 2016), and the redox modifications of the lipid membrane environment that surrounds the channel (Poteser et al., 2006). For instance, TRPC3 activation by 1-oleoyl-2-acetyl-sn-glycerol (OAG) or mechanical stretch has shown to induce ROS production in rat neonatal cardiomyocytes (Kitajima et al., 2011). Additionally, it has been described in human embryonic kidney (HEK) cells that nitric oxide (NO) activates TRPC5 channels through mechanisms that require oxidation of extracellular cysteines in response to the NO donor S-nitroso-N-acetyl-DL-penicillamine (Yoshida et al., 2006). Also, intracellular oxidation regulates TRPC5 activation by glutathionylation, nitrosylation, and hydroxylation reactions, respectively, in Cys176 and Cys178 in contact with the intracellular redox environment, resulting in a sustained increase in $[Ca^{2+}]i$ and consequent cellular toxicity and neurodegeneration (Hong et al., 2015). In addition, oxidative metabolism also regulates the expression of TRPC channels (Song et al., 2011). Together ROS generation and Ca^{2+} signaling through TRPC channels modulate cellular processes that allow physiological and pathological responses in several organs (Malczyk et al., 2016) including kidney (Kim et al., 2013), brain (Hong et al., 2015), and the immune system (López-Requena et al., 2019). These factors have been associated in the pathogenesis of several chronic neurological disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD), since ROS could activate cell death processes directly, through protein oxidation, lipids, and acting as second messengers in the cell death process (Gopalakrishna and Jaken, 2000; Nakamura and Lipton, 2009).

TRPV channels are activated by chemical ligands, such as capsaicin or cannabinoids, but also by noxious heat (>43°C),

low pH (<6) (Caterina et al., 1997; Tominaga et al., 1998) and voltage changes inducing depolarization (Cao, 2020). TRPVs are also activated by lipid signaling (Cortright and Szallasi, 2004; Jung et al., 2004), and eicosanoids, signaling molecules produced by the enzymatic or non-enzymatic oxidation of arachidonic acid or other similar polyunsaturated fatty acids (Hwang et al., 2000). Specifically, TRPV1 activation can be achieved, regulated, and enhanced by several inflammatory molecules throughout metabolites downstream of G-protein coupled, such as PIP_2 (Bhave et al., 2002), IP_3 and DAG (Burgess et al., 1989), protein kinases such as PKA (Vlachová et al., 2003), PKC (Bhave et al., 2002; Varga et al., 2006), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Jin et al., 2004; Rosenbaum et al., 2004), and arachidonic acid metabolites like 12-HPETE (Shin et al., 2002).

It has been described that DRG neurons express TRPV1 to transduce and modulate pain stimuli in response to ligands and temperature (Caterina et al., 1997; Basbaum et al., 2009). Furthermore, it has also been shown that Bradykinin can regulate nociceptors such as TRPV1 activity, an inflammatory response mediator, that simultaneously stimulates the synthesis of PLC and its downstream targets (PIP_3 and DAG), and arachidonic acid that further enhance cell excitation (Suh and Oh, 2005). During the inflammatory response, other pro-inflammatory mediators such as prostaglandins and sympathetic amines, sensitize nociceptors, including TRPV1, boosting pain sensation, or hyperalgesia (Zarpelon et al., 2013). A relationship between cytokines and oxidative stress has been found in hyperalgesia. For instance, NADPH oxidase leads to the production of superoxide anion by the TNF-α-induced NF-kB activation and consequentially causes overexpression of pro-inflammatory cytokines such as IL-1β (Possebon et al., 2014). Also, TNF-α and IL-1β activate cyclooxygenase-2 to produce prostanoids, which sensitize nociceptors, causing hyperalgesia (Verri et al., 2006).

TRPA1 channels have a wide range of natural and synthetic ligands (reactive electrophilic agonists) that induce channel gating by covalently bound to cysteine and lysine residues within the N-terminal and transmembrane domains, or promote the formation of C422–C622 disulfide bonds (Kimura, 2015). Also, polyunsaturated fatty acids (Viana, 2016), temperature (17–40°C) (Laursen et al., 2014; Moparthi et al., 2016) and changes in pH can activate TRPA1channels (Fujita et al., 2008; De La Roche et al., 2013; Zimova et al., 2018). De La Roche et al. (2013) reported activation of TRPA1, expressed in HEK 293T cells, with solutions above pH 5.4. However, it has been shown that in a Ca^{2+} dependent manner, pH between 7.4 and 8.5, also activates mouse TRPA1 channels heterologous expressed in HEK 293 cells (Fujita et al., 2008). Although the mechanism of how Ca^{2+} can modulate the sensitivity of the channel to more basic pH is still elusive, it has been shown that Ca^{2+} potentiates the activation and desensitization states of TRPA1 channels (Zimova et al., 2018). TRPA1 is a sensor for chemical irritants and a major contributor to chemo-nociception that is closely associated with TRPV1 channels, in terms of both expression and function (reviewed in Wang et al., 2019). Similarly to TRPV1, allogenic activators of TRPA1 channels are released from inflammatory environments or tissue injury sites to activate the channel (Chen and Hackos, 2015). For example, several lipid peroxidation products, oxidized lipids, and activators of the inflammasome, stimulate TRPA1 channels by an indirect mechanism involving H_2O_2 production (Trevisan et al., 2014). Additionally, endogenous lipidergic activators like nitrated fatty acids, produced by inflammatory processes, covalently bind to activate TRPA1 channels (Brewster et al., 2015).

TRPM activation mechanisms vary greatly among subfamily members, however, more than half of the members are sensitive to a wide range of temperatures, from cold to hot. For instance, TRPM4 and TRPM8 are activated by temperatures below 15 and 26°C, respectively (Talavera et al., 2005; Yao et al., 2011), while TRPM5 and TRPM2 are activated by temperatures above 35°C (Togashi et al., 2006). TRPM3 is the only member of this family that is activated by harmful heat, around 52°C in peripheral sensory neurons (Vriens et al., 2011). Some channels in this subfamily also respond to redox status, intracellular calcium, low temperatures, or ligands such as menthol. For instance, TRPM2 play a role in the transduction of oxidative stress stimuli (Oancea et al., 2011). In cortical neurons, TRPM2 channels are involved in the cytotoxic influx of Ca^{2+} that is induced by reactive oxygen species such as H_20_2 (Kaneko et al., 2006). TRPM2 also activated by-products of nucleotides metabolisms like ADP-ribose (ADPR) and nicotinamide adenine dinucleotide (NAD) (Nadler et al., 2001; Hara et al., 2002; Kraft and Harteneck, 2005). It is not clear whether ROS directly or indirectly activates TRPM2 downstream of ADPR or NAD, however, recent evidence shows that oxidative stress triggers the production of ADPR mitochondrial that is released to the cytosol to activate TRPM2 (Perraud et al., 2005). It has been also described that H_2O_2 production after DNA damage, especially during certain phases of the cell cycle, induces an accumulation of 2′-deoxy-ATP mediated by an increase in NAD synthesis and a decrease in reserves of cellular ATP (Fliegert et al., 2017). The increased ratio of 2′-deoxy-ATP to ATP facilitates the synthesis of 2′-deoxy-NAD and subsequent hydrolysis to 2′-deoxy-ADPR. It is known that increasing amounts of cellular 2′-deoxy-ADPR mediates TRPM2 activation with similar potency but greater efficacy than ADPR, making it a TRPM2 super-antagonist (Fliegert et al., 2017). These findings are in congruence with the suggestions that TRPM2 activation under an oxidative environment could be related to pathological cell death in neurodegenerative diseases (Xie et al., 2010). TRPM4 and TRPM5 channels are activated by Ca^{2+}, but they are not calcium-permeable (Oancea et al., 2011). The sensitivity of TRPM4 to intracellular Ca^{2+} is controlled by multiple signaling events, including ATP, PKC-dependent phosphorylation, calmodulin (CaM) binding, and membrane potential (Nilius et al., 2005). PIP_2, Ca^{2+}, and the voltage regulate the sensitivity of these channels, however, an increase in temperature in the range of 15 to 35°C further displaces the dependence of the voltage toward more negative potentials (Talavera et al., 2005). TRPM7 is also regulated by ROS and Ca^{2+} entry. Ca^{2+} has been considered as a relevant factor for the strong and lasting activation of TRPM7 in conditions of anoxia, oxidative stress, and metabolic imbalance, which could suggest mechanisms in which TRPM7 is involved and could induce even cell death (Aarts et al., 2003).

The PLC pathway, mediated by increased in intracellular calcium concentration, is an important mechanism involved in the modulation of some members of the TRPM family involved in depletion of PIP_2 and the desensitization of TRPM4, TRPM5, TRPM7, and TRPM8 channels. Specifically, for TRPM4 it has been reported to cause a shift to the left of its voltage dependence and increase its sensitivity to Ca^{2+} 100 times (Owsianik et al., 2006). TRPM8 activation is inhibited by Gq-coupled receptors that mediate PLC activation, however, depletion of Ca^{2+} store activates chemical signaling through lysophospholipids (LPLs), enhancing TRPM8 activity (Vanden Abeele et al., 2006). Also, exogenous PIP_2 (Liu and Qin, 2005), cold, or menthol (Rohács et al., 2005) activates TRPM8.

TRP CHANNELS AND NEURODEGENERATIVE DISEASES

Neuronal cell death rarely occurs in healthy brains, however, it can be triggered by internal/external factors in most neurodegenerative diseases (NDDs), where neurons initially lose their ability to maintain homeostasis due to changes in neuronal morphology, function, and viability (Dugger and Dickson, 2017; Chi et al., 2018). NDDs are categorized by their clinical features, anatomical structures affected, or molecular abnormalities (Kovacs, 2016). Although different in etiology, NDDs share common features, including mitochondria dysfunction, impaired energy metabolism, abnormal voltage-dependent anion channel activation, DNA damage, pro-inflammatory cytokines production, and disruption of cellular and axonal transport (Dugger and Dickson, 2017; Chi et al., 2018).

In the elderly, neurodegenerative diseases are a common and growing cause of mortality and morbidity, being AD and PD the most studied (Rahimi and Kovacs, 2014). AD is the most common form of dementia and makes up to 60–80% of all dementia cases worldwide, affecting an estimated 34 million people globally (Erkkinen et al., 2018). Meanwhile, PD affects 0.2 people per 100 of the population (independently of age), and almost 1–3% of the population older than 60 years (Tysnes and Storstein, 2017). Patients with AD or PD present learning and memory impairments, poor communication skills, irritability, symptoms of anxiety/depression, and progressive motor dysfunction (Batista and Pereira, 2016), and 40–85% of them suffer from painful conditions (Jost and Buhmann, 2019). Although the mechanisms that lead to these painful conditions are not fully understood, it is thought that neuropathological changes that occur in people with AD and PD dementia could alter pain perception (Van Kooten et al., 2016).

Calcium concentration level in neurons is exquisitely controlled to maintain cell homeostasis and to prevent neurodegeneration. The machinery that regulates intracellular Ca^{2+} levels is complex and includes several voltage-dependent plasma membrane calcium-conducting channels, glutamate receptors such as N-methyl-D-aspartate receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, calcium release activated channels, and TRPCs. In addition, calcium flow from the endoplasmatic reticulum (ER) is highly regulated by Ryanodine receptors, Inositol trisphosphate receptor, calcium-dependent kinases, and phosphatases (Brini et al., 2014). Alterations in Ca^{2+} homeostasis have been related to the appearance and progression of several NDDs, including AD and PD (Marambaud et al., 2009; Nevzati et al., 2014). Indeed, it has been reported that exposure to either Aβ peptides (Li et al., 2009) or α-synuclein oligomers (Danzer et al., 2007) induces neuronal death by activating Ca^{2+}dependent signaling pathways and metabolic derangements (Arundine and Tymianski, 2003), most likely by increasing mitochondrial Ca^{2+} levels and the release of proapoptotic factors (Orrenius et al., 2003; Beech, 2005

In physiological conditions, activation of G-coupled receptors at the plasma membrane induces the release of Ca^{2+} from the ER, which in turn stimuli the influx of extracellular Ca^{2+} through a diversity of plasma membrane channels. This process is known as store-operated Ca^{2+} entry (SOCE) (Putney, 1986). SOCE calcium fluxes are mediated by calcium selective ion channels ORAI (calcium release-activated calcium channel proteins) (Kraft, 2015) that allow the calcium release-activated calcium current (I_{CRAC}) and store-operated calcium current (I_{SOC}) mediated by relatively selective Ca^{2+} to non-selective cation channels, such as TRPC1/4/5 (Parekh and Putney, 2005; Yuan et al., 2007). It has been suggested that Orai binds to TRPC1 and the stromal interaction molecule 1 (STIM1) during SOCE activation, enhancing calcium currents (Liao et al., 2008; Zhang et al., 2016). In this regard, TRPCs play a role in $[Ca^{2+}]i$ regulation by modulating SOCE (Minke and Cook, 2002), which joint to other TRPs, such as TRPC3, TRPC4, TRPM2, and TRPM7, respond to oxidative stress (Selvaraj et al., 2012), and may contribute to neurodegeneration (**Figure 2**). Given the expression of TRP channels in brain regions damaged during the development of PD and AD, and their role in Ca^{2+} homeostasis and ROS/RNS sense, they are now considered key players in neuronal degeneration and potentially on altered pain perception (**Figure 3**) (Bernd and Appendino, 2007; Nilius and Flockerzi, 2014; Rojo et al., 2014).

TRPs IN PARKINSON'S DISEASE

PD is characterized by a marked loss of dopaminergic neurons (DNs) in substantia nigra (SN) (Cacabelos, 2017). Although the mechanism by which these neurons degenerate is not well known, mitochondrial dysfunction, oxidative stress, inflammation, altered calcium homeostasis, NO synthesis, protein aggregation, excitotoxicity, and glutathione (GSH) depletion (Mandel et al., 2003), and activation of microglia-mediated by glucocorticoid receptors (GR) (Maatouk et al., 2018), are related to degeneration of DNs (Channels, 2017). Considering that oxidative stress and changes in Ca^{2+} homeostasis are involved in PD, it has been suggested that TRP channels could mediate some of the mechanisms that lead to the development of the disease.

Kim et al. (2005) showed that capsaicin, a TRPV1 agonist, elicits cell death of mesencephalic DNs. Additionally, it has been reported that TRPV1 activation triggers Ca^{2+}-dependent

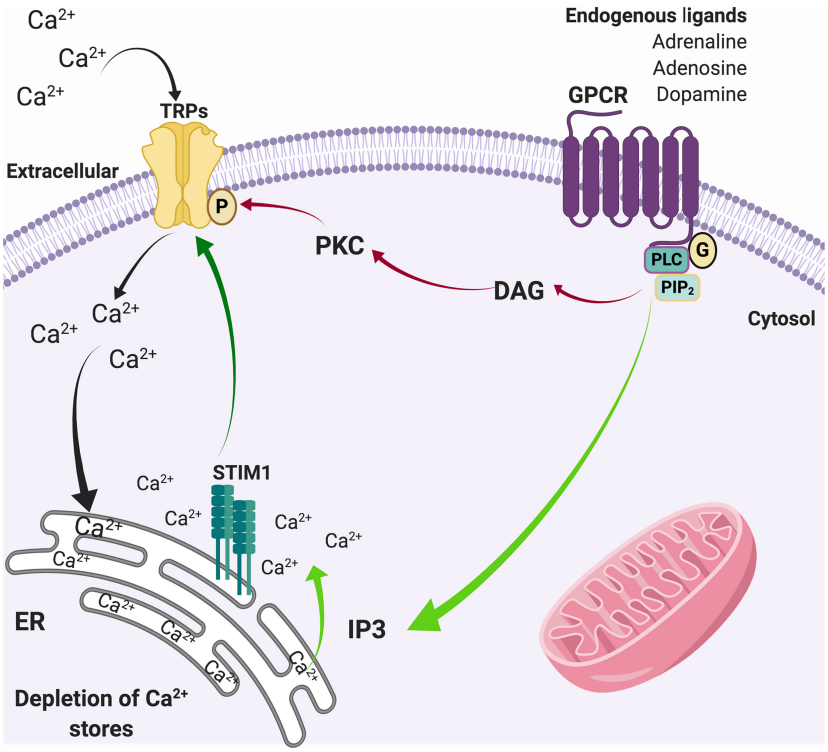

FIGURE 2 | SOCE through TRP channels. Activation of G-protein coupled receptors activates the phospholipase C pathway that induces the hydrolysis of PIP_2 to DAG (red arrows) that actives PKC, which in turn phosphorylates TRP channels. In parallel, the generation of IP_3 (green arrows) promotes the release of Ca^{2+} from the ER. The depletion of intracellular Ca^{2+} stores from the ER is sensed by STIM1, which also activates Ca^{2+} channels in the plasma membrane such as TRPs (dark green arrow), allowing the entry of Ca^{2+} from the extracellular medium to the cytosol (black arrows) to refill de ER deposits.

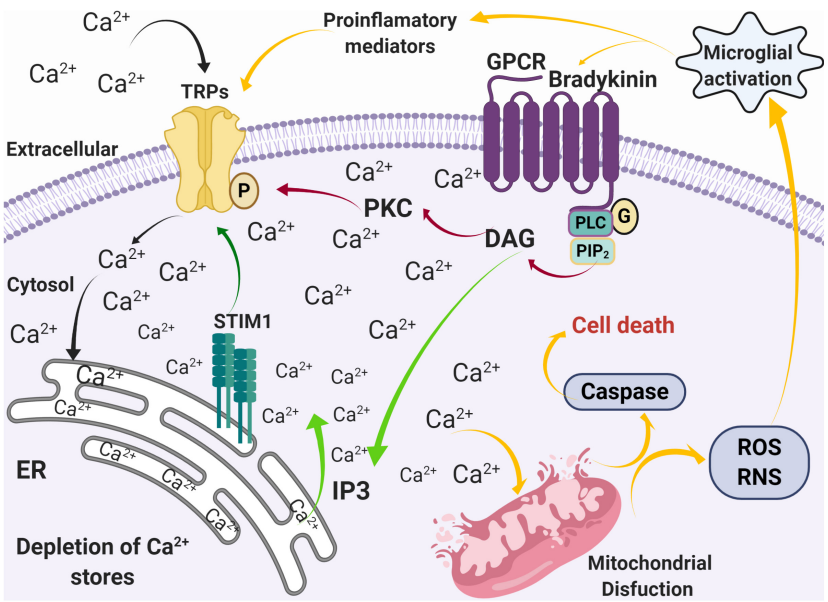

FIGURE 3 | Alterations in calcium homeostasis mediated by SOCE during inflammation and oxidative stress. Activation of G-coupled receptors by pro-inflammatory mediators, such as bradykinin, induces the release of Ca^{2+} from the ER stores through the PLC pathway (green arrows), followed by an influx of Ca^{2+} through Ca^{2+} permeable channels such as TRPs (black arrows). The increase $[Ca^{2+}]i$ then induces mitochondrial dysfunction that leads to caspase activation, ROS and RNS production, microglia activation, and production of pro-inflammatory mediators (yellow arrows).

cell death (Kim et al., 2006) and NADPH-oxidase-mediated production of ROS in microglia (Shirakawa and Kaneko, 2018), suggesting that a similar mechanism could operate in death of DNs in PD. For instance, TRPV1 antagonists such as capsazepine and iodo-resiniferatoxin inhibit DNs death *in vivo* and *in vitro* (Kim et al., 2005). Mechanistically, it is thought that TRPV1 activation induces an increase in $[Ca^{2+}]i$ that impairs mitochondrial function, induces cytochrome release, and caspase-3 cleavage. Consequently, activation of TRPV1 channels contributes to dopaminergic neuron damage *via* Ca^{2+} signaling and mitochondrial disruption (Kim et al., 2005). Although the nature of the endogenous ligands that induce the activation of TRPV1 in PD has not been elucidated, these channels are endogenously activated by anandamide, an endocannabinoid, which is increased in untreated PD patients (Pisani et al., 2010).

In contrast to the toxic role of TRPV1 activation on DNs, TRPC1 has been suggested as a protector and critical mediator of DNs survival (Sun et al., 2012). DNs are characterized by a pacemaker activity that is thought to be dependent on the activation of the Ca^{2+} channel $Ca_v1.3$ and Na^+ channels. Interestingly, Cav1.3-mediated cell death is prevented by translocation of stromal interacting molecule-1 (STIM1) induced by Ca^{2+}depletion of ER, allowing it to interact with and activate calcium permeable channels like TRPC1 to refill the ER Ca^{2+} store (Soboloff et al., 2012; Kraft, 2015). That process protects DNs against the Cav1.3-mediated cell death. Neurotoxins that mimic PD symptoms, such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), increase the activity of the Cav1.3 channel by downregulating the expression of TRPC1, which lead to a decrease in SOCE and the release of Ca^{2+} from ER to the cytosol in DNs and mesenchymal stem cells (Sun et al., 2018). It has been described that MPP^+ (1-methyl-4-phenylpyridinium), a toxic metabolite product of enzymatic activity of MAO-B on MPTP, kills DNs in SN (Choi et al., 2015). In this vein, it has been shown that Cav1.3 silencing or TRPC1 overexpression decreases caspase 3 and inhibits MPP^+-induced cell death. Therefore, TRPC1 expression facilitates STIM1-Cav1.3 interaction, and it is essential for the survival of DNs in PD (Sun et al., 2017). Moreover, Chen et al. (2013) found that the downregulation of Homer 1 protein inhibited the generation of ROS induced by MPP^+ in DNs, without affecting the activity of endogenous antioxidant enzymes; this inhibition was further potentiated by BAPTA-AM. Exposure of DNs to MPP^+ induces a rapid increase in cytosolic Ca^{2+} concentrations after its release from the ER, an effect that was prevented in DNs with low Homer1 expression (Chen et al., 2013).

Beyond its role in DNs-induced cell death, MPP^+ can directly activate microglia and promote the production of several pro-inflammatory mediators and iNOS (Kim et al., 2018; Lee et al., 2019). Once microglia are activated, the release of pro-inflammatory microglial cytokines and chemokines induce the death of dopaminergic (DA) neurons, evidencing the vulnerability of these neurons to glia-mediated neurotoxicity. In the MPTP model, M1 microglia have been associated with dopamine neurodegeneration by the induction of microglial NOS and NADPH oxidase (NOX) (Appel et al., 2015). Mizoguchi et al. found that brain derived neurotrophic factor (BDNF) induces a sustained elevation of $[Ca^{2+}]i$ through the overregulation of TRPC3, which is also crucial for the suppression of NO induced by BDNF-activated microglia. This signaling pathway has been linked to the inflammatory response that mediates DA death in PD (Mizoguchi et al., 2014).

Furthermore, Parkinsonian disorders are often associated with changes in the frequency and firing mode of GABAergic neurons (Zhou et al., 2008). In SN and Globus Pallidus internus, GABAergic neurons project and regulate the firing pattern of thalamic nuclei, superior colliculus, and brainstem motor nuclei, regulating the smoothness and coordination of movements (Zhou et al., 2008). TRPC3 channels selectively expressed in the SN GABA projection neurons regulate the firing pattern of these neurons. The expression of TRPC3 in SN maintains a constant influx of Na^+ that generates a tonic depolarized potential that contributes to the high frequency and regularity pattern of firing of these neurons (Zhou et al., 2008). However, it has been described that ROS-induced increased TRPC3 activity could lead to a more depolarized potential in GABAergic projecting neurons, contributing to the unbalance of disinhibition and inhibition cycles observed in PD (Zhou et al., 2008).

TRPM7, a Zn^{2+}, Ca^{2+}, and Mg^{2+} permeable channel, has been associated with NDDs given its regulation by intracellular Mg^{2+} levels and ROS (Nadler et al., 2001; Sun et al., 2015). PD animal models have shown that Mg^{2+} deficits increase the vulnerability of DNs to MPTP neurotoxicity (Muroyama et al., 2009). Furthermore, Mg^{2+}supplementation inhibits the toxicity of (methyl-4-phenylpyridium ion) by decreasing the death of DNs and maintaining the length of their neurites. These results are in agreement with the observation that TRPM7 is significantly decreased in the SN of PD patients and that long-term Mg^{2+} deficiencies significantly decrease the number of DNs in SN (Oyanagi et al., 2006). These results suggest that DNs utilize TRPM7 channels to regulate Mg^{2+} levels, and that loss of TRPM7 channel function may be involved in the development of PD (Landman et al., 2006).

It has been reported that PD patients have significantly elevated cortisol levels compared to control subjects of the same age (Bellomo et al., 1991; Ros-Bernal et al., 2011). Interestingly, expression of TRPM6 and TRPM7 can be regulated by glucocorticoids (GCs) in a tissue-dependent manner (Cuffe et al., 2015). In the brain, GC signaling is mediated by GRs well as by mineralocorticoid receptors expressed in neurons and glia (Sierra et al., 2008). A large number of studies indicate that activation of GRs by GC promotes inflammatory response (Bhattacharyya et al., 2010), particularly in microglia (Maatouk et al., 2018). For instance, inflammation caused by a low dose of Lipopolysaccharides (LPS) directly administrated in substantia nigra causes a specific loss of dopaminergic neurons (Castaño et al., 2002). Interestingly, pre-treatment with a low dose of dexamethasone (DXM, 1 mg/Kg) diminished nigrostriatal dopaminergic neurons damage in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP, 40 mg/Kg), while a high dose of DXM (10 mg/Kg) further aggravate loss of dopaminergic neurons (Kurkowska-Jastrzębska et al., 2004). However, physiological levels of GC and functional response of GRs are necessary to prevent neurodegeneration; indeed, it

has been reported that in the absence of GR, microglia-induced dopaminergic neuronal loss (Barcia et al., 2011).

TRPs IN ALZHEIMER DISEASE

Altered Ca^{2+} homeostasis has been considered one critical factor regulating neuronal death in AD (Small, 2009). For instance, mutations in presenilins, catalytic subunits of the gamma-secretase, have been linked to Ca^{2+} signaling dysregulation, proteolytic processing of amyloid precursor protein (APP), and thereby increasing production of Aβ peptide (Guo et al., 1997, 1999; Schneider et al., 2001; Banerjee and Hasan, 2005). Aggregation of the Aβ peptide may induce the release of Ca^{2+} stored in the ER, resulting in an overload of cytosolic Ca^{2+}. In response to the rise in $[Ca^{2+}]i$, endogenous levels of GSH are reduced, leading to a ROS accumulation within cells (Ferreiro et al., 2008). In addition, the deposition of Aβ also induces microglial activation (Seabrook et al., 2006) and the release of pro-inflammatory cytokines, initiating pro-inflammatory signaling pathways that subsequently contributes to neuronal damage and death (Wang et al., 2015). Pro-inflammatory cytokines also sensitized TRPV1 channels expressed in a variety of cells, such as microglia, astrocytes, pericytes, and neurons (Tóth et al., 2005), suggesting that these channels contribute to AD-related neuroinflammatory processes. Inhibition of TRPV1 dependent generation of ROS significantly diminishes the detrimental effect of activated microglia and the inflammatory response elicited by astrocytes upon stimulation with the Aβ peptide (Harada and Okajima, 2006; Benito et al., 2012). However, capsaicin activation of TRPV1 protects the hippocampus function by rescuing the effect of Aβ peptide on the hippocampal gamma oscillations (Balleza-Tapia et al., 2018). These differences compared to the response of TRPV1 after activation by capsaicin, could be accounted for by experimental conditions, likely related to β-amyloid concentrations used in both studies (Balleza-Tapia et al., 2018). Purely fibrillary beta-amyloid preparations have been reported to be more toxic in some experimental models (Kurudenkandy et al., 2014; Cohen et al., 2015), and this possibly induces pathological activation of inflammatory mechanisms, mediated by TRPV1 in primary astrocyte culture (Devesa et al., 2011; Tsuji and Aono, 2012).

In the brain, TRPA1 channels play an essential role in their development and function of non-neuronal cells, such as astrocytes (Shigetomi et al., 2012, 2013). Although AD is a complex disease in which several mechanisms may act, recent studies have evaluated the role of Ca^{2+} related signaling pathways in the etiology and development of the disease (Yamamoto et al., 2007; Takada et al., 2013). Lee et al. (2016) demonstrated *in vitro* that Aβ triggers a TRPA1-dependent Ca^{2+} influx and astrocytic activation. Additionally, ablation of TRPA1 in APP/PS1 transgenic mice slowed the progression of AD and improved learning and memory performance, and reduced Aβ plaques and cytokines (Lee et al., 2016). These results have been further supported by TRPA1 expression in HEK cells, where Aβ is also capable of inducing TRPA1 dependent Ca^{2+} signaling, that activate transcription factors such as NF-κB and NFAT and promote expression of pro-inflammatory cytokines (Lee et al., 2016).

Interestingly, loss-of-function or pharmacological inhibition of TRPM2 channels prevents microglial activation and TNF-α production induced by a wide range of Aβ42 concentrations (10–300 nM), proving a novel role of TRPM2 in microglial activation triggered by Aβ42 peptides (Alawieyah et al., 2018). Likewise, Ostapchenko et al. (2015) demonstrated that TRPM2 ablation in AD models decreases microglial activation, improves the expression of synaptic markers and reduces the deficits in memory observed in aging animals (Ostapchenko et al., 2015). Furthermore, it has been shown that TRPM2 endogenous expression in rat striatum neurons and activation by Aβ and oxidative stress is enough to drive cell death, suggesting that TRPM2 is an active transducer of ROS signaling that may contribute to neuronal death in AD (Fonfria et al., 2005). At a cellular level, ROS levels are regulated by a complex mechanism that involves antioxidant enzymes and small-molecule antioxidants such as GSH (Geon et al., 2015). GSH levels tend to be lower with age and have been considered as markers of cognitive impairment severity (McCaddon et al., 2003). Interestingly, in neuronal cultures that recapitulate aging, GSH supplementation significantly decreases TRPM2 expression and activity (Sita et al., 2018). Therefore, downregulation of the antioxidant defense plus the Aβ-induced production of ROS and cytokines in AD can lead to the activation of several TRP channels that can increase $[Ca^{2+}]i$, leading to excitotoxicity and apoptosis (Park et al., 2014).

Although some advances have made in understanding the role of TRP channels in neurodegenerative diseases, we are still far from having an integrated comprehension of the role of these channels in the etiology and development of these diseases. For instance, more studies are needed to unveil how all these channels work together either to degenerate or protect neurons in PD and AD.

TRPs INVOLVEMENT IN PAIN, ALZHEIMER'S, AND PARKINSON'S DISEASES

During the past decade it has been an increasing awareness of pain and pain management as important issues to address in the elderly (Ali et al., 2018) and patients with neurodegenerative diseases (Cravello et al., 2019). Pain symptoms in NDD patients include sleep disorders, musculoskeletal problems, reduced mobility, falls, malnutrition, cognitive impairment, increased drugs use, diminished social behavior, anhedonia, and depression (Cravello et al., 2019). Prevalence of painful symptoms in patients with AD range from 38 to 75%, and from 40 to 86% in PD (Batista and Pereira, 2016; Van Kooten et al., 2016; de Tommaso et al., 2017; Cravello et al., 2019).

Even when PD was previously considered as a purely motor disorder, now it is known that non-motor symptoms, including pain, occur throughout the course of the disease and significantly affect the quality of life (Jost and Buhmann, 2019).

Some nociceptive pain associated with PD is a secondary consequence of the motor impairment (abnormal muscular tone, spasms, rigidity, reduced active mobility, osteoarticular problems, and local inflammation), however, as many as 43% of Parkinson patients exhibits characteristics typical of neuropathic dysfunction (burning, tingling, formicating, decreased nocifensive flexion reflex, and lowered cold threshold) (Reichling and Levine, 2011; Skogar and Lokk, 2016; de Tommaso et al., 2017). Neuropathic pain has been recently studied in a model of nigro-estriatal pathway lesion, which induces allodynia and hyperalgesia in rats (Romero-Sánchez et al., 2019).

Similarly, it has been described that pain is more prevalent in AD patients, and that intensity of pain is also positively correlated with dementia severity (Cao et al., 2019). Typical cognitive impairment observed in AD also affect the assessment of a painful experience and the ability to describe it (Cravello et al., 2019). It has been reported that neural circuits mediating pain perception and its behavioral expression may be hyperactive or underactive in AD: Specifically, altered pain response seems to depend on the extension of the brain tissue damage, stage of the disease, and type of pain (acute stimuli or chronic medical conditions) (Monroe et al., 2012).

Recently, neuropathological changes occurring during the progress of dementias are being considered as possible causes of pain perception alterations (Cravello et al., 2019), and it has been suggested that primary neuropathic pain is not a simple consequence of nervous system deterioration but instead the result of the very same cellular processes that underlie neurodegenerative diseases (Reichling and Levine, 2011; Cravello et al., 2019). The neuropathological changes that occur in AD affect structures comprised in CNS processing affective-motivational (hippocampus, entorhinal cortex, cingulate gyrus, hippocampus, amygdala), cognitive-evaluative (prefrontal cortices), and sensory-discriminative (somatosensory cortex) aspects of pain (Monroe et al., 2012; Achterberg et al., 2013; Dugger and Dickson, 2017). Similarly, in PD, insufficient input from dopaminergic neurons to basal ganglia and motor and prefrontal cortices results in enhanced inhibitory inputs, which leads not only to body movement-related symptoms but also cognitive and emotional symptoms associated to altered pain perception (Chi et al., 2018).

Figure 4 shows brain's structures involved in pain perception, which include the prefrontal cortex, hippocampus, amygdala, entorhinal cortex, anterior cingulate cortex, basal ganglia, thalamus, insula, and sensory cortex (Fenton et al., 2015; Mano and Seymour, 2015; Cao et al., 2019), and TRP channels expressed in each one of these structures (Kauer and Gibson, 2009; Harteneck and Leuner, 2014; Nilius and Szallasi, 2015; Frias and Merighi, 2016; Katz et al., 2017). As described before, TRP channels have an unique role in nociceptive, neuropathic, and inflammatory pain as diverse members of their families are involved in pain pathways (Hung and Tan, 2018). For instance, members of TRPA, TRPV, and TRPM subfamilies have high expression levels in neurons mediating neuropathic pain (Naziroğlu, 2012). Interestingly, members of the TRPC and TRPM families are expressed in SN, basal ganglia, and hippocampus, brain structures that exhibit significant loss of neurons at the initial stages of the development of AD or PD, respectively. The specific role of TRPs on NDD-related pain symptoms have not been thoroughly studied. However, several lines of evidence indicate a relationship between pain, neurodegeneration and TRPs, particularly related to inflammation.

At the molecular level, it has been proposed that pain-related to NDDs is associated, not only to loss of selected neuronal population but to microglial activation, that response

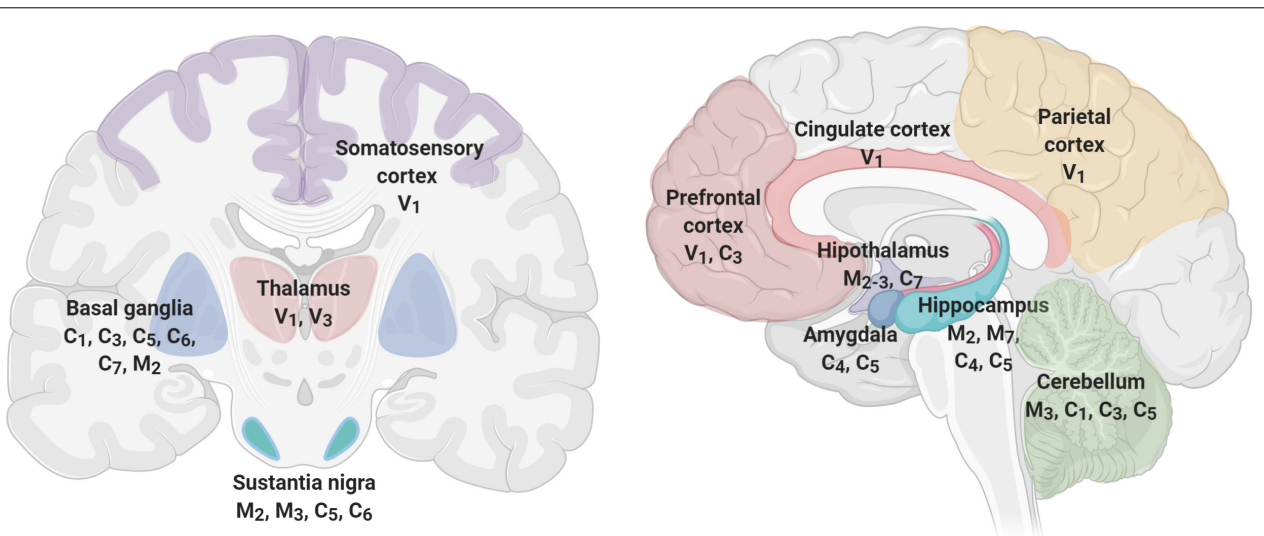

FIGURE 4 | TRP channels are expressed in brain structures involved in pain perception. Pain processing includes cortical (prefrontal, parietal, somatosensory, and cingulate), limbic (amygdala, hippocampus, thalamus, hypothalamus), and movement-related structures (Basal Ganglia, Substantia Nigra, and Cerebellum) that express several members of the TRP channels. TRP families are represented by capital letters as follow, C, TRPC; M, TRPM; V, TRPV; A, TRPA. Numbers indicates specific members of each family.

to noxious stimuli realizing inflammatory mediators such as pro-inflammatory cytokines, interleukins, and tumor necrosis factor alpha (TNFα) (Carniglia et al., 2017). Notably, chronic pain (inflammatory or neuropathic pain) related to neurodegeneration is also accompanied by neuroimmune activation and an escalated response that impairs homeostatic balance since anti-inflammatory mediators are not released, inducing further tissue damage, neuroinflammation, and neurodegeneration (Carniglia et al., 2017; Salter and Stevens, 2017; Inoue and Tsuda, 2018). The role of glial cells in the initiation, sensitization, and maintenance of chronic pain has been studied during the past two decades (Cravello et al., 2019), and it has been found that neuromodulators produced by microglia can rapidly alter synaptic plasticity, a driving force for the pathogenesis of pain after tissue or nerve injury (Chen et al., 2018).

During inflammatory pain, inflammatory molecules can change the TRP threshold activation, inducing mechanical allodynia, thermal hyperalgesia, and spontaneous pain. TRPV1, TRPA1, and TRPM2 channels have been intensely studied in pain sensation because they participate in the cellular signaling mechanism through which injury produces pain hypersensitivity. These channels can be activated by thermal stimuli and endogenous molecules derived from the inflammation process (Ma and Quirion, 2007; Hung and Tan, 2018). After an injury, inflammatory molecules such as eicosanoids, neuropeptides, and cytokines decrease the thresholds of sensory neurons, inducing sensitization in TRPV1 (Julius, 2013). As TRPA1 is highly regulated by oxidative stress and is targeted by different reactive species, so that they are activated during inflammatory progression, where ROS produced after tissue injury induces superoxidation of membrane phospholipids and activation of the channel (Julius, 2013; Mori et al., 2016; De Logu et al., 2017; Hung and Tan, 2018). The role of TRPM2 in pain generation is through their activation by reactive nitrogen species (Kaneko et al., 2006). Similar to TRPV1, TRPM2 suffers sensitization by H_2O_2 that lowered the temperature of activation (Kashio et al., 2012). Interestingly, it has been described that chronic pain is a risk factor to develop memory impairment, dementia, and other neuropsychiatric conditions (Moriarty and Finn, 2014; Whitlock et al., 2017).

TRP channels expressed in sensory neurons have an essential function in pain and inflammation transduction (Fernandes et al., 2012; Smani et al., 2015). Similarly, it has been reported that microglial TRP channels have a significant role in pain modulation as well as in AD and PD (Cravello et al., 2019) by regulating the levels of ROS, pro-inflammatory cytokines, and the homeostasis of Ca^{2+}. All these processes are connected with microglial activation, which is a cellular process proposed as a central player in both pain and neurodegenerative diseases (Miyake et al., 2014; Echeverry et al., 2016). For example, during inflammation, an upregulation of TRPM2 channels in microglia leads to an exacerbated inflammatory response mediated by ROS. This mechanism has been proposed as one of the primary inductors of inflammation and neuropathic pain (Haraguchi et al., 2012). However, It has also been shown that TRPV1 channels protect mesencephalic DA neurons by inhibiting microglia-originated oxidative stress, suggesting that TRPV1 channels may be novel targets for regulating the oxidative stress-mediated neurodegeneration observed in PD (Park et al., 2012).

TRP-dependent microglial activation involves the influx of Ca^{2+} and the activation of Ca^{2+} -mediated signaling pathways that induce the synthesis of pro-inflammatory molecules, including interleukins (IL-1β and IL-12), chemokines, prostaglandins (PGs), TNF-α, ROS, and NO. These molecules promote an exacerbated inflammatory response by the recruitment of other immune cells that conduce to neuronal damage. However, when the injurious stimuli are controlled, the inflammatory response is diminished by cytokines with anti-inflammatory activity such as transforming growth factor (TGF-β) and IL-10 by microglia. Therefore, the imbalance of microglial activation could exacerbate the pro-inflammatory response, leading to neuronal degeneration and cell death in AD and PD, and neuropathic and inflammatory pain (Suter et al., 2007; Ji et al., 2013; Beggs and Salter, 2016; Carniglia et al., 2017).

Some kinases have a described role in pain. It was reported that extracellular signal-regulated kinases 1/2 and 5 (ERK1/2 and ERK5) are expressed in microglia, and their phosphorylation is induced during neuropathic pain (Tatsumi et al., 2015; Carniglia et al., 2017). Furthermore, it was observed that neuropathic pain induced by nerve injury, promoted the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in spinal microglia. p38 MAPK is activated by multiple microglial receptors, inflammatory cytokines, membrane depolarization, and Ca^{2+} influx. This pathway regulates pro-inflammatory signaling networks as well as the production of diverse inflammatory molecules associated with pain facilitation, including the cytokines TNF-α and IL-1β (Ji and Suter, 2007; Lee and Kim, 2017). Moreover, it was confirmed that the inhibition of p38 MAPK decreases the release of pro-inflammatory cytokines, inducing relieve of mechanical allodynia in diverse models of neuropathic and inflammatory pain (Jin et al., 2003; Lee and Kim, 2017; Inoue and Tsuda, 2018). Interestingly, in addition to their role in pain, the p38 MAPK pathway has also been involved in the cellular mechanisms that regulate neurodegeneration (Lee and Kim, 2017; Kheiri et al., 2019). Activated p38 MAPK was observed in peripheral blood leukocytes and neuronal cells, as well as in postmortem brain from patients with AD (Sun et al., 2003; Kheiri et al., 2019).

p38 MAPK role in AD has been associated with both Tau protein and Aβ peptide, which are essential players in AD pathologies. For instance, Aβ peptide promotes the activation of p38 MAPK, which in turn, phosphorylates Tau protein in neuronal cells (Lee and Kim, 2017). In this vein, it has been described that Aβ peptides suppress nociception and inflammatory pain in APP overexpressing CRND8 transgenic mice (Shukla et al., 2013); this finding is in accordance with the finding that mice treated with a single intracerebroventricular injection of Aβ fragment (1–40) (400 pmol/mice) displayed increased pain tolerance (Pamplona et al., 2010). However, pain sensitivity could be altered in a more complex form since i.c.v. Aβ treated mice also display anxiogenic-like and depressive-like states, which are related to alterations in cognitive/emotional components of pain processing (Pamplona et al., 2010). Also, it

has been described that Tau depletion, *in-vivo* studies, negatively affects the main systems conveying nociceptive information to the CNS (Sotiropoulos et al., 2014). Tau-null (Tau−/−) mice display reduced C-fiber density and Aδ-fiber hypomyelination followed by diminished conduction properties sciatic nerves and decreased nociception but increased excitability of second-order spinal cord nociceptive neurons, resulting in heightened pain-like behaviors (Sotiropoulos et al., 2014; Lopes et al., 2016). These findings suggest that APP and Aβ peptides and Tau protein could affect in a complex way pain perception in AD patients.

Several reports also suggest that p38 MAPK is also involved in PD. It is proposed that oxidative stress in dopaminergic neurons prompted the activation of the p38 MAPK and c-Jun N-terminal kinase (JNK) signaling pathways that have linked to neuronal apoptosis in several models of PD (Oh et al., 2011; Sabens Liedhegner et al., 2011; Bohush et al., 2018). p38-MAPK activation has also been reported to contribute to mitophagy, a fundamental mechanism underlying α-synuclein accumulation associated with PD (Cheng et al., 2018).

TRP channels function has been related to p38 MAPK pathway activity. It has been reported that phosphorylated p38 MAPK stimulated by noxious cold colocalized in neurons that express TRPA1 channels (Mizushima et al., 2006). Additionally, stimulation of microglia with lipopolysaccharide and interferon γ (LPS/IFN γ) promoted the activation of TRPM2 channels and Ca^{2+} dependent signaling pathways, and the increase in p38 MAPK signaling (Miyake et al., 2014). Interestingly, the use of TRPM2 inhibitors inhibited the extracellular Ca^{2+} influx, affecting the activation of the p38 MAPK pathway. Similar results have been observed in TRPM2-KO microglia, where NO release was attenuated (Haraguchi et al., 2012). It is suggested that TRPM2 recruits the p38 MAPK pathways for NO production induced by LPS/INFγ. Furthermore, phosphorylation of p38 MAPK was abolished in TRPM2-knockout microglia, indicating that this process is selectively dependent on TRPM2 signaling. Similarly, lisophosphatidylcholine (LPC), an endogenous inflammatory phospholipid that induces TRPM2 translocation to the plasma membrane, also promotes Ca^{2+} influx and microglia activation. It has been demonstrated that LPC increases phosphorylation of p38 MAPK in microglia, which was eliminated in TRPM2-KO. From these results, it is feasible to propose TRPM2 channels as potential therapeutic targets to inhibit excessive microglial activation, neuroinflammation, and, therefore, pain through modulation of p38 MAPK phosphorylation (Miyake et al., 2014; Jeong et al., 2017; Shirakawa and Kaneko, 2018).

Considering that the p38 MAPK pathway is a central player in neurodegeneration and pain, several recent studies have been focused in search of p38 MAPK activity modulators, and some molecules have shown anti-inflammatory activity (Jeong et al., 2017; Kheiri et al., 2019). However, cross-reactivity with other kinases and the appearance of cardiovascular, psychiatric, and hepatic side effects have halted the use of these molecules, suggesting that it is necessary to study further the mechanism by which p38 MAPK could be modulated to avoid the adverse side effects observed (Ji and Suter, 2007; Kheiri et al., 2019).

In addition to the regulation of p38 MAPK phosphorylation in microglia, TRP channels also play a role in the generation of peripheral pain through oxidative stress. Oxidative stress-mediated by lipid peroxidation has been observed in both neurological and peripheral pain. It has been proposed that selenium could act as neuroprotector through a mechanism that involves TRP channels inhibition, which in turn, induces modulation of ROS overproduction and Ca^{2+} influx (Nazıroğlu et al., 2020). Selenium is an inhibitor of TRPM2 channels, which reduces oxidative stress in the cytosol (Zeng et al., 2012). Besides TRPM2, selenium also acts as TRPA1 and TRPV1 inhibitor, suggesting that selenium could be used as a modulator of neuropathic pain through TRP channel modulation (Nazıroğlu et al., 2020).

Despite high rates of painful comorbidities, lower use of analgesics among individuals with dementia has been reported (Van Kooten et al., 2016). Detriment in pain management seems to occur in part due to challenging pain assessment in patients with compromised cognition and impaired communication skills, as well as barriers to analgesics (Shen et al., 2018).

Currently, several families of agents have been of clinical utility to treat pain. The most common analgesic drug prescribed for mild to moderate pain is paracetamol (also known as acetaminophen); however, for peripheral or central neuropathic pain, this analgesic drug has poor effectiveness. Opioids, anticonvulsants, nonsteroidal anti-inflammatory drugs (NSAIDs), topical medications, and more recently, third-generation antidepressants have been used to treat pain related to nerve injury (Lynch and Watson, 2006; Yaksh et al., 2015). However, important drugs safety and side effects limit their use; this is particularly important in the case of opioids, which are the most effective pain killers but have high potential to induce addiction and may cause sedation and respiratory depression (Moran and Szallasi, 2018). Clinical daily work shows that the use of painkillers, opioids, antidepressants, or anticonvulsive drugs are often not sufficient to treat pain in neurodegenerative diseases, so it has been suggested that in selected individuals, refractory to conventional treatment of pain, cannabinoid management could be attempted (Jost and Buhmann, 2019). It has been recently shown that cannabinoids provide promising multitarget approach for the treatment of pain and neurodegeneration since they regulate the activity of TRP channels, which are considered non-cannonical endocannabionoid receptors. In this vein, it has been shown that cannabidiol, cannabinol, cannabigerol, or cannabidiolic acid binds TRPs, including TRPV1–4, TRPA1, and TRPM8 (Shirakawa and Kaneko, 2018; Muller et al., 2019; Starkus et al., 2019).

Since TRPs are involved in the progression of neurodegenerative diseases and have a role in pain, they are remarkable potential targets for the treatment of both pain and neurodegenerations (Zündorf and Reiser, 2011; Naziroğlu, 2012; Maiese, 2017; Echeverry et al., 2016; Belrose and Jackson, 2018). Recent evidence regarding the involvement of TRP channels in several diseases has led to the identification of TRP channels as potential drug targets to manage pain. For instance, capsaicin, an agonist of TRPV1, has been used in clinical trials

to control neuropathic pain conditions (Kiani et al., 2015; Derry et al., 2017), however, its use would be limited by two major adverse effects of TRPV1 channel agonists/antagonists: (a) dysregulation of body temperature, and (b) long-lasting compromise of temperature sensation leading to burning injuries. Agents targeting TRPM8, TRPV2, TRPV3, TRPV4, and TRPA1 have also been tested with mixed results. Interestingly, in animal models, TRPA1 deletion or inhibition reduces pain associated with inflammation, as well as inflammation *per se* (Moilanen et al., 2015, 2016; Horváth et al., 2016). A role for TRPA1 channels in neurogenic inflammation has been suggested (Moran and Szallasi, 2018); indeed, a Phase 2 clinical trial has have reported that the Glenmark's GRC 17536 TRPA1 channel antagonist significantly reduce pain scores in a pre-specified subset of patients with painful diabetic neuropathy and intact sensory responses without notable side effects (Moran and Szallasi, 2018). Recently, TRPM2 inhibitors have been proposed as a potential candidate to treat neurodegeneration and pain, and several novel molecules targeting TRPM2 (8Br-ADPR, 8-Ph-2'-deoxy-ADPR and novel ADPR analogs capable of selectively inhibiting TRPM2) appear as potential candidates to develop novel therapeutic agents (Belrose and Jackson, 2018). Notably, a cell-permeable peptide tat-M2NX that inhibits TRPM2 provides protection from ischemic stroke in adult mice decreases infarct volume with a clinically relevant therapeutic window (when provided either prior to the infarct or 3 h following the insult) (Shimizu et al., 2016).

Since TRP channels are involved in numerous physiological processes, attention should be paid to potential side effects of drugs able to block TRP channels their function. Concerns predominantly relate to the roles of TRP channels in temperature sensation and regulation, immune function, and insulin release (Belrose and Jackson, 2018). Ultimately, assessment of the risk-benefit profile of TRPs as therapeutic targets will require the development of specific compounds with favorable pharmacokinetic and pharmacodynamic properties and identification of specific patient populations that would benefit the most (Belrose and Jackson, 2018). In this vein, it would be worth testing selective drugs targeting TRPs to manage neurodegeneration and treat associated symptoms such as pain and cognitive/motor dysfunction. The evidence suggests that the effectiveness of pharmacological agents regulating TRP channel activity to treat neuropathological processes and pain deserves further research. Evaluation of the risks and benefits of TRPs' use as therapeutic targets will need the development of compounds with favorable pharmacological properties and identification of specific patient populations that would benefit the most. In this regard, it would be worthy of testing selective drugs targeting TRPs to manage neurodegeneration and treat associated symptoms, such as pain and cognitive/motor dysfunctions. Furthermore, given that TRPs are involved in the progression of neurodegenerative diseases and have a role in pain, it is feasible to propose that these channels could act as central players that connect both processes, making TRP channels potential targets to treat pain in NDDs patients (Zündorf and Reiser, 2011; Naziroğlu, 2012; Echeverry et al., 2016; Maiese, 2017). Direct evidence describing the role of TRPs on pain related to NDDs development is still required, however, indirect evidence suggests that this subject deserves further research and supposes and interesting field of research.

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