ADENOSINERGIC SYSTEM AND CENTRAL NERVOUS SYSTEM DISORDERS

EDITED BY: Francisney Pinto Nascimento, Paula Agostinho and Rui Daniel Prediger

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ADENOSINERGIC SYSTEM AND CENTRAL NERVOUS SYSTEM DISORDERS

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REVIEW
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Caffeine and Parkinson's Disease: Multiple Benefits and Emerging Mechanisms

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by dopaminergic neurodegeneration, motor impairment and non-motor symptoms. Epidemiological and experimental investigations into potential risk factors have firmly established that dietary factor caffeine, the most-widely consumed psychoactive substance, may exerts not only neuroprotective but a motor and non-motor (cognitive) benefits in PD. These multi-benefits of caffeine in PD are supported by convergence of epidemiological and animal evidence. At least six large prospective epidemiological studies have firmly established a relationship between increased caffeine consumption and decreased risk of developing PD. In addition, animal studies have also demonstrated that caffeine confers neuroprotection against dopaminergic neurodegeneration using PD models of mitochondrial toxins (MPTP, 6-OHDA, and rotenone) and expression of α-synuclein (α-Syn). While caffeine has complex pharmacological profiles, studies with genetic knockout mice have clearly revealed that caffeine's action is largely mediated by the brain adenosine A_{2A} receptor (A_{2A}R) and confer neuroprotection by modulating neuroinflammation and excitotoxicity and mitochondrial function. Interestingly, recent studies have highlighted emerging new mechanisms including caffeine modulation of α-Syn degradation with enhanced autophagy and caffeine modulation of gut microbiota and gut-brain axis in PD models. Importantly, since the first clinical trial in 2003, United States FDA has finally approved clinical use of the A_{2A}R antagonist istradefylline for the treatment of PD with OFF-time in Sept. 2019. To realize therapeutic potential of caffeine in PD, genetic study of caffeine and risk genes in human population may identify useful pharmacogenetic markers for predicting individual responses to caffeine in PD clinical trials and thus offer a unique opportunity for "personalized medicine" in PD.

Keywords: caffeine, Parkinson's disease, α-synuclein, adenosine A_{2A} receptor, autophagy, gut microbiota

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by clinical presentation of motor impairment and non-motor symptoms. The hallmarks of PD pathology are selective degeneration of dopaminergic neurons in the midbrain and by the prominent α-Syn-containing proteinaceous inclusions, Lewy body (Dauer and Przedborski, 2003;

Kalia and Kalia, 2015; Postuma and Berg, 2016; Wong and Krainc, 2017). PD affect 1% of the world population age 65 and older, but with the aging population and increases in life expectancy in modern society, the number of PD cases is projected to double by 2030 (Dorsey et al., 2007; Lees et al., 2009). Dopamine replacement, such as L-dopa, is the mainstay treatment to control motor symptoms (Rascol et al., 2002; Lang and Obeso, 2004; Goetz et al., 2005), but chronic L-dopa treatment is associated with loss of its efficacy and the onset of debilitating motor complications (dyskinesia, wearing-off and ON-OFF) (Ahlskog and Muenter, 2001). Furthermore, with the discovery of α-Syn as the main pathogenesis gene and the main component of Lewy body (Lashuel et al., 2013; Kalia and Kalia, 2015; Wong and Krainc, 2017), wide-spread distribution of α-Syn aggregates in the brain provide the pathological basis for non-motor symptoms of PD (e.g., cognitive dysfunction, fatigue, balance impairment, sleep disturbance, autonomic dysfunction) which are increasingly recognized as a key component of the disease (Chaudhuri et al., 2006; Postuma and Berg, 2016). Importantly, despite of the extensive studies, there are no successful treatments currently available that can slow or halt this chronic neurodegeneration (Shoulson, 1998; Mattson, 2004; Olanow, 2004; Lopez-Diego and Weiner, 2008; Mestre et al., 2009).

In the absence of an effective disease modifying treatment to slow down or hold PD course, epidemiological and experimental investigations into potential risk factors (such as dietary factor caffeine) that may modify PD pathology and confer therapeutic benefits become compelling. Caffeine is probably the most widely psychoactive substance and regularly consumed by >50% of the world's adult population, largely for its psychostimulant (and cognitive enhancement) effect, reducing fatigue and enhancing performance. The regular human consumption of caffeine was previously estimated to be 70–350 mg/person/day or 5 to 8 mg/kg/day (equivalent to three couples of coffee) which is absorbed by the small intestine within 45 min of ingestion and produces peak plasma concentration of 0.25 to 2 mg/l (or approximately 1 to 10 μM) within 1–2 h, distributes throughout all bodily tissues and produces overall psychostimulant effects, plasma caffeine levels are usually in the range of 2–10 mg/L in coffee drinkers (Nehlig et al., 1992; Fredholm et al., 1999b). Caffeine-containing drinks, such as coffee, tea, and cola, are very popular; as of 2014, 85% of American adults consumed some form of caffeine daily, consuming 164 mg on average (Mitchell et al., 2014). Caffeine intake doses determined not to be associated with adverse effects by Health Canada (comparators: 400 mg/day for adults [10 g for lethality], 300 mg/day for pregnant women, and 2.5 mg/kg/day for children and adolescents), 2.5 mg caffeine/kg body weight/day remains an appropriate recommendation (Wikoff et al., 2017). In healthy adults, caffeine's half-life is between 3 and 7 h, caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system, in particular, by the CYP1A2 isozyme, into three dimethylxanthines, including paraxanthine, theobromine, and theophylline, each of which has its own effects on the human body. Caffeine was found to present in all rat tissues after administration for 10 days and accumulated for 25 days. The caffeine level was high in brain, liver and kidney and widely distributed and accumulated in these organs (Che et al., 2012). Because caffeine is both water- and lipid-soluble, it readily crosses the blood brain barrier. Once in the brain, caffeine may act at multiple molecular targets to produce complex pharmacological actions, ranging from adenosine receptor antagonism, to phosphodiesterase inhibition, to GABA receptor blockade and calcium release (Fredholm et al., 1999a). However, genetic knockout studies have demonstrated that caffeine's primary action in the brain is as an antagonist of adenosine receptors (mainly $A_{2A}R$) (Chen et al., 2013).

In this review, we first summarized the neuroprotective, motor and cognitive benefits of caffeine in PD patients and PD models. We then described the potential mechanisms underlying caffeine's protective effects, including modulation of neuroinflammation and of newly emerging mechanisms associated with autophagy and gut microbiota. Lastly, we discussed possible genetic polymorphisms of caffeine-associated genes in influencing caffeine drug responses and its clinical implications.

MULTIPLE BENEFITS OF CAFFEINE IN PD TREATMENT

Potential Neuroprotective Effects of Caffeine in PD Patients and PD Models

The first evidence for the potential neuroprotective effect of caffeine came from the Honolulu Heart Program, a large prospective study of 8004 Japanese-American men over a 30 years follow-up study. The study revealed that daily consumption of 784 mg/kg or more of coffee during the mid-life reduce the risk for developing PD at age of 65-year old by 5-folds compared to non-coffee drinkers after age- and smoking-adjustment (Ross et al., 2000). This inverse relationship between consumption of caffeine and the risk of developing PD was further supported by the Health Professionals' Follow-Up Study and the Nurses' Health Study – involving 47,351 men and 88,565 women, and subsequently also by at least three additional large prospective studies, including Finnish Mobile Clinic Health Examination Survey (Saaksjarvi et al., 2008) and the NeuroGenetics Research Consortium (Powers et al., 2008) and Danish case-control study involving idiopathic PD (Kyrozis et al., 2013). A meta-analysis of 13 study involving total 901,764 participants for coffee intake found a non-linear relationship was found between coffee intake and PD risk, with maximum protection effect at approximately 3 cups/day (Qi and Li, 2014). Systemic analysis of 120 observation studies have firmly established that regular human consumption of caffeine is associated with reduced risk for PD (Ross et al., 2000; Ascherio et al., 2001, 2003; Saaksjarvi et al., 2008; Grosso et al., 2017) and does not impose significant adverse effects on the cardiovascular system, bone status, or the incidence of cancer (Fredholm et al., 1999b; Winkelmayer et al., 2005; Higdon and Frei, 2006; van Dam et al., 2006; Cadden et al., 2007; Daly, 2007). Interestingly, this inverse relationship of coffee consumption and risker for developing PD is largely attributed to caffeine since that the consumption of caffeinated (but not decaffeinated) coffee is

associated with the reduced risk of developing PD in the Health Professional Follow-up Study (Ascherio et al., 2001). Notably, this inverse relationship is strong and consistent in men in the Health Professional Follow-up Study (Grosso et al., 2017) and in postmenopausal women who never used hormone replacement therapy in the Cancer Prevention Study II Nutrition Cohort (CPS II-Nutrition) (Palacios et al., 2012), but uncertain in women and postmenopausal women ever users of hormone replacement therapy. Thus, caffeine and estrogen interaction modify the risk of developing PD. More recently, the Harvard Biomarkers Study (HBS) conducted a cross-sectional, case-control study in 566 subjects consisting of idiopathic PD patients and healthy controls, which highlighted the robustness of lower caffeine intake and plasma urate levels as factors inversely associated with idiopathic PD (Bakshi et al., 2020).

Furthermore, the neuroprotective effect of caffeine from epidemiological investigation is further supported by mounting evidence from animal studies demonstrating that caffeine confers neuroprotection against dopaminergic neurodegeneration in neurotoxin PD models using mitochondrial toxins (MPTP, 6-OHDA and rotenone) (Chen et al., 2001; Ikeda et al., 2002; Xu et al., 2002; Aguiar et al., 2006) and α-Syn transmission mouse model through intracerebral injection of α-Syn fibers (Luan et al., 2018). For example, acute or chronic treatment with caffeine attenuates MPTP-induced dopaminergic neurotoxicity and neurodegeneration (Chen et al., 2001; Xu et al., 2002). It is interesting to note that in a chronic MPTP infusion model of PD caffeine can still confer protection against dopamine neurodegeneration even when caffeine was administered after the onset of the neurodegenerative process (i.e., 14 days after MPTP infusion) (Sonsalla et al., 2012). Furthermore, our recent study demonstrated that caffeine can protect against A53T mutant α-Syn induced pathological alterations in intact animals using the α-Syn fibril model of PD, an effect associated with the enhanced activity of autophagy (specifically macroautophagy and CMA) (Luan et al., 2018) (see below for the more discussion).

Importantly, similar to caffeine, pharmacological blockade or genetic deletion of adenosine A_{2A} receptor ($A_{2A}R$, the main pharmacological target of caffeine in the brain) also protected against dopaminergic neurodegeneration in animal models of PD (Chen et al., 2001; Ikeda et al., 2002; Kachroo and Schwarzschild, 2012), suggesting that the protective effects of caffeine are likely due to its action on the $A_{2A}R$. Moreover, recent studies also show that $A_{2A}R$ modulated α-Syn aggregation and toxicity in SH-SY5Y cells (Ferreira et al., 2017a), and $A_{2A}R$ blockade rescued synaptic and cognitive deficits in α-Syn transgenic mice model of PD (Ferreira et al., 2017b). These animal studies provide a neurobiological basis for the inverse relationship between caffeine consumption and the reduced risk of developing PD, and support the clinical potential for caffeine and $A_{2A}R$ antagonists as a disease-modifying drug target for PD.

Two important caveats need to be taken into the consideration. First, caffeine exerts synergistic neuroprotection with eicosanoyl-5-hydroxytryptamide (EHT) in animal models of PD, co-administration of these two compounds of coffee orally have synergistic effects in protecting the brain against α-Syn mediated toxicity through maintenance of protein phosphatase 2A in an active state (Yan et al., 2018). Second, in methamphetamine-induced neurotoxicity both *in vitro* and *in vivo*, caffeine has been shown to increase toxicity of methamphetamine in SH-SY5Y neuroblastoma cell line through inhibition of autophagy (Pitaksalee et al., 2015) and potentiate 3,4-methylenedioxymethamphetamine (MDMA) -induced dopamine neuron degeneration in substantia nigra pars compacta, possibly involving an increase in dopamine release and excess ROS generation (Sinchai et al., 2011; Frau et al., 2016). Therefore, caffeine has been consistently shown to exert a neuroprotective effect in multiple neurotoxin (including MPTP, 6-OHDA, rotenone) and alpha-synuclein models of PD, but has been reported to exacerbate methamphetamine-induced neurotoxicity. Additional studies are needed to clarify these neurotoxicity context-dependent effects of caffeine.

Motor Benefit of Caffeine in PD Patients and PD Models

The symptomatic effect of caffeine in PD was first tested in 1970s (Shoulson and Chase, 1975), but has been revisited by several clinical studies recently. The motor benefit of caffeine were documented in a pilot open-label, 6-week dose-escalation study (Altman et al., 2011) and a 6-week randomized controlled trial of caffeine (200–400 mg daily) involving 61 PD patients (Postuma et al., 2012). These clinical studies suggest that caffeine improved objective motor deficits in PD with the reduced total Unified PD Rating Scale score and the objective motor component. Furthermore, coffee consumption (>336 mg/day) is associated with the reduced hazard ratio for the development of dyskinesia compared with subjects who consumed <112 mg/day in the Comparison of the Agonist Pramipexole with Levodopa on Motor Complications of Parkinson's Disease (CALM-PD) and CALM Cohort extension studies (Wills et al., 2013). Based on these positive findings, caffeine was recently investigated for motor and disease-modification involving 121 PD patients PD in a phase 3, 5-years (planned), two-arm, double-blind RCT, with a primary outcome focused on motor symptoms and disease-modification as a secondary outcome[1]. Unfortunately, with the primary outcome analysis after 6 months demonstrating no significant symptomatic benefit of caffeine (200 mg twice daily) (Postuma et al., 2017), the study was terminated earlier than the planned.

Based on the concentrated expression of $A_{2A}R$ in the striatum and the A2AR is the key molecular target of caffeine, caffeine (and $A_{2A}R$ antagonists) has been proposed 20 years ago to improve motor activity in PD (Garcao et al., 2013; Morato et al., 2017). Indeed, preclinical studies using rodent and non-human primate models of PD demonstrate motor benefits caffeine and $A_{2A}R$ antagonists in PD (Richardson et al., 1997; Chen, 2003; Schwarzschild et al., 2006; Jenner et al., 2009), leading to clinical pursuit of $A_{2A}R$ antagonists as a leading non-dopaminergic treatment for motor deficits in PD. Since 2001, more than 25 clinical trials were conducted to evaluate the safety and clinical efficacy of $A_{2A}R$ antagonists in PD patients; among these, eight double blind, placebo controlled, phase IIb and III trials of

[1]https://clinicaltrials.gov/ct2/show/NCT01738178

istradefylline (KW-6002, > 4000 PD patients), one phase IIb trial with preladenant (SCH 420814, 253 PD patients), one phase IIb trial with tozadenant (337 PD patients), all reported motor benefits in advanced PD patients as an add-on therapy with L-DOPA (Hauser et al., 2011). The culmination of the two decade-long clinical studies of the effects of istradefylline in more than 4,000 PD finally led to the U.S. Food and Drug Administration (FDA) approval of the $A_{2A}R$ antagonist Nourianz® (istradefylline) developed by Kyowa Hakko-Kirin Inc., Japan, as an add-on treatment to levodopa in Parkinson's disease (PD) with "OFF" episodes in August 2019. Istradefylline is the first non-dopaminergic drug approved by United States FDA for PD in the last two decades and this approval paves the way to foster novel therapeutic opportunities for $A_{2A}R$ antagonists including caffeine for neuroprotection or reversal of mood and cognitive deficits in PD.

Caffeine and Cognitive Improvement in PD

Parkinson's disease is primarily characterized by cardinal motor symptoms but cognitive changes also occur both in the early and later stages of the disease process. In fact, ∼30% of PD patients have dementia and an additional 25% of non-demented PD patients developed mild cognitive impairments that are characterized by frontostriatal cognitive deficits such as alterations in executive function, attention, working and episodic memory (Majbour and El-Agnaf, 2017). Convergent evidence from human and animal studies supports the existence of DA-dependent cognitive deficits in PD (Lewis et al., 2003). The cognitive symptoms seen early in PD include the decreased executive function (e.g., planning and decision making), working memory deficit and impaired procedural learning, leading to cognitive inflexibility that are largely attributed to the frontostriatal dysfunction (Knowlton et al., 1996; Kehagia et al., 2010). Early cognitive deficits are extremely troubling to patients and reduce their quality of life. Currently there is no effective treatment for mild cognitive impairments in PD. In this regard, it is important to note that at least six longitudinal studies support an inverse relationship between caffeine consumption and decreased memory impairments associated with aging as well as a reduced risk of developing dementia and Alzheimer's disease, including the Maastricht Aging Study (Hameleers et al., 2000; van Boxtel et al., 2003), the Canadian Study of Health and Aging (CSHA) (Lindsay et al., 2002), the FINE study (van Gelder et al., 2007), the French Three Cities study (Ritchie et al., 2007), the Cardiovascular risk factors, Aging and Dementia (CAIDE) study (Eskelinen et al., 2009), the Honolulu-Asia Aging Study (Gelber et al., 2011). Furthermore, in a cross-sectional study involving 196 early-stage, treatment-naïve PD patients, coffee drinking was significantly associated with a reduced severity of the mood/cognition domain of NMSS in patients with PD ($p = 0.003$) (Cho et al., 2018). These epidemiological findings raise the possibility of caffeine as therapeutic treatment for cognitive impairments in PD.

Indeed, preclinical studies with $A_{2A}R$ antagonist effect on cognition in normal and MPTP-treated non-human primates (NHP) provide the experimental evidence that $A_{2A}R$ antagonists including caffeine can improve cognitive impairments in PD models (Chen et al., 2013; Chen, 2014). Recent preclinical studies in rodents and non-human primates demonstrated that $A_{2A}R$ antagonists not only enhance working memory (Zhou et al., 2009), reversal learning (Wei et al., 2011), set-shifting (Mingote et al., 2008), goal-directed behavior (Li et al., 2016), and Pavlovian conditioning (Wei et al., 2014) in normal animals, but also reverse working memory impairments in animal models of PD (Ko et al., 2016) and Huntington's disease (Li et al., 2015), traumatic brain injury (Ning et al., 2013, 2015, 2019) as well as Alzheimer's disease (AD) (Dall'Igna et al., 2007; Cunha and Agostinho, 2010; Laurent et al., 2014; Faivre et al., 2018). Furthermore, we recently demonstrated a pro-cognitive effect in normal as well as MPTP-treated Cynomolgus monkeys (Li et al., 2018b). The demonstrated treatment paradigm of the $A_{2A}R$ antagonist KW6002 for spatial working memory enhancement in non-human primate model of PD provide required preclinical data to facilitate the design of clinical trial of $A_{2A}R$ antagonists including caffeine for cognitive benefit in PD patients (Li et al., 2018b). Notably, recent clinical trials of $A_{2A}R$ antagonists for motor benefits in PD did not evaluate their possible effects on cognitive impairment in PD patients (Chase et al., 2003; Aarsland et al., 2010). With the approval of istradefylline, it will now be possible to evaluate the ability of $A_{2A}R$ antagonists to reverse cognitive deficits in PD patients in clinical phase IV trials.

MECHANISMS OF NEUROPROTECTION BY CAFFEINE IN PD

Multiple mechanisms have been proposed to account for the neuroprotective effects of caffeine, including modulation of glutamatergic excitotoxicity and neuroinflammation via adenosine receptors (Chen et al., 2013). Furthermore, recent investigation into the autophagy and gut microbiota in PD pathogenesis raise the exciting possibilities that caffeine may modify autophagy (through metabolism-related action of caffeine) and gut microbiome (with caffeine direct action on gut microbiota) to influence PD development.

Caffeine Modulates Neuroinflammation in PD

Neuroinflammation is critically involved in the pathogenesis of PD. Increasing evidence showed that neuroinflammation response regulated by reactive microglia played an important role in the neurodegeneration of DA neurons (Hirsch and Hunot, 2009; Tansey and Goldberg, 2010; Hirsch et al., 2012). α-Syn, in extracellular aggregated form, can bind to Toll-like receptor 2 (TLR2), CD11b receptors and integrin β1 subunit on microglia to trigger massive microglial activation and neuroinflammation, contributing to consequent neuronal death (Lee et al., 2010; Tansey and Goldberg, 2010; Fellner et al., 2013; Yasuda et al., 2013; Sacino et al., 2014). The involvement of neuroinflammation in PD was further suggested by the observation of the increased number of reactive microglial cells and an upregulation of major histocompatibility

complex class II (MHC-II) in PD patients (McGeer et al., 1988). Caffeine can exert an anti-neuroinflammatory effect under various pathological conditions. Caffeine administration (daily intraperitoneal injection) reduces lipopolysaccharide (LPS)-induced microglia activation in three regions of the hippocampus, in a dose-dependent manner (Brothers et al., 2010) and abrogate LPS-induced neuroinflammation, and synaptic dysfunction in adult mouse brains (Badshah et al., 2019). As a critical neuroprotective factor in PD, caffeine may control microglia-mediated neuroinflammatory response associated PD (Madeira et al., 2017). Indeed, daily intraperitoneal administration of caffeine attenuates microglia reactivity and prevents blood-brain barrier (BBB) dysregulation in the MPTP mouse model, leading to decreased dopaminergic neuronal loss (Xu et al., 2002; Chen et al., 2008). Furthermore, even when introduced in the later phases of the neurodegenerative process, caffeine is also able to attenuate the inflammatory process and microglial cell expression of CD68 (a marker of reactive microglia), which suggests its ability to arrest or delay neuroinflammation and neurodegeneration (Chen et al., 2008). Consistent with this view, by using an α-Syn fibril model of PD, we recently found that chronic caffeine treatment attenuated α-Syn-induced microglial activation and astrogliosis in the striatum in mice (Luan et al., 2018). In addition, caffeine has been shown to protect dopaminergic neurons by activation of the anti-oxidative signaling pathways, such as the nuclear factor erythroid 2-related factor 2 (Nrf2)-Keap1 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha) (Zhou et al., 2019).

The mechanisms underlying anti-neuroinflammation by caffeine may involve the antagonism of the $A_{2A}R$, the major molecular target (Chen et al., 2001; Kalda et al., 2006). $A_{2A}Rs$ affords neuroprotection through the control of microglia reactivity and neuroinflammation. Notably, pharmacological blockade or genetic deletion of $A_{2A}R$ produces similar anti-neuroinflammatory and neuroprotective effects as with caffeine in several experimental models of PD (Chen et al., 2001; Ikeda et al., 2002; Kalda et al., 2006; Hu et al., 2016; Luan et al., 2018). For example, enhanced reactive astrogliosis and NF-κB p65 activation around the injection site in hippocampus in an α-Syn transmission mouse model of PD, and these α-Syn-triggered neuroinflammatory responses were largely prevented in $A_{2A}R$ KO mice (Hu et al., 2016). In the well-established α-Syn fibril model of PD, chronic caffeine treatment largely reverted the α-Syn-induced microglial activation and astrogliosis in the striatum in mice (Brambilla et al., 2003; Boison et al., 2010; Luan et al., 2018). Moreover, in $A_{2A}R$ antagonists also control neuroinflammation (through p38), of synaptopathy (Canas et al., 2009) and β-amyloid processing (Cao et al., 2009) in AD models. Thus, caffeine may exert anti-neuroinflammation and neuroprotection effect in PD by targeting the $A_{2A}Rs$.

Caffeine May Modulate PD Pathology by Regulating Autophagy Activity

Autophagy is a high conserved defense and protective mechanism in eukaryotic cell to achieve degradation of abnormal proteins and damaged organelles by three types of autophagy processes: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). PD is essentially a protein misfolding disease or conformational disease; characterized by abnormal accumulation and aggregation of misfolded α-Syn (Kalia and Kalia, 2015). Increasing evidence demonstrates that aberrant regulation of autophagy contributes to the aggregation of α-Syn and α-Syn-induced neurodegeneration in PD (Ebrahimi-Fakhari et al., 2012; Poehler et al., 2014; Xilouri et al., 2016; Hou et al., 2020; Senkevich and Gan-Or, 2020). First, several studies found that alteration of autophagy-lysosome pathway activity, including both CMA and macroautophagy, existed in brains of postmortem PD patients and experimental models of PD (Alvarez-Erviti et al., 2010; Cerri and Blandini, 2019; Lamonaca and Volta, 2020; Qin et al., 2020; Wan et al., 2020), revealing the direct correlation between autophagy and pathological process of PD. Second, abnormally aggregated α-Syn inclusions are mainly degraded by autophagy (Webb et al., 2003) and defects or deficiency of autophagy can lead to accumulation of intracellular misfolded amyloid α-Syn aggregates, thus causally linking autophagy to PD pathological process (Xilouri et al., 2016; Guo et al., 2020). Third, α-Syn overexpression can impede autophagy by reducing autophagosome formation in human Neuroblastoma SH-SY5Y (Nascimento et al., 2020), and contribute to many different pathologies seen in PD (Winslow and Rubinsztein, 2011). Notably, the pathogenic A53T and A30P α-Syn mutants selectively bound to lysosome-associated membrane protein type 2A (LAMP2A) with high affinity, blocked CMA activity at the LAMP2A level, ultimately resulting in complete damage to this pathway (Cuervo et al., 2004). Collectively, autophagy plays an important role in the pathogenesis of PD, thus targeting autophagy may represent a promising strategy for treatment of PD (Rivero-Rios et al., 2016; Moors et al., 2017; Lu et al., 2020).

Both caffeine and $A_{2A}R$ signaling can regulate autophagy activity under different conditions in several cell types (Sinha et al., 2014; Liu et al., 2016). Caffeine can induce macroautophagy caused by a starvation response and confer a cytocidal effect on Zygosaccharomyces bailii food spoilage yeast) in combination with benzoic acid (Winter et al., 2008). Higher concentrations of caffeine (10 mM) enhance autophagic flux in various tumor cell lines (HeLa, SH-SY5Y, and PC12D cells). Caffeine can induce apoptosis by enhancement of autophagy in PC12D cells through PI3K/Akt/mTOR/p70S6 signaling pathway (Saiki et al., 2011). Using both genetic and pharmacological inhibitors of autophagy, researchers directly linked caffeine-induced autophagy with oxidative lipid metabolism both in HepG2 cells and in mice liver; and further demonstrated that autophagy was associated with caffeine-induced hepatic fat clearance in a mouse model of non-alcoholic fatty liver disease, indicating that caffeine surprisingly is a potent stimulator of hepatic autophagic flux (Ray, 2013; Sinha et al., 2014). Accordingly, caffeine-enhanced autophagic flux in hepatic stellate cells was stimulated by endoplasmic reticulum (ER) stress via the IRE1-α signaling pathway, and autophagy trigged by caffeine instigated cell apoptosis (Li et al., 2017). Acute high-caffeine

exposure also significantly reduced skeletal myotube diameter by increasing autophagic flux in differentiated C2C12 mouse skeletal myoblasts cells (Bloemberg and Quadrilatero, 2016; Hughes et al., 2017). Notably, caffeine increased autophagy by promoting the calcium-dependent activation of AMP-activated protein kinase (AMPK) in mammalian skeletal muscle cells (Mathew et al., 2014), and prevent skin from oxidative stress-induced senescence through the activation of autophagy. This caffeine-induced autophagy, mainly mitophagy, was mediated by $A_{2A}R$/SIRT3/AMPK pathway, protecting skin from oxidative stress-induced senescence both *in vitro* and *in vivo* models (Li et al., 2018a). Therefore, caffeine also has the potential in protection of skin disease. Consistent with these studies, a recent research found that caffeine directly enhanced autophagy in concentration- and time-dependent manners in primary cultured thymocytes, which was dependent on $A_{2A}R$ signaling (Liu et al., 2019). Taken together, these studies from various cell models and in different tissues and organs *in vivo* demonstrate that caffeine enhances autophagy which is related to its therapeutic effects on diverse human diseases, including tumors, aging, liver fibrosis, and skin disease.

Using an α-Syn fibril model of PD, we recently have provided the first evidence that caffeine can attenuate abnormal α-Syn aggregation and neurotoxicity by re-establishing autophagy activity in animal models of PD (Luan et al., 2018). Specifically, chronic caffeine treatment did not affect autophagy processes in the normal mice striatum, but did selectively reverse α-Syn-induced defects in macroautophagy and CMA (Luan et al., 2018). Thus, caffeine may represent a novel pharmacological therapy for PD by targeting autophagy pathway. This study collaborates with the previous study showing that caffeine-induced autophagy protected against human prion protein (PrP) peptide (106–126)-triggered apoptosis in a SH-SY5Y neuroblastoma cell line. Therefore, autophagy enhanced by caffeine may be a valid therapeutic strategy for neurodegenerative diseases such as PD and prion (Moon et al., 2014; Corti et al., 2020).

Caffeine May Influence PD Pathology by Modulating Gut Microbiota

The gut microbiota in the human gastrointestinal (GI) tract is estimated to contain 10 times more microbial cells than human cells, and approximately 100–200-times more protein coding genes than the human genome (Qin et al., 2010). The gut microbiota critically influence various aspects of human biology, including the absorption and metabolism of nutrients, vitamins, medications, and toxic compounds; the development and differentiation of the intestinal epithelium and immune system, the maintenance of tissue homeostasis, and the prevention of pathogens invasion (Sommer and Backhed, 2013). The gut microbiota also plays an important role in gut-brain communication, and the neuroimmune system to maintain brain homeostasis, thus influencing brain function and behavior (Carabotti et al., 2015). In healthy subjects, the intestinal microbiota is generally stable over time, but compositional changes might occur following antibiotic usage or dietary modifications (Lozupone et al., 2012).

Mounting evidence suggest that the intestinal microbiota may be the triggering factor of PD pathology. Specifically, the gut microbiota encoded proteins and their metabolites can initiate accumulation of misfolding of α-Syn in the enteric nervous system through molecular mimicry and intestinal mucosal immunoinflammatory mechanisms, which thereafter could act in a prion-like fashion and spread along the gut-brain axis via vagus nerve, eventually leading to the development of PD pathology (Friedland, 2015; Klingelhoefer and Reichmann, 2015; Pellegrini et al., 2018; Ho et al., 2019; Miraglia and Colla, 2019; Cirstea et al., 2020; Zheng et al., 2020). Multiple lines of preclinical and clinical evidences support the role of gut microbiota dysfunction in various aspects of PD pathogenesis: (i) according to the widely accepted Braak Staging hypothesis about the pathogenesis of PD, α-Syn pathology may begin in the intestine and spread to the brain through the vagus nerve. This hypothesis has been supported by two lines of key clinical findings. (a) Idiopathic constipation is one of the strongest risk-factors for PD since up to 80% of PD patients develop gastrointestinal dysfunction, in particular constipation, in the early stages of PD, preceding the onset of motor symptoms by years (Schapira et al., 2017). (b) Full truncal vagotomy is associated with a decreased risk for subsequent PD, suggesting that the vagal nerve may be critically involved in the pathogenesis of PD (Svensson et al., 2015). (ii) The gut microbiota of patients with PD is altered depending on clinical motor phenotype and related to progress of PD; thus the gut microbiome may be a potential PD biomarker (Scheperjans et al., 2015a; Hopfner et al., 2017; Gerhardt and Mohajeri, 2018; Qian et al., 2018; Nuzum et al., 2020; Pietrucci et al., 2020; Ren et al., 2020). (iii) Gut microbiota is necessary to promote α-Syn pathology, neuroinflammation, neurodegeneration, and characteristic motor features in validated mice model of PD, and specific microbial metabolites are sufficient to promote PD symptoms, suggesting a casual and functional role in PD pathogenesis (Sampson et al., 2016; Sun et al., 2018; Klann et al., 2020; Koutzoumis et al., 2020). (iv) The human gut microbiota metabolizes the PD medication Levodopa (L-dopa), potentially reducing drug availability and causing side effects (Maini Rekdal et al., 2019). Collectively, these findings support that gut microbiota is critical contributor to PD pathogenesis and may represent a promising therapeutic target for the treatment of PD (Lubomski et al., 2019).

As the most important environmental and dietary factor of PD, how does long-term drinking caffeine affect the diversity of gut microbiota? Moreover, how do the human intestinal microorganism and their encoded enzymes influence the metabolism of caffeine? Caffeine is initially absorbed in the stomach and small intestine but is further fermented in the colon by gut microbiota (Scheperjans et al., 2015b). Recently, caffeine consumption is reportedly related to the colonic mucosa-associated gut microbiota (Gurwara et al., 2019); long-term coffee intake is associated with fecal microbial composition in humans, and regular consumption of coffee appears to be associated with changes in some intestinal microbiota groups in which caffeine, as the main dietary factor influencing PD

development, may play a role (Gonzalez et al., 2020). Intestinal microorganisms also play a role in the metabolism of caffeine as caffeine was degraded in the gut of *H. hampei*, and that experimental inactivation of the gut microbiota eliminates this activity, suggesting that the detoxification of caffeine in *H. hampei* is mediated by the insect's gut microbiota (Ceja-Navarro et al., 2015). Several recent studies have shed light on the relationship between coffee consumption, caffeine and gut microbiota, as well as GI function in PD (Derkinderen et al., 2014; Scheperjans et al., 2015b). Some effects of coffee on the gastrointestinal tract promote gastro-oesophageal reflux, stimulation of gallbladder contraction and an increase of colonic motor activity (Boekema et al., 1999). Coffee consumption is also inversely associated with the prevalence of self-reported constipation (Murakami et al., 2006). Notably, coffee and caffeine regulates the composition and abundance of intestinal flora under different pathologies. For example, coffee caused an increase of anti-inflammatory Bifidobacteria and a decrease of Clostridium spp. and Escherichia coli that invade the gut mucosa in PD (Khokhlova et al., 2012). Furthermore, chronic coffee consumption attenuated the increase in Firmicutes (F)-to-Bacteroidetes (B) ratio and Clostridium Cluster XI normally associated with high-fat feeding and augmented the levels of Enterobacteria (Cowan et al., 2014). Coffee or its components caffeine can also affect the gut microbiome and short-chain fatty acids (SCFAs) profile in Tsumura Suzuki obese diabetes (TSOD) mice and thereby improve hepatic inflammation. Specifically, daily intake of coffee or its components did not repair the gut dysbiosis in TSOD mice, rather, altered the percentages of six microbial genera changed in these mice, including Blautia, Coprococcus, and Prevotella, which have been implicated in inflammation or obesity (Clarke et al., 2012; Nishitsuji et al., 2018). Other studies suggest that the diversity and structure of the gut microbiota is not sensitive to caffeine, however, when the predicted metagenome functional content of the bacterial communities was analyzed, the caffeine treatments did induce a dramatic decrease of the aromatic amino acid decarboxylase gene (Scorza et al., 2019).

Consistent with these findings, our preliminary meta-genome analysis of the gut microbiota of A53T-α-Syn transgenic mice model of PD by chronic intervention of caffeine indicated that chronic caffeine treatment for one month had no significant effect on gastrointestinal function, but apparently normalized the structure and imbalance of the gut microbiota of PD model mice (unpublished data). Additional studies are warranted to study the interaction between caffeine and intestinal flora in the body, and determine whether the beneficial effects of coffee consumption on PD are mediated through the modulation of the microbiota-gut-brain axis.

GENETIC STUDIES ON THE INTERACTION BETWEEN CAFFEINE AND PD RISK GENES

Epidemiological investigation coupled with genetic analyses of the genetic and environmental interaction in development of PD has provided several important insights into the interaction between caffeine and several genes associated with PD pathogenesis such as NMDA-glutamate-receptor subunit, LRRK2 and estrogen in PD: (i) A recent genome-wide association and interaction study (GWAIS) uncover a complex interplay between genetic (GRIN2A, encoding an NMDA-glutamate-receptor subunit) and environmental factors (caffeine consumption) in etiology of PD, as a PD genetic modifier in inverse association with caffeine intake (Hamza et al., 2011). (ii) A study with patient-control study Swedish population has revealed an association of a single nucleotide polymorphism, GRIN2A_rs4998386, and its interaction with caffeine intake with PD (Yamada-Fowler et al., 2014). Thus, the interaction between caffeine and glutamate receptor genotypes may contribute to the protective effects of coffee drinking/caffeine intake in PD. (iii) Furthermore, a recent case control study of 812 subjects consisting of PD and healthy controls showed that caffeine intake would significantly reduce the risk of PD by 15-folds in those carrying PD gene risk variant (LRRK2 R1628P) (Kumar et al., 2015). Metabolomic analyses of the LRRK2 Cohort Consortium (LCC) samples identified caffeine, its demethylation metabolites, and trigonelline as prominent markers of resistance to PD linked to pathogenic LRRK2 mutations, more so than to idiopathic PD (Crotty et al., 2020). Exploratory analysis on potential interactions of smoking and caffeine intake with 10 genome-wide association studies of SNPs (at or near the SNCA, MAPT, LRRK2, and HLA loci) further reveal that a combined smoking and caffeine intake exposure is associated with a significant interaction with rs2896905 at SLC2A13, near LRRK2 (Gao et al., 2012). In addition, the Parkinson's Epidemiology and Genetics Association Studies in US (PEGASUS) consortium (involving 3000 subjects of five independent well-characterized patient-control series) uncovered an association between an *Adora2a* variant (rs7165183 and rs5996696) and a reduced risk of PD (Popat et al., 2011), with the strongest coffee-PD association among those with homozygous carriers of the *CYP1A2* polymorphisms and slow metabolizers of caffeine. These genetic studies support the protective effect of caffeine intake on PD through the interaction between caffeine and GRIN2A, LRRK2, and $A_{2A}R$. These findings raise the exciting possibility of selecting patient subpopulations by these genetic polymorphisms of the GRIN2A, LRRK2, $A_{2A}R$, and CYP1A1 genes.

AUTHOR CONTRIBUTIONS

Both authors wrote and revised the manuscript. J-FC carried out the overall responsibility.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Notoginsenoside R1 Improves Cerebral Ischemia/Reperfusion Injury by Promoting Neurogenesis via the BDNF/Akt/CREB Pathway

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Notoginsenoside R1 (R1), a major component isolated from *P. notoginseng*, is a phytoestrogen that exerts many neuroprotective effects in a rat model of ischemic stroke. However, its long-term effects on neurogenesis and neurological restoration after ischemic stroke have not been investigated. The aim of this study was to evaluate the effects of R1 on neurogenesis and long-term functional recovery after ischemic stroke. We used male Sprague-Dawley rats subjected to middle cerebral artery occlusion/reperfusion (MCAO/R). R1 was administered by intraperitoneal (i.p.) injection immediately postischemia. We showed that R1 significantly decreased infarct volume and neuronal loss, restored neurological function, and stimulated neurogenesis and oligodendrogenesis in rats subjected to MCAO/R. More importantly, R1 promoted neuronal proliferation in PC12 cells *in vitro*. The proneurogenic effects of R1 were associated with the activation of Akt/cAMP responsive element-binding protein, as shown by the R1-induced increase in brain-derived neurotrophic factor (BDNF) expression, and with the activation of neurological function, which was partially eliminated by selective inhibitors of BDNF and PI3K. We demonstrated that R1 is a promising compound that exerts neuroprotective and proneurogenic effects, possibly via the activation of BDNF/Akt/CREB signaling. These findings offer insight into exploring new mechanisms in long-term functional recovery after R1 treatment of ischemic stroke.

Keywords: ischemic stroke, notoginsenoside R1, neurogenesis, neurological recovery, oligodendrogenesis

INTRODUCTION

Stroke is the second most common cause of human death and the leading cause of human disability with high morbidity worldwide (O'Donnell et al., 2010; Abdulhak et al., 2014). Due to the narrow window for administering rtPA (recombinant tissue plasminogen activator) treatment, only a small percentage of patients receive rtPA treatment during this therapeutic window (4.5 h) after the onset of stroke (Dibajnia and Morshead, 2013; Dirnagl and Endres, 2014). Therefore, new drugs that target the subacute and chronic stages of stroke are urgently needed. In the ischemic area, neurons die

quickly due to a series of biochemical changes. Therefore, enhancing the survival of newborn neurons is an attractive strategy for promoting neurogenesis after ischemic stroke (Wang et al., 2016b).

Ischemic stroke induces substantial neurogenesis in the two specific regions of the adult brain: the subventricular zone (SVZ), which lines the lateral ventricular wall (LV), and the subgranular zone (SGZ) of the dentate gyrus (DG) (Kempermann and Gage, 1999; Ming and Song, 2011); these regions are the sites where most of the neural progenitors in the adult mammalian brain are located (Gross, 2000). Neuronal stem cells located in the SVZ mainly produce committed progenitor cells that migrate into the olfactory bulb (OB) through the rostral migratory stream and differentiate into local interneurons (Sharma and Reed, 2013); progenitor cells located in the SGZ migrate mainly to the granular cell layer and differentiate into neurons (Eriksson et al., 1998). After ischemic injury, the migration of newborn neurons is not confined to these constant sites (Jin et al., 2004). In response to ischemic injury, endogenous neuron precursors gradually migrate to the striatum, the hippocampus CA1 region and ischemic core area of the cerebral cortex, where they can merge with brain circuits and complete neural repair processes (Arvidsson et al., 2002; Jin et al., 2003). Newborn neurons have crucial physiological functions in learning and memory, plasticity and mood regulation. Therefore, a drug that effectively promotes the survival and formation of newborn neurons would provide a novel therapeutic strategy for ischemia-induced neurological damage; the search for such a drug remains the focus of both basic and clinical research.

Panax notoginseng is a famous traditional Chinese herb that has great clinical value for regulating cardiovascular (Lei and Chiou, 1986) and cerebrovascular (Su et al., 2014) diseases in China. Notoginsenoside R1 (R1), a major component isolated from *P. notoginseng*, is a novel phytoestrogen that exerts many neuroprotective effects in a rat model of ischemic stroke through the suppression of oxidative stress (Meng et al., 2014), apoptosis (Zou et al., 2017) and endoplasmic reticulum stress (ERS) (Wang et al., 2016a). Our previous studies mainly revealed the neuroprotective effects of pretreatment with R1 at the acute stage of stroke in rats (Meng et al., 2014), and its long-term effects on neuronal regeneration and neurological restoration after ischemic stroke have not been investigated.

In the present study, we used *in vivo* and *in vitro* models of cerebral ischemic/reperfusion (I/R) injury for MCAO/R in rats and oxygen-glucose deprivation/reoxygenation (OGD/R) in PC12 cells. The primary purpose of the present study was to evaluate the effects of R1 on neurogenesis and long-term functional recovery after ischemic stroke. Moreover, the mechanisms by which R1 facilitated neurogenesis in rats subjected to MCAO/R we were elucidated.

MATERIALS AND METHODS

Materials

R1 (molecular weight = 933.15; purity >98%) was purchased from Shanghai Winherb Medical S and T Development (Beijing, China). A positive drug dl-3-n-butylphthalide (NBP) was obtained from CSPC NBP Pharmaceutical Co., Ltd. Edaravone was provided by Kunmingshenghuo Pharmaceutical Co., Ltd. Triphenyltetrazolium chloride (TTC) and 1,5-DAN hydrochloride were purchased from Sigma-Aldrich (MO, United States). 5-Ethynyl-2′-deoxyuridine (EdU) was purchased from Invitrogen (Grand Island). An EdU cell proliferation kit was purchased from Beyotime Biotechnology (Shanghai, China). All the primary antibodies used in the experiments were provided by Abcam (Cambridge, UK). The ANA-12 and LY294002 inhibitors and the ELISA kits for BDNF, nerve growth factor (NGF), and neurotrophin-4 (NT-4) were acquired from HaiTai TongDa Sci Tech Ltd (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, United States).

Animals

Male Sprague-Dawley (SD) rats (purchased from Beijing Vital River Laboratories, Beijing, China) weighing 250–280 g were used in this study. All rats care and experimental procedures were reported in accordance with the Laboratory Animal Ethics Committee of the Institute of Medicinal Plant Development, Peking Union Medical College and complied with NIH Guidelines for the Care and Use of Laboratory Animals (approval number: SYXK 2017–0020). All rats were maintained in ventilated cages at a temperature of 20–25°C and a relative humidity of 30–50% under a 12 h light/dark cycle and were given free access to food and water.

MCAO Surgery

The SD rats were anesthetized with ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}) intraperitoneally (i.p.) by using the MCAO procedure. Cerebral I/R was induced by MCAO/R as previously described (Meng et al., 2014). After MCAO surgery, the wound was disinfected with iodine, and then the wound was sutured with sterile surgical suture to reduce the bleeding. We also injected tramadol (2.5 mg kg^{-1}) by tail intravenous to relieve the pain caused by the operation. The sham-operated rats were manipulated using the same surgical procedure, but the MCA was not occluded. The body temperature was maintained at 37 ± 0.5°C until rats woke up using a heating pad (sunbeam, United States). The researcher who conducted all the subsequent analyses was blinded to the treatment that the rats had received.

Drug Treatment

The drug was dissolved in 0.9% normal saline prior to administration. The drug was administered by i. p. injection. To select the drug dosages, R1 was given at doses of 10 mg kg^{-1}, 20 mg kg^{-1} and 40 mg kg^{-1} for 7 days after MCAO surgery. To detect neurogenesis, the optimal drug dosage was given for 28 days after MCAO surgery. At 28 days post-injection, serum and brain tissues were harvested to investigate the mechanism underlying the R1-mediated regulation of ischemic stroke in rats subjected to MCAO/R. Schematic graphic of drug treatment refers to **Figure 1**.

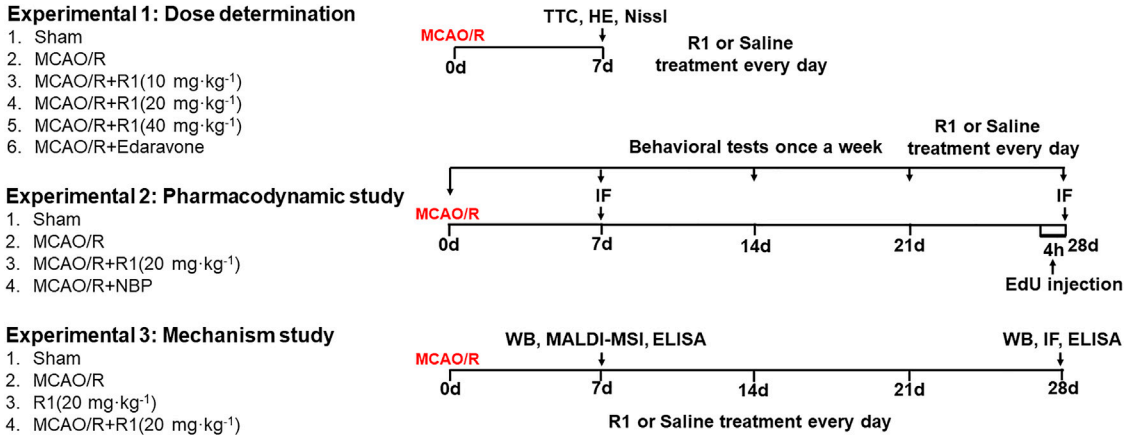

FIGURE 1 | Schematic graphic of the rat experimental design. On the left is the group information, and on the right is the timeline of the experimental process.

Neurological Score

Neurological behavior was investigated at 1, 7, 14, 21, and 28 days after I/R by two blinded investigators using a 5-point scale as previously published (Meng et al., 2014). The neurological function was scored according to a series of scales from 0 to 4. The highest score represents the most severe neurological deficits.

TTC Staining

TTC staining was conducted 7 days after I/R based on previously described methods (n = 5 for each group) (Meng et al., 2014). Cerebral infarct area was quantified by an image analysis system (Image-Pro Plus 5.0). The infarct volume can be obtained by multiplying the total infarct area by the thickness of the brain sections. Calculating the corrected infarct volume is contribute to compensate for the error caused by brain edema (Meng et al., 2014).

Novel Object Recognition Task

The novel object recognition test (NORT) was performed as previously described (Camarasa et al., 2010; Zhang et al., 2018). To assess nonspatial memory, the rats were familiarized with an open-field box (50 × 50 × 50 cm, length × width × height) 28 days after MCAO/R to reduce the contribution of stress and anxiety. In the first phase, a learning trial was conducted for 10 min. In this phase, two objects of the same shape, color and size were placed symmetrically in the open field approximately 6 cm from the walls. In the second phase, one of the objects was replaced with a novel object of a dissimilar shape and color. During the 10 min recall trial, these two objects were presented in the same box 1 h after the first trial. All the objects and boxes were wiped down with 75% ethanol after each individual trial to avoid olfactory cues. Notebook computers recorded the time spent exploring the same object (TA1) and the novel object (TB1). The discrimination index was evaluated according to the following expression (TA1-TB1/TA1+TB1).

Cylinder Tests

The cylinder tests were performed as previously described (Huotarinen et al., 2018). To assess asymmetric forelimb use during spontaneous vertical movements, the rats were tested individually in a 25 cm diameter glass cylinder, and videos were recorded for 5 min. The number of times the paw contralateral to the lesion contacted the supporting wall was counted and was expressed as a percentage of all the supporting wall contacts in each session (Francardo et al., 2014).

Histopathology Staining

Histopathological staining (7 days postreperfusion) was conducted based on previously described methods (Wang et al., 2016c). The brain samples were embedded in paraffin and coronally dissected into 5 μm-thick sections. Then paraffin sections were stained with H&E and Nissl staining to reveal the histopathological lesions.

EdU Injection and Immunofluorescence Staining

The relative numbers of proliferating cells were identified by double immunofluorescence staining with the relevant primary antibodies and EdU, which is a marker of cell proliferation. At 1 and 4 weeks after reperfusion, some rats received a single intraperitoneal injection of EdU (100 mg kg^{-1}). After 4 h, these rats were transcardially perfused with PBS, and the tissue was fixed in 4% paraformaldehyde solution overnight (n = 3 per group). For double immunofluorescence staining, the frozen sections were incubated for 15 min in 0.3% Triton X-100 to disrupt the cell membrane, and then, the sections were incubated with anti-neuron-specific nuclear protein (NeuN, 1:500; Abcam, Cambridge, UK), anti-Doublecortin (DCX, 1:500; Abcam, Cambridge, UK), anti-Nestin (1:500; Abcam, Cambridge, UK) and anti-adenomatous polyposis *coli* (APC, 1:500; Abcam, Cambridge, UK) in blocking serum overnight at 4°C. After washing, the sections were incubated with FITC-conjugated secondary antibodies for 1 h and sealed with a coverslip. Then, the sections were incubated with a BeyoClick™ EdU-

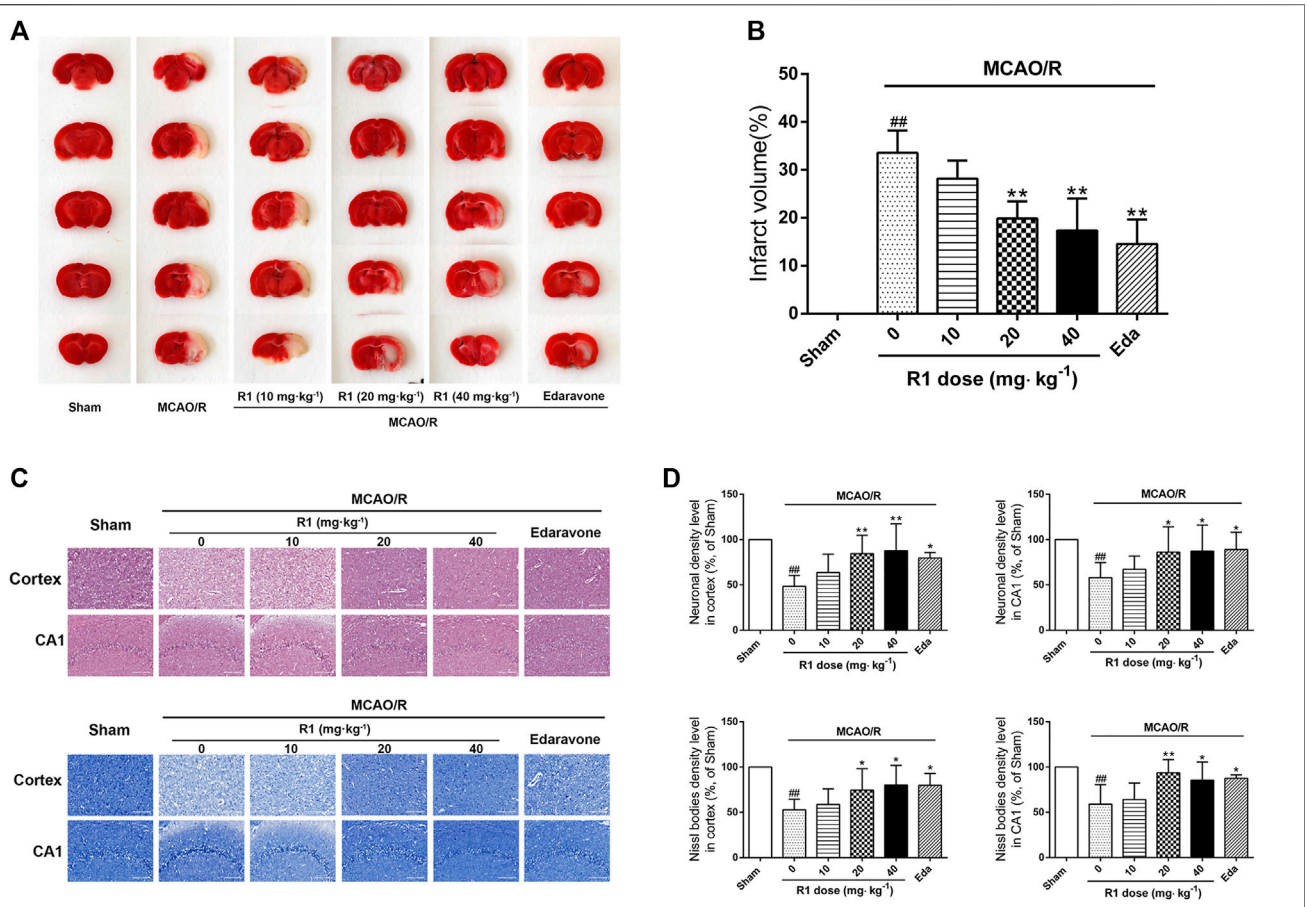

FIGURE 2 | R1 reduces the infarction volume and neuronal loss in rats subjected to MCAO. R1 and edaravone were both intraperitoneally injected immediately after MCAO surgery **(A)** Effects of R1 on infarction volumes (n = 5) **(B)** Quantitative analysis of cerebral infarct volumes **(C)** H&E staining and Nissl staining of the cortex and hippocampal CA1 regions of each group (n = 5) **(D)** Relative density (% of sham) of the H&E and Nissl staining in the cortex and hippocampal CA1 regions in all the groups. Scale bar = 200 μm. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MCAO/R group.

594 reaction cocktail for 30 min for EdU staining. Images were observed using TissueFAXS (Zeiss, Germany). Immunofluorescent positive cells were counted in three sections per rat. Results were expressed as the average numbers of positive cells in unit area per section of three rat brains.

Mass Spectrometry Imaging

Frozen sections (10 μm) of the brain tissues (n = 5 per group) were taken for MALDI-MSI using a Leica CM1950 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) −20°C and placed the thawed brain tissue slices on electrically conductive glass slides coated with indium tin oxide (ITO). The MALDI MSI experiments were implemented using the Autoflex Speed™ MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) as described reported previously (Liu et al., 2017).

Cell Culture and Drug Preparation

PC12 cells were differentiated into neural cells by incubation with NGF (50 ng/ml; New England Biolabs, MA, United States) and were cultured in DMEM supplemented with 10% horse serum and 5% FBS at 37°C in an incubator. In all the experiments, PC12 cells in the exponential phase were used. The R1 stock solution (100 mM) was prepared with DMSO. The indicated concentrations of R1 were prepared immediately before use.

Oxygen-Glucose Deprivation/ Reoxygenation and Drug Treatment

OGD/R was conducted in PC12 cells to mimic cerebral I/R injury *in vitro*. This procedure was conducted according to a previously described method with slight modification (Lu et al., 2011; Meng et al., 2014). Briefly, the PC12 cells were cultured in glucose-free Locke's medium under hypoxic conditions for 4 h. Then, the cells were moved from the anaerobic chamber (TYPE c; coy Laboratory Products, Inc, Grasslake, MI, United States) to a normoxic environment, the medium was replaced with normal medium, and the cells were allowed to reoxygenate for 12 h. In the R1-treated group, the PC12 cells subjected to ODG/R were treated with R1 (12.5–100 μM) for 12 h. In the inhibitor-treated group, the cells were preincubated with 10 μM ANA-12 and 10 μM LY294002 for 0.5 h prior to treatment with R1.

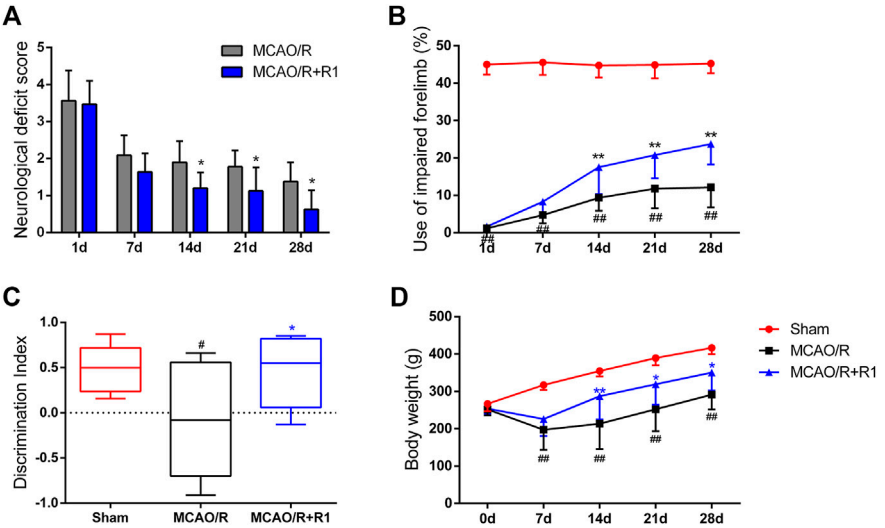

FIGURE 3 | R1 restores long-term neurological function in rats subjected to MCAO. The behavioral tests were conducted on days 1, 7, 14, 21 and 28 after MCAO surgery **(A)** Zea-Longa score **(B)** cylinder tests **(C)** novel object recognition tests on day 28 after MCAO surgery **(D)** The body weight of the rats in each treatment group over 28 days. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p< 0.05$ and $^{\#\#}p< 0.01$ vs. Sham group; $^{*}p< 0.05$ and $^{**}p< 0.01$ vs. MCAO/R group.

Cell EdU Staining

Cell proliferation was examined with an EdU cell proliferation kit according to the recommended procedure. The photographs were acquired with a fluorescence microscope (Leica DM4000, Frankfurt, Germany).

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions to quantify the expression of BDNF (HT100026), NGF (HT100169), and NT-4 (HT100171) in the serum and brain tissue.

Western Blot Analysis

Western blot analysis was conducted as previously described (Meng et al., 2014). Right cortex tissues were collected from each rat ($n = 3$), and the total protein was extracted using a protein extraction reagent supplemented with protease and phosphatase inhibitor cocktails (ComWin Biotech, China). The total protein concentration of each sample was determined by a BCA kit (ComWin Biotech, China). Equal amounts of protein were separated using SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes. These membranes were blocked before being incubated overnight at 4°C with the appropriate primary antibodies: cnpase (ab183500, 1:1000), MBP (ab209328, 1:1000), Vimentin (ab92547, 1:10,000), SYP (ab32127, 1:1000), PSD95 (ab76115, 1:1000), MAP-2 (ab32454, 1:2000), Tau-1 (ab75714, 1:1000), BDNF (ab108319, 1:1000), *p*-TrkB (Tyr705) (ab229908, 1:1000), TrkB (CST4603, 1:1000), *p*-CREB (Ser133) (ab32096, 1:1000), CREB (ab32515, 1:1000), *p*-Akt (Ser473) (CST4060, 1:1000), Akt (CST4685, 1:1000), and β-actin (EXP0036 F, 1:2000). Then, the membranes were washed three times and incubated with the appropriate secondary antibodies. The blots were visualized using enhanced chemiluminescence western blot detection kits (ComWin Biotech, China). The blot densities were calculated by ImageJ software.

Statistical Analysis

Experimental data were obtained from three independent experiments and are expressed as the means ± standard deviations (SDs). All analyses were statistically evaluated using SPSS17 software (IBM Corporation, New York, NY, United States). One-way ANOVA followed by Tukey test or two-way ANOVA followed by Bonferroni's multiple comparison test, if these data were normally distributed. The Kruskal-Wallis test was used if these data were not normal distributed. A p value of less than 0.05 was considered statistically significant.

RESULTS

R1 Attenuates the Infarction Volumes and Neuronal Loss After Ischemia

MCAO was performed on the right side. 7 days after ischemia, the infarction volumes and neuronal numbers were altered. After treatment for 7 days, 20 and 40 mg kg^{-1} R1 significantly reduced the infarction volumes and neuronal loss (**Figures 2A–D**). At a dose of 20 mg kg^{-1}, R1 exhibited more remarkable effects of Nissl body loss on the hippocampal CA1 region. Thus, subsequent studies in the rats were performed with this dose of R1.

R1 Ameliorates the Long-Term Neurological Dysfunction After Ischemia

To further evaluate the effect of R1 treatment on the long-term recovery of neurological function, a series of neurological assessments, including the Zea-Longa score, cylinder tests and novel object recognition tests,

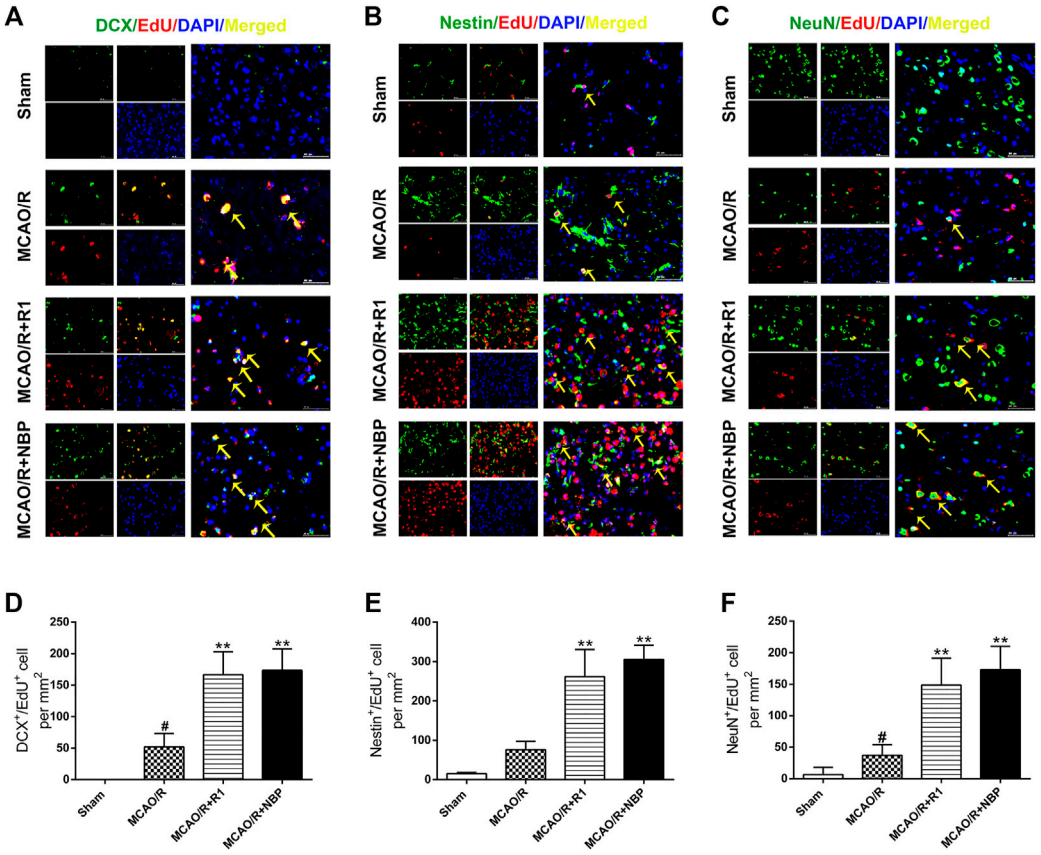

FIGURE 4 | R1 enhances neural reconstruction by stimulating neurogenesis after ischemic stroke. Representative images of the infarction area costained with antibodies against **(A)** DCX (green, marker of migrating and immature neurons) and EdU (red, marker of proliferating cells) on day 7 after R1 treatment, yellow arrows indicate migrating neuroblasts (EdU^+/DCX^+ cells) **(B)** Nestin (green, marker of proliferating NPCs) and EdU (red) on day 7 after R1 treatment, yellow arrows indicate proliferating NPCs ($EdU^+/Nestin^+$ cells) **(C)** NeuN (green, marker of mature neurons) and EdU (red) on day 28 after R1 treatment, yellow arrows indicate newly formed mature neurons ($EdU^+/NeuN^+$ cells). DAPI (blue) indicates the nucleus, Scale bar = 50 μm **(D-F)** Quantitative analysis of **(A-C)** in the striatum region respectively after R1 treatment. $n = 3$ brains per group. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ vs. Sham group; $^{*}p< 0.05$, $^{**}p< 0.01$ vs. MCAO/R group.

were performed throughout the 28 days observation period. All the rats subjected to MCAO presented consistent, substantial neurological deficits 1 day after MCAO surgery, and neurological function gradually improved during the 28 days. The neurological deficit scores of the rats in the R1 treatment group were significantly lower than those of the rats in the MCAO/R group 14 days after treatment (**Figure 3A**). The rats treated with R1 also exhibited significantly improved function of the impaired forelimb beginning at 14 days compared with the rats subjected to MCAO/R (**Figure 3B**). Moreover, in the NORT, R1 significantly increased the abilities of the rats to distinguish novel from familiar objects, as determined by the discrimination index (**Figure 3C**), indicating that R1 has the potential to improve recognition memory. Concomitantly, compared with the MCAO/R group, the R1-treated group exhibited an increase in body weight (**Figure 3D**).

R1 Promotes Regenerative Neurogenesis After Ischemia

After MCAO surgery, the neuron structures and numbers were destroyed. To confirm whether R1 possesses long-term therapeutic effects postischemia on the migration and proliferation of newborn neurons, we performed DCX/EdU, Nestin/EdU and NeuN/EdU double staining to identify neuroblasts (migrating and immature neurons) (Gleeson et al., 1999), proliferating NPCs (Arvidsson et al., 2002) and newborn mature neurons, respectively. The striatum and cortex regions in the brain are commonly considered to be sensitive to cerebral ischemia (Zhang et al., 2018). In the sham group of our study, almost no DCX^+/EdU^+, $Nestin^+/EdU^+$ or $NeuN^+/EdU^+$ cells were detected in the striatum region. However, in the rats subjected to MCAO, the numbers of DCX^+/EdU^+, $Nestin^+/EdU^+$ and $NeuN^+/EdU^+$ cells were significantly increased in the infarcted area of the ipsilateral hemisphere. Furthermore, the numbers of DCX^+/EdU^+ (**Figures 4A,D**), $Nestin^+/EdU^+$ (**Figures 4B,E**) and $NeuN^+/EdU^+$ (**Figures 4C,F**) double-positive cells were obviously increased after R1 treatment compared with MCAO treatment.

R1 Stimulates Oligodendrogenesis and Preserves Myelin After Ischemia

Axon diameter and myelin thickness and spacing determine the rate of neuronal conduction along the axon. Oligodendrocyte

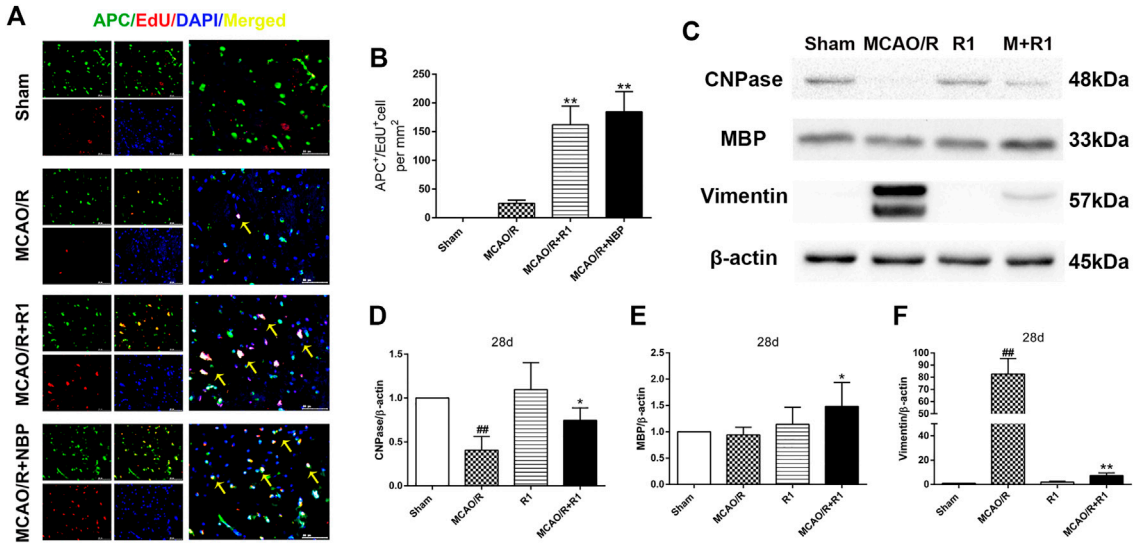

FIGURE 5 | R1 facilitates oligodendrogenesis and preserves myelin after ischemic stroke **(A)** Representative images of the infarction area costained with antibodies against APC$^+$ (green, marker of oligodendrocytes) and EdU (red, marker of proliferating cells) on day 28 after R1 treatment, yellow arrows indicate proliferated oligodendrocytes (EdU$^+$/APC$^+$ cells), DAPI (blue) indicates the nucleus, Scale bar = 50 μm **(B)** Quantitative analysis of proliferating oligodendrocytes (EdU$^+$/APC$^+$ cells) in the cortex region after R1 treatment. n = 3 brains per group. Representative images of immunoblotting **(C)** and quantification of cnpase (cyclicnucleotide 30-phosphohydrolase, **D**), MBP (myelin basic protein, **E**), and vimentin **(F)** in the infarct cortex region of the Sham, MCAO/R, R1, and MCAO/R + R1 groups on day 28 after MCAO surgery. n = 3 in each group. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ vs. Sham group; $^{*}p< 0.05$, $^{**}p< 0.01$ vs. MCAO/R group.

precursor cells (OPCs) generate myelin-forming oligodendrocytes, which are essential for myelin regeneration and functional recovery after cerebral ischemia (Kang et al., 2010; Hughes et al., 2013; Hill et al., 2018). By double labeling with APC, which a marker of mature oligodendrocytes (Han et al., 2015), and EdU, we found that the group treated with R1 for 28 days exhibited significantly increased numbers of APC$^+$/EdU$^+$ cells in the infarcted area compared with the group subjected to MCAO/R (**Figures 5A,B**). Concomitantly, R1 treatment for 28 days elevated the protein expression of cnpase and MBP (Saneto and de Vellis, 1985), which are markers of immature oligodendrocytes. Moreover, the dramatic increase in the protein expression of the microglia and reactive astrocyte marker vimentin (Jiang et al., 2012) after ischemia was markedly reduced by R1 (**Figures 5C–F**).

R1 Increases Neurotrophic Factor Expression and Promotes Synaptic Formation After Ischemia

Neurotrophic factors play a major role in regulating neurite sprouting and regeneration in response to nerve injuries (Fornaro et al., 2020). By ELISA, we found that the group treated with R1 exhibited clearly increased protein levels of various neurotrophic factors, such as BDNF, NGF and NT-4, compared with the group subjected to MCAO (**Figures 6A–F**). BDNF is a neurotrophic factor known to regulate neuronal survival and growth and to actively participate in synaptic transmission and plasticity in various brain regions (Kang and Schuman, 1995; Ding et al., 2011). To determine how BDNF activity is affected by R1 after MCAO surgery, we located the main source of BDNF by double immunofluorescence staining. We reported that a few NeuN-positive neurons expressed BDNF 28 days after MCAO surgery. However, NeuN-positive neurons predominately expressed BDNF after R1 treatment for 28 days (**Figure 6G**). Moreover, the crucial question is whether R1 can promote synaptic formation after ischemic injuries. Here, we detected the protein expression of SYN (synaptophysin), PSD95 (postsynaptic density protein 95), MAP2 (a somato-dendritic marker) and Tau-1 (an axonal marker). We found that rats treated with R1 for 28 days exhibited dramatically higher expression of SYN, PSD95, MAP-2 and Tau-1 than those treated with saline (**Figure 6H–L**).

R1 Promotes Regenerative Neurogenesis Through Akt/CREB Activation by Upregulating BDNF Expression

Mature BDNF specifically activates its receptor, tyrosine receptor kinase B (TrkB), to promote the survival, growth, migration, differentiation and maturation of NPCs (Keifer et al., 2009; Song et al., 2015). We aimed to assess if BDNF and the downstream effectors of TrkB signaling are involved in the mechanisms underlying the R1-mediated promotion of neurogenesis. Western blotting assays of the ischemic cortical tissue revealed that the rats treated with R1 for 7 and 28 days exhibited

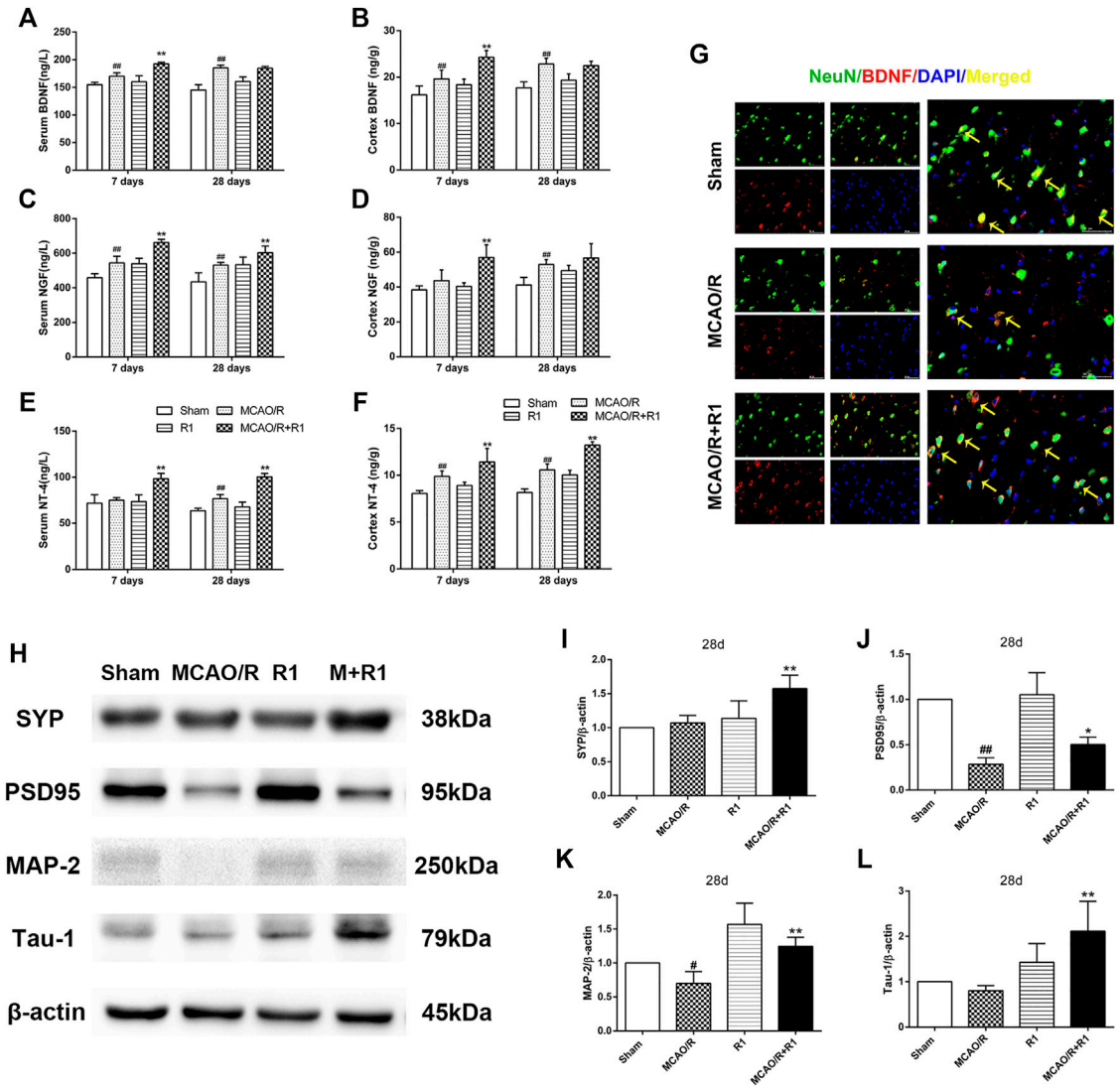

FIGURE 6 | R1 increases neurotrophic factor expression and restores disrupted neural synaptic function after ischemic stroke. The serum and cortex tissue levels of neurotrophic factors, such as BDNF **(A, B)**, NGF **(C, D)**, and NT-4 **(E, F)** were detected by ELISA. **(G)** Representative images of the infarction area costained with antibodies against BDNF (red) and NeuN (green) on day 28 after R1 treatment, yellow arrows indicate NeuN-positive neurons expressed BDNF, DAPI (blue) indicates the nucleus, Scale bar = 50 μm. Representative images of immunoblotting **(H)** and quantification of the relative protein levels of SYN **(I)**, PSD95 **(J)**, MAP-1 **(K)** and Tau-1 **(L)** in the infarct cortex region of the Sham, MCAO/R, R1, and MCAO/R + R1 groups on day 28 after MCAO surgery. $n = 3$ in each group. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ vs. Sham group; $^{*}p< 0.05$, $^{**}p< 0.01$ vs. MCAO/R group.

dramatically increased BDNF expression compared with the rats subjected to MCAO (**Figures 7A,B**). Akt and CREB have been confirmed as downstream targets of BDNF (Clarkson et al., 2015). We also found that 7 and 28 days after ischemia, *p*-TrkB/TrkB, *p*-CREB/CREB and *p*-Akt/Akt were significantly decreased due to ischemic injury compared with the sham rats. In addition, at 7 and 28 days, the rats treated with R1 exhibited markedly increased *p*-TrkB/TrkB (**Figures 7A,C**), *p*-CREB/CREB (**Figures 7A,D**) and *p*-Akt/Akt (**Figures 7A,E**) expression compared with the rats subjected to MCAO.

The pathogenesis of neurological disorders, such as stroke, can disrupt the function or expression of neurotransmitters (Abg Abd Wahab et al., 2019; Vogt, 2019). Neurotransmitter release from synapses is affected by BDNF (Ding et al., 2011). We wondered if R1 can promote neurotransmitter release after ischemic injuries. By matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI–MSI), we found that glutamate, N-acetylaspatate and K^+ profoundly were decreased 7 days after ischemic injury. The rats treated with R1 exhibited significantly higher levels of glutamate, N-acetylaspatate and K^+ in the ischemic cortex region that the rats subjected to MCAO (**Figures 7F–K**), suggesting that R1 regulated synaptic communication by modulating neurotransmitters (Shariatgorji et al., 2019).

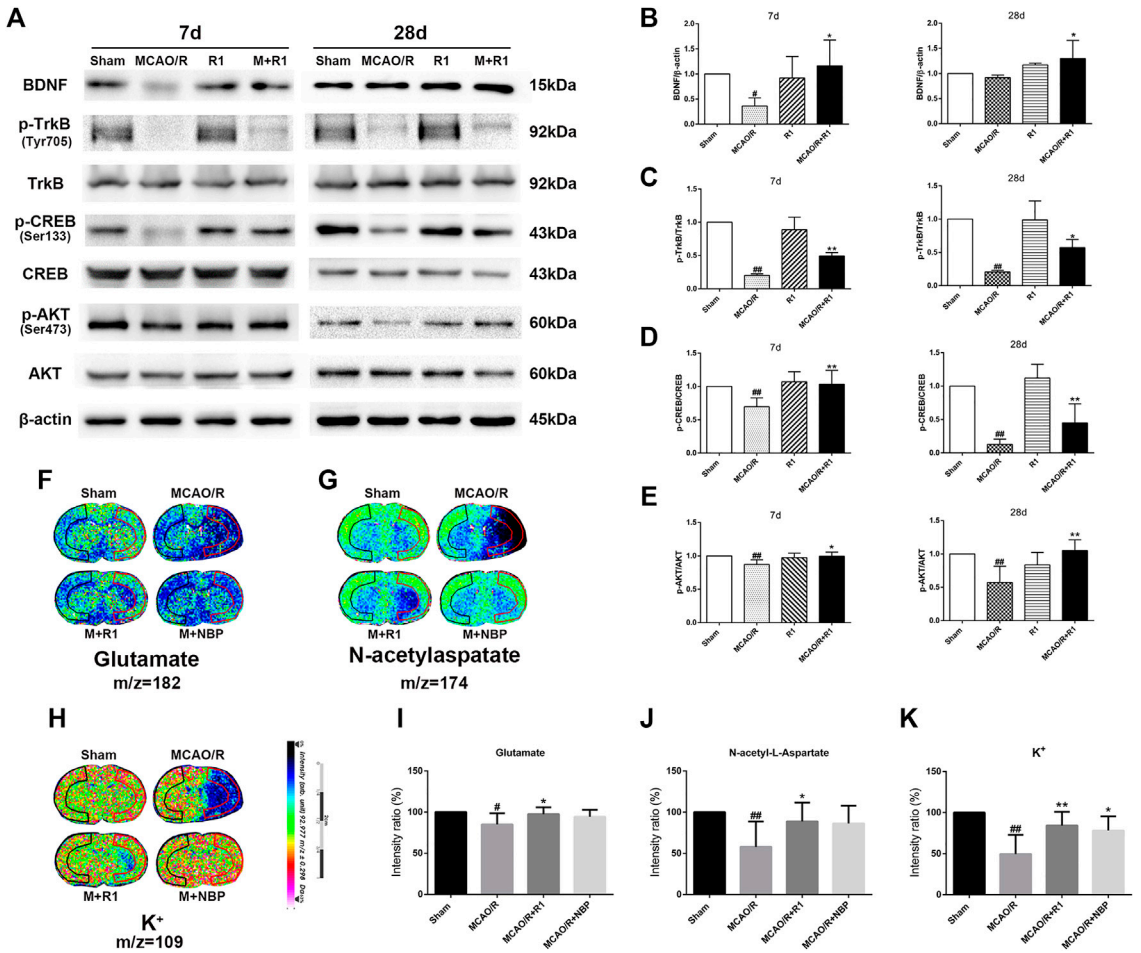

FIGURE 7 | R1 activates the BDNF/Akt/CREB signaling pathway to promote the recovery of neurological function. Representative images of immunoblotting **(A)** and quantification of the relative protein levels of BDNF **(B)**, *p*-TrkB/TrkB **(C)**, *p*-CREB/CREB **(D)** and **(E)** *p*-Akt/Akt in the infarct cortex region of the Sham, MCAO/R, R1, and MCAO/R + R1 groups on days 7 and 28 after MCAO surgery. $n = 3$ in each group. *In situ* MALDI MSI of glutamate **(F)**, N-acetylaspatate **(G)** and K^+ **(H) (I-K)** Quantitative measurement of the corresponding indicators in the ischemic cortex. Scale bar = 2 cm, $n = 5$ in each group. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R group.

R1 Promotes Neuronal Proliferation by BDNF and Akt Activation *in Vitro*

To better determine the effect of R1 on neuronal proliferation and the potential mechanism involved, we incubated R1 (the concentration reached up to 100 μM) with PC12 cells (after the cell attachment rate was approximately 70%) for 12 h and examined neuronal proliferation by EdU staining. R1 (25, 50 and 100 μM) more notably promoted neuronal proliferation by increasing the proliferation rate of EdU^+ cells (**Figures 8A,C**). To confirm whether the BDNF and Akt signals participated in the effect of R1 on neuronal proliferation, the neurons were pretreated with pharmacological inhibitors of the BDNF receptor TrkB (ANA-12) and PI3K (LY294002) before the addition of R1. Collectively, both ANA-12 and LY294002 inhibited the increased neuronal proliferation due to R1 (**Figures 8B,D**). Furthermore, both ANA-12 and LY294002 resulted in the loss of R1-induced neurogenesis after OGD/R injury and the R1-mediated preservation of BDNF, TrkB, CREB and Akt expression after OGD/R challenge, as shown by the western blotting results (**Figures 8E–I**).

DISCUSSION

Our previous studies have shown the neuroprotective effects of pretreatment with R1 during the acute stage of stroke in rats (Meng et al., 2014). In this study, we systematically investigated various doses of R1, injected i. p, and found that R1 decreased infarct volumes and ameliorated neurological deficits 7 days after ischemia in rats, suggesting that these doses produced optimal efficacies for evaluating neurological restoration after ischemic stroke.

We have demonstrated that R1 exerts potent neuroprotective effects via the suppression of NADPH

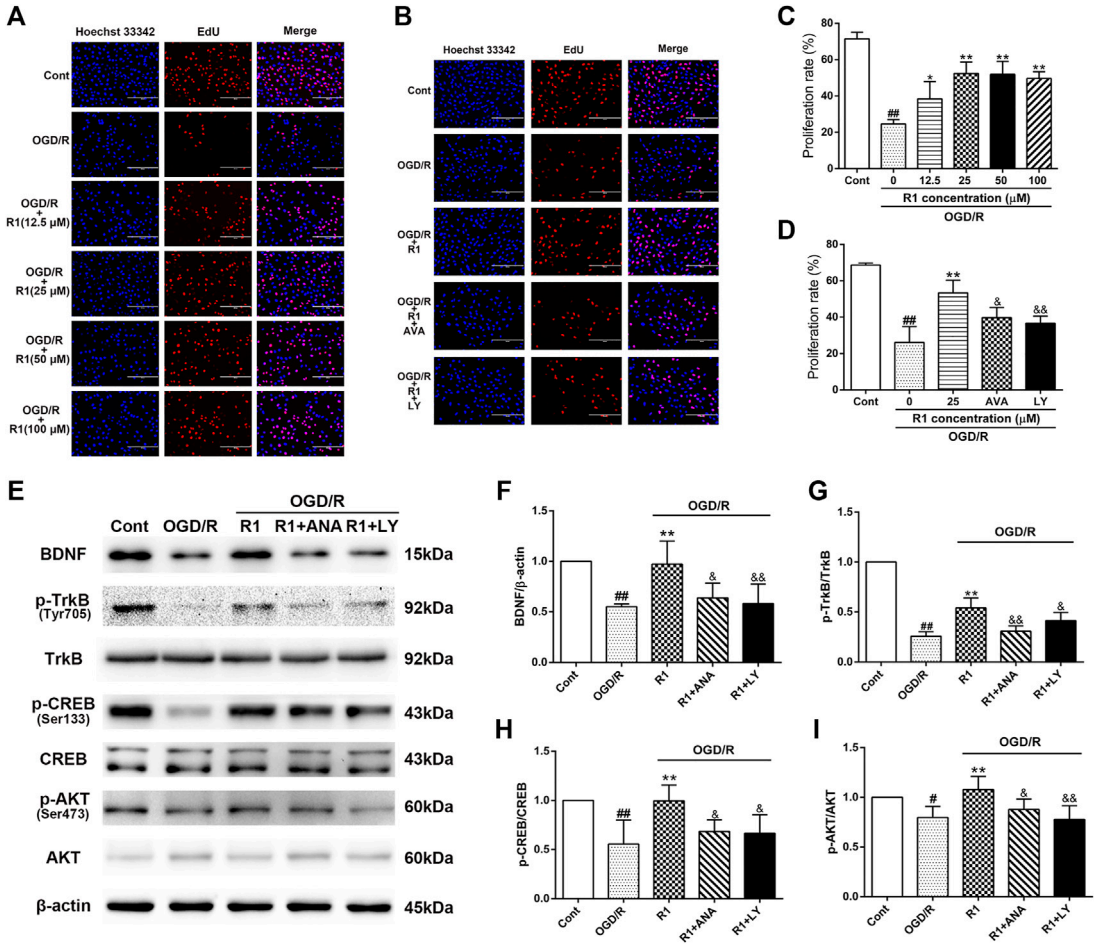

FIGURE 8 | R1 promotes neuronal proliferation though the BDNF and PI3K signaling pathways *in vitro* **(A)** R1 promotes neuronal proliferation. PC-12 cells were treated with or without R1 (drug concentration reached up to 100 μM) for 12 h. Images of proliferating neurons detected by EdU staining. Nuclei were visualized by DAPI staining (blue). Scale bar = 200 μm **(B)** PC-12 cells were pretreated with or without R1 (25 μM) in the presence or absence of ANA-12 (10 μM) or LY294002 (10 μM) for 12 h. Images of proliferating neurons detected by EdU staining. Nuclei were visualized by DAPI staining (blue). Scale bar = 200 μm **(C, D)** Proliferation rate of EdU^+ cells was analyzed. Representative images of immunoblotting **(E)** and quantification of the relative protein levels of BDNF **(F)**, *p*-TrkB/TrkB **(G)**, *p*-CREB/CREB **(H)** and *p*-Akt/Akt **(I)** in PC-12 cells. $n = 3$ in each group. Data are expressed as the mean ± SD and were analyzed by ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OGD/R group; and $p < 0.05$, and $p < 0.01$ vs. R1 group.

oxidase- and mitochondrion-derived superoxide and the inhibition of oxidative stress level *in vivo* and *in vitro* (Meng et al., 2014). In this study, we focused on the long-term recovery of neurogenesis and oligodendrogenesis postischemic stroke in rats. R1 promoted the recovery of long-term neurological function and stimulated neurogenesis. These preliminary results provide a theoretical basis for the generation of newborn neurons in ischemic brains. To the best of our knowledge, newborn neurons play a key role in neural plasticity, learning and memory, and emotional regulation, and their dysregulation is involved in a variety of brain disorders (Christian et al., 2014; Anacker and Hen, 2017). Combined with our previous research (Meng et al., 2014), these results suggest that R1 has both neuroprotective and neurorestorative effects that lead to improved neurological function at both the acute and chronic phases postischemia.

Neuronal stem cells (neural progenitor cells, NPCs) present in the hippocampus SVZ and SGZ have the ability to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes (Libert et al., 2008). After (hypoxic) ischemic brain injury, the rapid proliferation of young precursors enhances neurogenic capacity. In our present study, there was a significant increase in the number of DCX^+/EdU^+ cells and $Nestin^+/EdU^+$ cells in the striatum after R1 treatment, demonstrating that R1 stimulated the proliferation of NPCs and enhanced the formation and migration of neuroblasts. The vast majority of newborn neurons die within 2–5 weeks after ischemia due to the harmful niche environment, a lack of adequate trophic support, and the failure to form connections with other neurons, and only a small fraction of these newborn neurons can differentiate into mature neurons (Doeppner et al., 2011; Ryu et al., 2016). Our data also show that the number of $NeuN^+/EdU^+$ mature neurons in the periinfarct region was increased by R1 treatment. These results indicated that R1 treatment not only directly protects

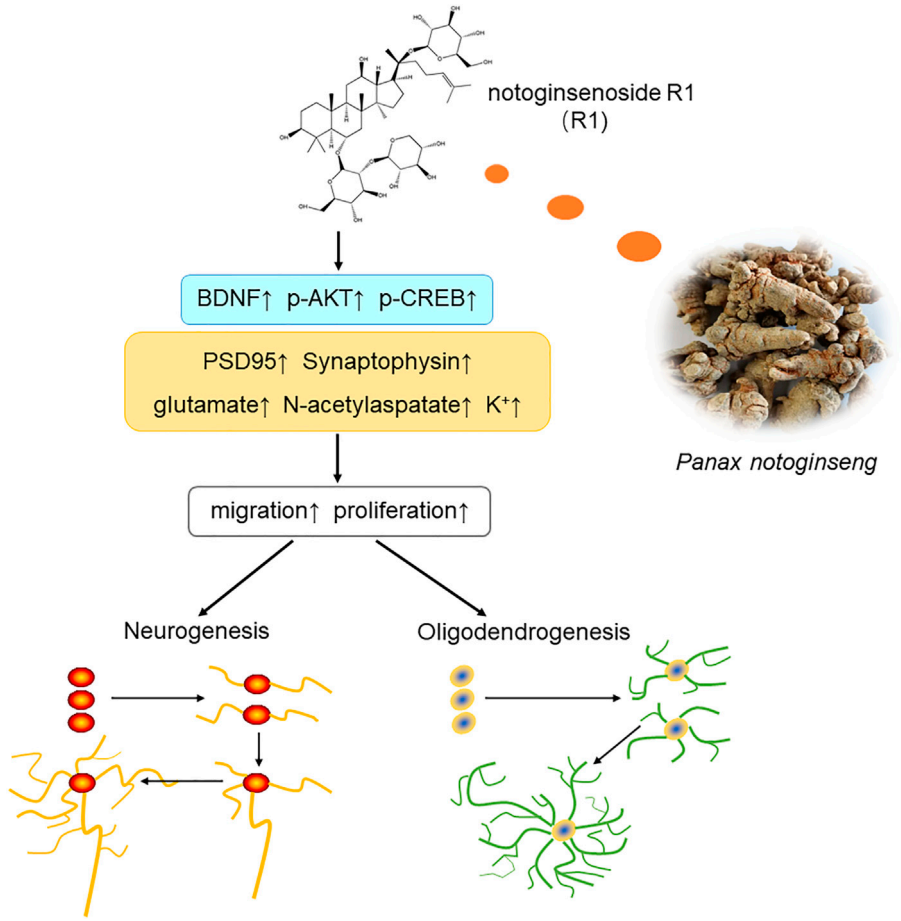

FIGURE 9 | Schematic diagram of the mechanisms underlying the R1-induced promotion of neurological recovery after ischemia-reperfusion injury. Overall, R1 enhances neurogenesis and oligodendrogenesis by promoting NPC proliferation, migration and the survival of newborn neurons. Therefore, R1 stimulates the expression of neural synaptic factors and facilitates the release of neurotransmitters. R1 activates the BDNF/Akt/CREB signaling pathway, thereby accelerating neurogenesis to promote neurological recovery.

newborn neurons but also supports the long-term survival of newborn neurons by preserving the local microenvironment.

Axons and dendrites are the two main structural and functional units of differentiated mature neurons, and both synaptogenesis and dendritic remodeling are related to increases in neurological activity in the cerebral cortex (Dimyan and Cohen, 2011). Importantly, neurons that release neurotransmitters at the synapse can be considered the vocabulary of the neuronal language (Vogt, 2019). In our results, R1 upregulated the levels of various synapse and neurotransmitters, namely, PSD95, SYN, glutamate, N-acetylaspatate and K^+, and increased the protein expression of mature dendrite and axon markers, namely, MAP-2 and Tau-1, respectively, which further showed that R1 is beneficial for promoting neuronal differentiation.

Oligodendrocytes originate from OPCs and eventually differentiate into myelin cells (Bergles and Richardson, 2015). The differentiation of oligodendrocytes and the subsequent myelination of axons can maintain axonal integrity and neuron survival, and the lack of this well-coordinated axon-glia interaction easily causes neuropsychiatric disorders (Herbert and Monk, 2017; Saab and Nave, 2017). Our work showed that the proliferation of APC^+ cells after stroke was stimulated by R1 treatment. We also observed increased protein levels of cnpase, an immature oligodendrocyte-specific protein, and MBP, or myelin basic protein. These findings indicated that R1 has the ability to promote oligodendrocyte production and remyelination after I/R injury. These benefits provide therapeutic potential for treating multiple sclerosis, spinal cord injury and other demyelinating diseases (Najm et al., 2015; Saab and Nave, 2017).

Many neurotrophic factors, including BDNF, NGF, and NT-4 have been demonstrated to protect neural stem cells (NSCs) and to promote neurogenesis after cerebral ischemia (Edelbrock et al., 2018). Our current study revealed that R1 treatment significantly increased the expression of BDNF, NGF and NT-4 after ischemic stroke, and these results are similar to a previous report on the promotion of neurogenesis following the overexpression of adenoviral-transduced BDNF mRNA (Benraiss et al., 2001). Because the occurrence of ischemic stroke will increase the

permeability of the blood brain barrier (BBB), a large number of inflammatory substances enter the brain to induce brain edema, accompanied by the production of newborn neurons. However, 80% of newborn neurons die due to lack of nutritional support or lack of timely drug intervention (Arvidsson et al., 2002). Our current study showed that R1 treatment remarkably alleviated BBB disruption (**Supplementary Figure S1**) and facilitated neurogenesis after I/R injury by increasing BDNF expression. Part of the reason was that the increased BDNF expression of R1 provides a microenvironment suitable for survival of newborn neurons. Simultaneously, the massive production of new neurons will repair the brain damage caused by ischemia, and then improve the permeability of the BBB.

BDNF specifically binds to TrkB receptor, which can promote neuron differentiation, maturation and synaptic plasticity during development or after injury (Edelbrock et al., 2018). These functions of BDNF/TrkB signaling are achieved by a combination of three downstream signaling cascades: the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase-C gamma (PLCγ) pathways (Edelbrock et al., 2018). Moreover, the activation of BDNF/TrkB signaling results in the phosphorylation of the transcription factor CREB (Shang et al., 2019; Du et al., 2020). Blocking BDNF signaling leads to reduced CREB transcription, which is essential for synaptic plasticity and learning and memory (Mowla et al., 1999). Western blot analysis confirmed that R1 upregulates the protein expression of BDNF, *p*-TrkB, *p*-Akt and *p*-CREB and that ANA-12 (TrkB inhibitor) and LY-294002 (PI3K inhibitor) significantly inhibited the R1-induced neural migration and proliferation *in vitro*. The data suggest that R1 promotes the recovery of neurological function after stroke via the BDNF/Akt/CREB signaling pathway. A previous study showed that Akt phosphorylates CREB, resulting in CREB-mediated gene expression, including the expression of BDNF, that is essential for neuron survival (Lu et al., 2013; Zhang et al., 2018). More specifically, we confirmed that BDNF, Akt and CREB participate in one regulatory loop. These findings were supported by the inhibition of TrkB (by ANA-12) and PI3K (by LY-294002), which resulted in the impairment of the R1-induced *p*-Akt and BDNF protein expression that ameliorated the I/R-induced neurofunction deficits.

CONCLUSION

In summary, the current study showed that treatment with R1 promoted neurogenesis and oligodendrogenesis after ischemic stroke. The mechanisms by which R1 restored neurological function involved the upregulation of Akt/CREB by increasing the expression of BDNF (**Figure 9**). These findings demonstrated that R1 is a promising new treatment for the long-term recovery of neurological function after ischemic stroke by promoting neurogenesis and oligodendrogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

All rats care and experimental procedures were reported in accordance with the Laboratory Animal Ethics Committee of the Institute of Medicinal Plant Development, Peking Union Medical College and complied with NIH Guidelines for the Care and Use of Laboratory Animals (approval number: SYXK 2017-0020).

AUTHOR CONTRIBUTIONS

GS and XS conducted the study. TZ designed the detailed experiments, performed the study, and collected and analyzed data. LW, WX, XM, and YF took part in the animal experiments in this study. TZ wrote the manuscript and LW helped to revise it. All authors discussed, edited, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.615998/full#supplementary-material

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Astrocytes and Adenosine A_{2A} Receptors: Active Players in Alzheimer's Disease

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Astrocytes, through their numerous processes, establish a bidirectional communication with neurons that is crucial to regulate synaptic plasticity, the purported neurophysiological basis of memory. This evidence contributed to change the classic "neurocentric" view of Alzheimer's disease (AD), being astrocytes increasingly considered a key player in this neurodegenerative disease. AD, the most common form of dementia in the elderly, is characterized by a deterioration of memory and of other cognitive functions. Although, early cognitive deficits have been associated with synaptic loss and dysfunction caused by amyloid-β peptides (Aβ), accumulating evidences support a role of astrocytes in AD. Astrocyte atrophy and reactivity occurring at early and later stages of AD, respectively, involve morphological alterations that translate into functional changes. However, the main signals responsible for astrocytic alterations in AD and their impact on synaptic function remain to be defined. One possible candidate is adenosine, which can be formed upon extracellular catabolism of ATP released by astrocytes. Adenosine can act as a homeostatic modulator and also as a neuromodulator at the synaptic level, through the activation of adenosine receptors, mainly of A_1R and $A_{2A}R$ subtypes. These receptors are also present in astrocytes, being particularly relevant in pathological conditions, to control the morphofunctional responses of astrocytes. Here, we will focus on the role of $A_{2A}R$, since they are particularly associated with neurodegeneration and also with memory processes. Furthermore, $A_{2A}R$ levels are increased in the AD brain, namely in astrocytes where they can control key astrocytic functions. Thus, unveiling the role of $A_{2A}R$ in astrocytes function might shed light on novel therapeutic strategies for AD.

Keywords: astrocyte reactivity, amyloid-β protein, synaptic plasticity, cognitive deficits, adenosine A_{2A} receptors, Alzheimer's disease

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ADENOSINE SIGNALING AND ASTROCYTE-NEURON COMMUNICATION

One pathway underlying astrocyte-neuron interactions in CNS is the purinergic signaling, mainly operated by ATP and adenosine that constitute two superimposed signaling systems (Agostinho et al., 2020). ATP released by astrocytes, a subtype of glial cells, is a significant source of adenosine in the brain, being the extracellular adenosine levels regulated by a set of

ectonucleotidases, in particular by astroglial ecto-5′-nucleotidase (CD73), which is the last and rate-limiting step in the extracellular formation of ATP-derived adenosine (Brisevac et al., 2015). Moreover, adenosine can also be released directly *via* equilibrative nucleoside transporters (ENT), such as ENT-1 and ENT-2, being the intracellular levels of this nucleoside controlled by the activity of adenosine kinase (ADK), which is mainly expressed in astrocytes and metabolize the conversion of adenosine into AMP (Boison et al., 2010). Adenosine is considered both a neuromodulator and a stress signal, and its functions are mediated by four subtypes of adenosine receptors: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R, which are G-protein-coupled receptors, each with a unique pharmacological profile. These metabotropic receptors can recruit different enzymatic activities and/or changes of ion channels function to mediate modulatory actions, with A_1R and A_3R being considered inhibitory and $A_{2A}R$ and $A_{2B}R$ as facilitatory (reviewed in Cunha, 2016, 2019; Agostinho et al., 2020). Briefly, the activation of A_1R and A_3R, through the action of Gi proteins, inhibits adenylyl cyclase (AC) activity and leads to increased activity of phospholipase C that further triggers its downstream signaling pathways, whereas $A_{2A}R$ and $A_{2B}R$ activation, through Gs proteins instigation, increases the production of cAMP to activate protein kinase A (PKA) signaling pathways (Jacobson and Gao, 2006; Cunha, 2016; Agostinho et al., 2020).

Adenosine's effects in the brain are mostly mediated by A_1R and $A_{2A}R$, which prime role is the modulation of synaptic activity, interfering with information transmission within neuronal circuits (reviewed in Fredholm et al., 2005; Cunha et al., 2008; Agostinho et al., 2020). Both A_1R and $A_{2A}R$ are mostly located in synapses in particular in excitatory (glutamatergic) synapses, although they are also present in gamma-aminobutyric acid (GABA)ergic, cholinergic, dopaminergic, serotoninergic, or noradrenergic synapses (reviewed in Cunha, 2016). In excitatory synapses, adenosine under basal conditions can inhibit synaptic transmission *via* A_1R-mediated activity mainly located in presynaptic terminals (Rebola et al., 2003, 2005). In contrast, $A_{2A}R$ are only recruited upon higher frequencies of nerve stimulation, triggering plastic changes of synaptic efficiency that enhance glutamate release as well as *N*-methyl-D-aspartate receptor (NMDAR) activation (Rebola et al., 2008), in order to facilitate synaptic plasticity (reviewed in Cunha, 2016). Synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD), is considered the neurophysiological basis of memory (Martin et al., 2000; Neves et al., 2008). Accordingly, a study using optogenetic tools showed that $A_{2A}R$ activation, through a phospho-CREB signaling in the hippocampus is sufficient to impair memory function (Li et al., 2015). Moreover, there is evidence supporting a correlation between the adenosine sources and the type of adenosine receptors that are activated, insofar as it was described that A_1R are mainly activated by the tonus of adenosine, formed from the catabolism of ATP released from astrocytes (Pascual et al., 2005) and by postsynaptic adenosine efflux (Lovatt et al., 2012), whereas adenosine derived from synaptically-released ATP, due to CD73 action, mainly activates neuronal, mainly postsynaptic, $A_{2A}R$ (Cunha et al., 1995; Rebola et al., 2008; Augusto et al., 2013; Carmo et al., 2019; Gonçalves et al., 2019).

Adenosine can also act as an astrocytic modulator, regulating astrocytic metabolism (Lemos et al., 2015), Ca^{2+} waves (Kawamura and Kawamura, 2011; Kanno and Nishizaki, 2012), and neurotransmitter uptake ability (Nishizaki et al., 2002; Cristóvão-Ferreira et al., 2013; Matos et al., 2013). In relation to adenosine signaling in astrocytes, it has been documented that all the different subtypes of adenosine receptors are present in astrocytes, although A_1R and $A_{2A}R$ have been the most studied (Daré et al., 2007; Boison et al., 2010; Agostinho et al., 2020). Under physiological conditions, the role of adenosine signaling in astrocytes is mostly hypothetical, since it is based on the effects of A_1R and $A_{2A}R$ in cultured astrocytes; additionally, the role of these receptors in pathological conditions, controlling the morpho-functional changes of reactive astrocytes, seems to be better supported by experimentation, although still far from being established. Among the functions known until now, A_1R in astrocytes mediate an immunosuppressive effect, whereas astrocytic $A_{2A}R$ can trigger transcriptional deregulation (Paiva et al., 2019) and mediate astrocyte reactivity (Brambilla et al., 2003; Ke et al., 2009), control glutamate release and consequently synaptic transmission (Nishizaki et al., 2002; Cervetto et al., 2017), regulate glutamate uptake by controlling the levels of glutamate transporters and the activity of Na^+/K^+-ATPase (Nishizaki et al., 2002; Matos et al., 2013). Moreover, it was shown that astrocytic $A_{2A}R$ and Gs-coupled signaling regulate memory consolidation in mice (Orr et al., 2015). Altogether, these findings highlight the relevant role of astrocytic $A_{2A}R$ in regulating synaptic plasticity and memory, suggesting that $A_{2A}R$ in astrocytes might also be a good candidate to normalize memory in case of pathology associated with cognitive deficits, such as in Alzheimer's disease (AD).

ASTROCYTES' ROLE IN ALZHEIMER'S DISEASE

In the last years, the neurocentric view of AD, which considers neurons as the major players in this neurodegenerative process, has been challenged due to increasing evidences supporting a role of astrocytes in this age-related disease. Although the pathological potential of astrocytes in AD was first recognized in 1910 by Alois Alzheimer, who described the presence of glial cells in close association with dying neurons and reported that glial cells made part of senile plaques (reviewed in Serrano-Pozo et al., 2011; Rodríguez-Arellano et al., 2016), the role of astrocytes in AD pathology has been underexplored.

Alzheimer's disease pathology is associated with an abnormal production of amyloid-beta (Aβ) peptides that accumulate extracellularly over time, as amyloid plaques. Aβ peptides are generated through the sequential proteolytic processing of amyloid-beta precursor protein (AβPP) by β-secretase (BACE1) and γ-secretase. Other neuropathological features of AD are the intracellular formation of intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein, together with synaptic dysfunction and loss that progress to neuronal

death (Haass and Selkoe, 2007; Selkoe and Hardy, 2016). Several studies have shown that in early AD, corresponding to the first symptoms of cognitive deficits, soluble Aβ oligomers trigger an extensive proliferation of astrocytes with a reactive phenotype. Reactive astrocytes, besides functional changes, exhibit alterations of their morphology that encompass a hypertrophy of the cell soma and the shortening and/or thickening of astrocytic processes, as well as the upregulation of several astrocytic structural proteins, such as glial fibrillary acidic protein (GFAP), or cell signaling proteins, like S100β (Ceyzériat et al., 2018; Escartin et al., 2021). Astrocyte reactivity usually persists and aggravates over time and parallels Aβ deposition, being common to observe reactive astrocytes surrounding amyloid plaques in the hippocampus of AD mouse models (Olabarria et al., 2010), as well as in human AD brains (Kashon et al., 2004). Astrogliosis *in vivo* can be assessed by positron-emission tomography detection of ^{11}C-deuterium-L-deprenyl (^{11}C-DED); deprenyl is a selective inhibitor of monoaminoxidase-B (MAO-B) localized in astrocytes, and an increase in the ^{11}C-DED signal reflects astrocyte hypertrophy (Fowler et al., 1997; Verkhratsky et al., 2019). On the other hand, the astrocytes located away from amyloid plaques are usually atrophic (Rodríguez et al., 2009; Olabarria et al., 2010). Astrocyte atrophy is detected in several AD mice models as well in in postmortem tissues of patients with advanced (Braak V–VI) stages of AD (see review, Verkhratsky et al., 2019), corresponding to a reduction of astrocytes territories with a decrease in coverage of synaptic contacts and other neuronal structures, and is accompanied by a loss of astrocyte function. These morphofunctional astrocyte changes can also lead to early cognitive deficits through dwindling neuronal support and synaptic dysfunction (Verkhratsky et al., 2019).

Studies performed in mouse models of AD have provided important information about the astrocytic alterations along AD progression. A reduction of volume and surface area of astrocytes, and a decrease in their processes, corresponding to a process of astrodegeneration, has been observed in AD mouse models. Triple transgenic AD mice, 3×TgAD, exhibit astrodegeneration before Aβ deposition in the medial prefrontal cortex, entorhinal cortex, and hippocampus (Yeh et al., 2011; Kulijewicz-Nawrot et al., 2012; Rodríguez et al., 2014). Astrocyte atrophy was also reported in the hippocampus of other transgenic AD (PDAPP) mouse model at early phases, before formation of amyloid plaques (Beauquis et al., 2014). In contrast, at later stages of AD, the presence of Aβ deposits triggers a secondary astroglial response, crresponding to a robust reactive astrogliosis in areas surrounding the amyloid plaques (Olabarria et al., 2010; Rodríguez-Arellano et al., 2016; Verkhratsky et al., 2016). This prompts the hypothesis that both this distal atrophy of astrocytes and the proximal astroglial reactivity contribute to the development of AD pathology.

Although, astrogliosis was long considered a broad secondary reaction to pathological conditions, reactive astrocytes can cause harmful effects to other brain cells, either as a consequence of loss of their normal homeostatic functions or due to a gain of toxic functions, linked to a decreased capacity of these cells to remove pathogens and amyloid proteins, in particular Aβ, or to phagocyte dystrophic synapses and cell debris (Barreto et al., 2011; Rodríguez-Arellano et al., 2016; Garwood et al., 2017). There is evidence that reactive astrocytes contribute to amyloid plaque formation, and consequently AD pathology, by different mechanisms, including: (i) hampered phagocytic capacity that is mediated by actin-regulated phagocytosis and/or by several membrane receptors (e.g., lipoprotein receptor-related ligand 1, receptor for advanced glycation end products (RAGE), scavenger receptors); (ii) reduced capacity to degrade the internalized Aβ, which involves the production of proteases, such as neprilysin, insulin-degrading enzyme, and enzymes of the ubiquitin-proteasome system (reviewed in Wyss-Coray et al., 2003; Miners et al., 2008; Ries and Sastre, 2016; Rodríguez-Arellano et al., 2016); and (iii) increased Aβ production by astrocytes due to BACE1 upregulation and, subsequent, amyloidogenic processing of AβPP triggered by proinflammatory conditions, mainly by IL-1β and TNF-α (Blasko et al., 2000; Sastre et al., 2003; Zhao et al., 2011). Although it was reported that reactive astrocytes produced Aβ in less quantity than neurons, astrocytes mostly produce N-truncated Aβ species, which are highly prone to aggregation and more toxic than the species produced by neurons (Oberstein et al., 2015; Frost and Li, 2017). Besides having a key role in amyloid pathology, reactive astrocytes can also participate in tau pathology (reviewed in Kovacs, 2020). A recent study reported that hippocampal astrocytes in the dentate gyrus of AD patients exhibit hyperphosphorylated tau, and this abnormal tau impairs Ca^{2+} oscillation and mitochondria motility, distribution, and function in astrocytes, contributing also to reduce: (i) adult neurogenesis; (ii) parvalbumin-expressing neurons; (iii) inhibitory synapses; and (iv) hilar gamma oscillations, which were accompanied by a weakened spatial memory performance (Richetin et al., 2020). Remarkably, since the presence of tau is not detectable in astrocytes under physiological conditions, the origin of this protein in astrocytes in AD-like conditions has been speculated, being proposed that during AD progression, tau translation might occur from mRNA present in astrocytes and astrocytic uptake of extracellular tau, released in the interstitial fluid by neurons, as well as intercellular propagation of tau through exosomes (see Richetin et al., 2020, and references within). In agreement, it was reported that astrocyte reactivity correlates with NFT density in the brains of AD patients (Overmyer et al., 2000; Gallardo and Holtzman, 2019). These findings contribute to sustain that astrogliosis is more directly associated with NFT, and hyperphosphorylated tau, since reactive astrocyte responses increase linearly with NFT burden and distribution, but not with amyloid pathology that tends to reach a plateau (Ingelsson et al., 2004; Serrano-Pozo et al., 2011).

Since the 1990s, several studies pointed out that astrocytes enwrap synaptic terminals and exchange information with them, responding to synaptic activity and regulating synaptic transmission (Parpura et al., 1994; Araque et al., 1999; Halassa et al., 2007; Covelo and Araque, 2016). Thus, astrocyte reactivity, due to Aβ accumulation and tau dyshomeostasis, may compromise synaptic plasticity and consequently memory. Accordingly, immunohistochemistry studies, assessing GFAP or S100β upregulation and distribution in AD patient's brain,

reported that the degree of astrogliosis is correlated with cognitive decline (Beach and McGeer, 1988; Mrak et al., 1996; Kashon et al., 2004). Studies performed in cultured astrocytes exposed to Aβ peptides showed that these cells became reactive and had a decreased glutamate uptake capacity, due to a downregulation of glutamate transporters, mainly of GLT-1 (Matos et al., 2012b; Zumkehr et al., 2015), being also shown that $A\beta_{1-42}$ peptide promotes GLT-1 internalization (Scimemi et al., 2013). These alterations could contribute not only to excitotoxicity but also to impair synaptic plasticity occurring in AD conditions. Accordingly, it was reported that genetic reduction of GLT-1 levels accelerates the onset of cognitive deficit in a double (AβPPswe/PS1ΔE9) transgenic AD mouse model (Mookherjee et al., 2011), whereas the pharmacological upregulation of GLT-1 ameliorates the pathological tau accumulation, restores synaptic proteins and rescues cognitive decline, with minimal effects on Aβ pathology, in 3xTgAD mice (Zumkehr et al., 2015).

Moreover, Aβ accumulation causes an increased astrocytic excitability, mediated by sporadic Ca^{2+} signals that spread over to other astrocytes in the form of Ca^{2+} waves, which were shown to impact on synaptic transmission (Kuchibhotla et al., 2009). The hyperactive Ca^{2+} signaling might trigger an aberrant release of gliotransmitters, mainly through exocytosis involving the fusion of SNARE proteins of vesicles with the plasma membrane (Araque et al., 1999; Genoud et al., 2006). These alterations in astrocyte excitability and in gliotransmitters release (e.g., ATP, glutamate, D-serine) sustain the possibility of an abnormal metaplasticity, i.e., the regulation of synaptic plasticity by astrocytes, which might underlie the cognitive deficits observed in most mouse models of AD (Jones, 2015). The increased levels of glutamate and of Aβ oligomers, occurring in early AD phases, affect NMDAR subunits, NR2A and NR2B, which are crucial for synaptic plasticity, being the increased NR2B levels particularly associated with LTP inhibition and metaplasticity inversion in hippocampal slices of a transgenic AD mouse model (AβPP23) with impaired spatial working memory (Balducci et al., 2010). Moreover, soluble Aβ oligomers can engage the astrocytic α7 nicotinic acetylcholine receptors to induce glutamate release from astrocytes that in turn activate extrasynaptic NMDAR in neurons, causing a reduction in miniature excitatory postsynaptic currents (Talantova et al., 2013). In line with these findings, it is believed that the beneficial role of memantine (an NMDAR antagonist), in patients with moderate to severe AD, is mainly mediated through the blockade of extrasynaptic NMDAR activated by excess glutamate (Reisberg et al., 2003). GABA is the major inhibitory transmitter in the adult mammalian brain, and AD patients have increased GABA levels in their cerebrospinal fluid (Samakashvili et al., 2011) and antagonists of $GABA_A$ receptors improve hippocampal LTP and memory in an AD (APP/PS1) mouse model (Yoshiike et al., 2008). In line with these findings, it was reported that hippocampal reactive astrocytes of different AD mouse models (APP/PS1 and 5xFAD) excessively produce GABA and release GABA abundantly through bestrophin 1 (Best1), a channel that in non-reactive astrocyte mediates the release of glutamate (Jo et al., 2014). Moreover, these authors also showed that the abnormal release of GABA by reactive astrocytes reduces the spike probability of granule cells by acting on presynaptic GABA receptors, leading to an impairment of synaptic plasticity and memory in AD mouse models (Jo et al., 2014). Another study also reported that in human AD brains, hippocampal astrocytes have a higher GABA content, and that 5xFAD mice modeling AD have astrocytes also with higher GABA levels and release through astrocyte-specific GABA transporters, GAT3/4 (Wu et al., 2014).

In conclusion, astrocytes support neuronal function, in particular synaptic plasticity, in many ways, and it is plausible that the dysfunction of these glial cells contributes to cognitive deficits associated with AD. In the last years, most of the therapies developed for AD were directed to avoid Aβ formation and accumulation or to normalize synaptic plasticity, as, for example, by inhibiting acetylcholinesterase to normalize acetylcholine levels in the synaptic cleft and by NMDAR antagonism. These strategies have been shown to be little effective, thus there is a need to find novel targets to delay the onset of synaptic and memory deficits in AD (Mangialasche et al., 2010; Morsy and Trippier, 2019).

A possible valid target for AD management is astrocytes, more precisely the manipulation of their functions, and a promising candidate to interfere with the ability of astrocytes to control synaptic function and memory is adenosine receptors, in particular adenosine $A_{2A}R$. This stems from observations that: (i) $A_{2A}R$ are located in astrocytes, where they critically control Na^+/K^+-ATPase (Matos et al., 2012b, 2013), the main energizing system to sustain membrane-dependent processes in astrocytes; (ii) astrocytic $A_{2A}R$ control glutamate uptake by GLT-1, a process de-regulated in an AD mouse model (Matos et al., 2013); and (iii) astrocytic $A_{2A}R$ are upregulated in AD animal models and patients (Matos et al., 2015, 2012b; Orr et al., 2015). This is of particular importance in view of the convergence of epidemiological and animal studies showing that caffeine intake is inversely correlated with memory deterioration in aging and in AD, an effect mimicked by the selective $A_{2A}R$ blockade (Cunha and Agostinho, 2010; Agostinho et al., 2020). Noteworthy, our group showed that the pharmacological activation of $A_{2A}R$ (Pagnussat et al., 2015) or the optogenetic activation of neuronal $A_{2A}R$ intracellular signaling in the hippocampus is actually sufficient to impair memory (Li et al., 2015), and these receptors in astrocytes were also shown to regulate memory processes, since the genetic ablation of astrocytic $A_{2A}R$ enhances memory performance of aged mice modeling AD (Orr et al., 2015).

ADENOSINE $A_{2A}R$ SIGNALING IN AD: WHAT IS THE ROLE OF ASTROCYTIC $A_{2A}R$?

$A_{2A}R$ signaling, besides having a discrete role in normal brain function, is mainly able to modulate the development or progression of several brain diseases, including AD (Gomes et al., 2011; Cunha, 2016; Franco and Navarro, 2018). Accordingly, the antagonism of $A_{2A}R$ has been shown to confer neuroprotection in several injurious and pathological brain conditions and can recover memory deficits in animal models of AD, which prompts $A_{2A}R$ as a therapeutic target for this disease

(Arendash et al., 2006; Dall'lgna et al., 2007; Canas et al., 2009; Laurent et al., 2016; Viana da Silva et al., 2016; Silva et al., 2018). The neuroprotective effect of $A_{2A}R$ blockade against different brain pathologies, is mimicked by caffeine (Arendash et al., 2006; Dall'lgna et al., 2007; Takahashi et al., 2008; Espinosa et al., 2013; Laurent et al., 2014; Cunha, 2016). The regular consumption of caffeine/coffee, a non-selective adenosine receptor antagonist that at dose usually consumed by humans (around 200–300 mg caffeine or 3–5 cups of coffee/day) acts particularly as an $A_{2A}R$ antagonist (Fredholm et al., 2005), is inversely correlated with the incidence of AD and later dementia in humans (Maia and de Mendonça, 2002; Eskelinen et al., 2009), and is also protective against cognitive decline in AD mouse models (Arendash et al., 2006; Dall'lgna et al., 2007; Cao et al., 2009; Han et al., 2013; Chen, 2014; Laurent et al., 2014). Curiously, it was also reported that caffeine enhances the consolidation of long-term memories in humans, 24 h after a one-dose (200 mg) administration (Borota et al., 2014). Moreover, our group demonstrated that in rodent AD models, consisting in the intracerebroventricular Aβ injection, both caffeine and the selective $A_{2A}R$ antagonist SCH58261 prevent Aβ-induced cognitive impairments and synaptotoxicity (Dall'lgna et al., 2007; Cunha et al., 2008; Canas et al., 2009). A similar protective effect of the $A_{2A}R$ antagonist SCH58261 was also observed in AD transgenic (3xTgAD and APP/PS1) mouse models (Viana da Silva et al., 2016; Silva et al., 2018). These data were complemented with studies in rat primary-cultured neurons where it was observed that the pharmacological $A_{2A}R$ blockade attenuates Aβ-induced neuronal death through a reduction in $A_{2A}R$-mediated p38 mitogen-activated protein kinase (MAPK) activation and preservation of hippocampal synaptosome function (Canas et al., 2009).

All these findings reporting that the blockade of $A_{2A}R$ prevents synaptic dysfunction and cognitive deficits, mainly memory loss, in conditions of AD, support that the modulation of synaptic function may constitute an interesting strategy to improve memory dysfunction related to neurodegenerative processes (Coleman et al., 2004; Wishart et al., 2006; Canas et al., 2018).

In accordance with the putative role of $A_{2A}R$ as a therapeutic target in AD, it was reported that $A_{2A}R$ levels are increased in the hippocampus of AD patients, in astroglial cells (Angulo et al., 2003; Orr et al., 2015), and also in the frontal cortex of AD (Albasanz et al., 2008). A recent study showed that aging also caused a significant upsurge of $A_{2A}R$ in hippocampal neurons of aged humans, a phenotype aggravated in AD patients (Temido-Ferreira et al., 2020). Regarding A_1R, there are evidences from positron emission tomography (PET) studies that their levels are decreased in the hippocampus of AD patients (Fukumitsu et al., 2008), whereas increased levels of A_1R were detected in postmortem AD frontal cortex (Albasanz et al., 2008) and in degenerating neurons with neurofibrillary tangles and in dystrophic neurites of senile plaques (Angulo et al., 2003). Likewise, in AD mice and in aged mice, a cortical and hippocampal upsurge of $A_{2A}R$ was reported mainly in glutamatergic synapses (Diógenes et al., 2007; Canas et al., 2009; Costenla et al., 2011), and recently, $A_{2A}R$ overexpression was reported to be sufficient to drive age-like memory impairments in young rats and to uncover a hippocampal LTD-to-LTP shift, which is a signature of memory impairment (Temido-Ferreira et al., 2020). Furthermore, the activation of $A_{2A}R$ in endothelial cells was shown to increase blood–brain barrier (BBB) permeability in mice, facilitating the penetration of macromolecules into the brain, such as proinflammatory and neurotoxic factors, which might contribute to AD pathology (Carman et al., 2011). Curiously, a gene-based association study reported that the gene encoding $A_{2A}R$ (ADORA2A) is associated with hippocampal volume in humans, being its minor allele, rs9608282, related with larger hippocampal volumes and better memory (Horgusluoglu-Moloch et al., 2017).

Although the above-described findings reinforce a link between $A_{2A}R$ and cognitive deficits associated with AD, the impact of astrocytic $A_{2A}R$ in AD-associated cognitive deficits has surprisingly been underexplored. In the brain of AD patients, it was reported that $A_{2A}R$ levels are increased in astrocytes but not in microglia (Orr et al., 2015). Moreover, cultured astrocytes exposed to $A\beta_{1-42}$ also exhibited an upregulation of $A_{2A}R$, which is related with a reduced capacity of astrocytes to uptake glutamate (Matos et al., 2012b) that can trigger excitotoxicity. This decrease in glutamate uptake is caused by the downregulation of glutamate transporter, GLT-1, in astrocytes, whose activity is dependent on Na^+/K^+ activity that is regulated by $A_{2A}R$ (Matos et al., 2013). Interestingly, the genetic lowering of GLT-1 expression in AD mice (APP/PS1) causes an earlier onset of cognitive deficits (Mookherjee et al., 2011). The upregulation of $A_{2A}R$ in cultured astrocytes strongly modulates the transcriptome of these cells, affecting mainly genes related with neuroinflammation, angiogenesis and cell activation; some of the changes were reversed by a selective $A_{2A}R$ antagonist (Paiva et al., 2019). Although there is evidence suggesting that $A_{2A}R$ blockade restrains astrocyte reactivity (Hindley et al., 1994; Brambilla et al., 2003; Minghetti et al., 2007, see also Cunha, 2016), the pathophysiological impact of astrocytic $A_{2A}R$ upsurge in reactive astrocytes in conditions of brain disorders, in particular of AD, remains to be defined. It was described that $A_{2A}R$ in astrocytes regulate Ca^{2+} efflux from the endoplasmic reticulum and glutamate release (Kanno and Nishizaki, 2012) and also ATP release (unpublished data from our group, Madeira D, poster #726, T05-063B, *XIV European meeting on glial cells in health and disease*) as well as GABA transport (Cristóvão-Ferreira et al., 2013), which support that astrocytic $A_{2A}R$ regulate the secretory capacity of these cells and, thus, impact on astrocyte-neuron communication. Therefore, taking in account the role of $A_{2A}R$ in controlling key astrocytic functions and the upregulation of $A_{2A}R$ in AD, it might be helpful to develop strategies, genetic or pharmacological, directed to tinker specifically with astrocytic $A_{2A}R$ to halt AD-associated cognitive decline.

CONCLUSION AND FUTURE PERSPECTIVES

Although it is known for over a century that astrocytes display substantial morphological alterations in AD brains,

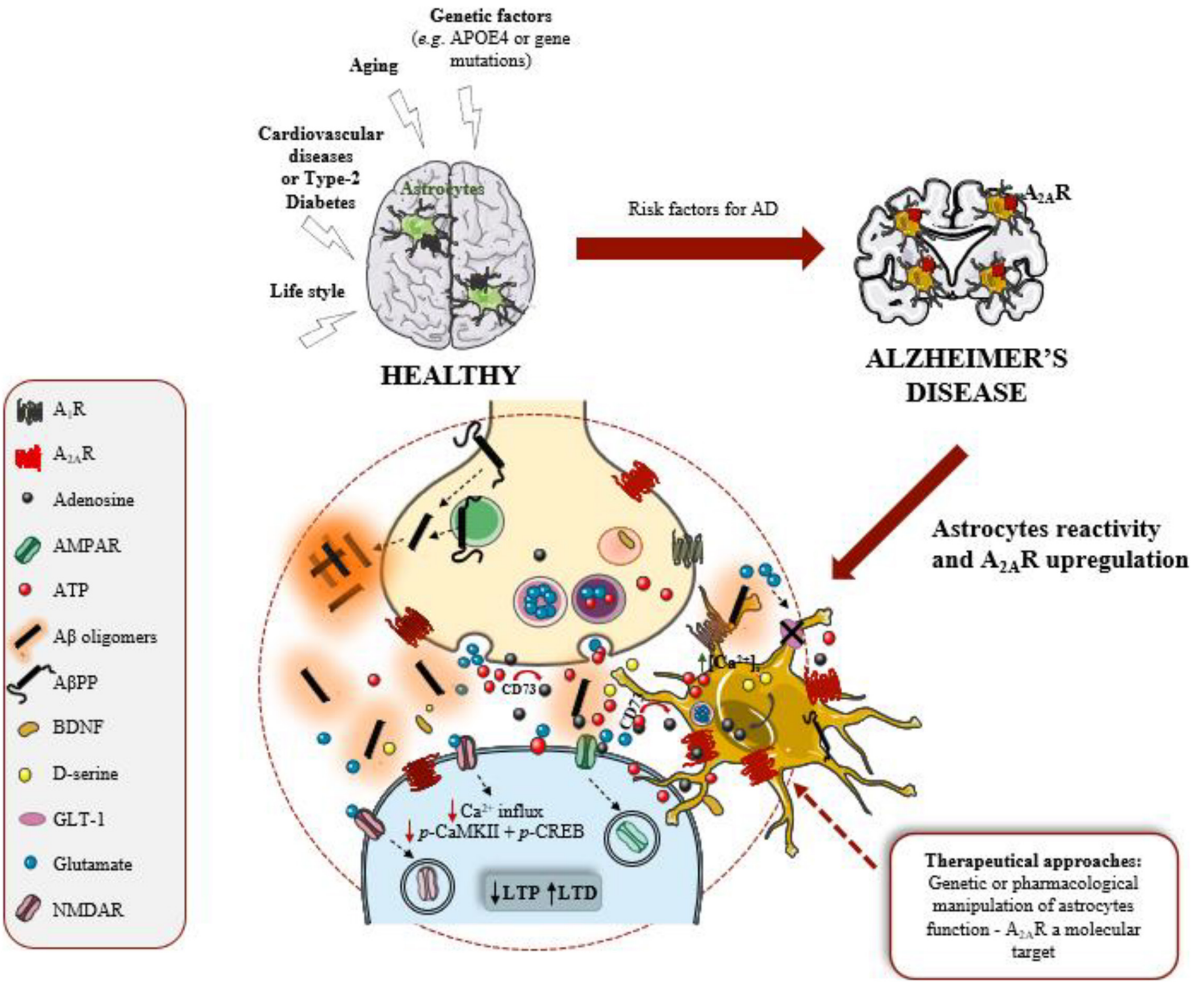

FIGURE 1 | Astrocytes and $A_{2A}R$ are active players in Alzheimer's disease (AD). Adenosine A_{2A} receptors ($A_{2A}R$) are upregulated in AD brain, namely in astrocytes. These glial cells are considered the third active element of the synapse, regulating synaptic plasticity, mainly long-term potentiation (LTP) and long-term depression (LTD) that are events mediated by AMPAR and NMDAR (receptors for glutamate). Astrocytes became reactive and dysfunctional in response to amyloid-β peptide (Aβ) overproduction, derived from amyloid-β precursor protein (AβPP) proteolytic cleavage. Astrocyte dysfunction negatively impacts on synaptic plasticity, the neurophysiological basis of memory. $A_{2A}R$ regulate key functions of astrocytes, such as intracellular Ca^{2+} levels $[Ca^{2+}]_i$, glutamate uptake by the transporter (GLT-1) and the release of ATP, and consequently regulate synaptic plasticity. Thus, $A_{2A}R$ in astrocytes might be a therapeutical target to manage AD.

neuronal damage has been considered the paramount pathological event causing cognitive decline. In recent years, this "neurocentic" view of AD has been changing, with the growing evidences that astrocytic morphological changes also reflect functional alterations with impact in AD pathology. Astrocytes are distributed throughout the brain in an optimal arrangement to establish chemical and physical interactions with neuronal synapses. Thus, changes in astrocyte morphology and function disturb synaptic contacts, function, and plasticity and, consequently, contribute to early cognitive deficits in AD (**Figure 1**). Understanding which astrocyte-neuronal signaling pathways are disrupted could lead to the development of more effective therapies as well as to the identification of novel biomarkers for synaptopathies, such as AD (Allen and Barres, 2009; Agostinho et al., 2020). Currently, a great research challenge has been the development of tools and strategies to detail the molecular pathways underlying the diverse functions of different astrocyte subpopulations, in particular of human astrocytes that are larger, more ramified and more heterogenous than rodent astrocytes. Recent studies using refined labeling strategies based on astrocytic promoters, and intersectional fluorescence-activated cell sorting-based strategy, as well as single-cell RNA sequencing provided great advances in revealing distinct spatial distributions of distinct astrocyte populations, possessing distinct morphologies and physiologies (Morel et al., 2019; Batiuk et al., 2020). However, it remains to be explored how the morphology and function of different astrocytic subpopulations are influenced by local environment, mainly by signal instigators of disease as well as the molecular signals involved in astrocyte communication with other brain cells. Filling these gaps of knowledge will set the stage for tackling

astrocytic functions as targets to delay the onset of synaptic and memory deficits in AD.

Adenosine exerts two parallel modulatory roles in the brain, acting as a homeostatic modulator and also as a neuromodulator at the synaptic level. Its effects are mediated by G protein-coupled receptors, being the subtypes A_1R and A_{2A}R the most abundant and studied. A_{2A}R are considered to mediate excitatory effects and to be more involved in neurodegeneration, contrasting to A_1R (reviewed in Lopes et al., 2020). Furthermore, increasing evidences show that A_{2A}R levels are not only upregulated in neurons but also in astrocytes, in the brain of AD patients and of AD mouse models. Moreover, it was shown that the astrocytic A_{2A}R upregulation contributes to memory loss in AD (Orr et al., 2015). These findings are relevant in view of the convergence of epidemiological and animal studies showing that caffeine intake is inversely correlated with memory deterioration in aging and AD, an effect mimicked by the selective A_{2A}R blockade (Cunha, 2016). As discussed in this review, we are only beginning to unveil the role of adenosine signaling in the control of astrocyte-neuron communication. However, there are already a set of evidences that reinforces the interest of exploring the therapeutic potential of astrocytic A_{2A}R. In the future, an ambitious challenge will be getting strategies, genetic or pharmacological, directed to A_{2A}R in astrocytes that allow control of their functions to be introduced into clinical practice as novel drugs to AD.

AUTHOR CONTRIBUTIONS

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REVIEW
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Metabolic Aspects of Adenosine Functions in the Brain

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Adenosine, acting both through G-protein coupled adenosine receptors and intracellularly, plays a complex role in multiple physiological and pathophysiological processes by modulating neuronal plasticity, astrocytic activity, learning and memory, motor function, feeding, control of sleep and aging. Adenosine is involved in stroke, epilepsy and neurodegenerative pathologies. Extracellular concentration of adenosine in the brain is tightly regulated. Adenosine may be generated intracellularly in the central nervous system from degradation of AMP or from the hydrolysis of S-adenosyl homocysteine, and then exit via bi-directional nucleoside transporters, or extracellularly by the metabolism of released nucleotides. Inactivation of extracellular adenosine occurs by transport into neurons or neighboring cells, followed by either phosphorylation to AMP by adenosine kinase or deamination to inosine by adenosine deaminase. Modulation of the nucleoside transporters or of the enzymatic activities involved in the metabolism of adenosine, by affecting the levels of this nucleoside and the activity of adenosine receptors, could have a role in the onset or the development of central nervous system disorders, and can also be target of drugs for their treatment. In this review, we focus on the contribution of 5′-nucleotidases, adenosine kinase, adenosine deaminase, AMP deaminase, AMP-activated protein kinase and nucleoside transporters in epilepsy, cognition, and neurodegenerative diseases with a particular attention on amyotrophic lateral sclerosis and Huntington's disease. We include several examples of the involvement of components of the adenosine metabolism in learning and of the possible use of modulators of enzymes involved in adenosine metabolism or nucleoside transporters in the amelioration of cognition deficits.

Keywords: adenosine, 5′-nucleotidases, adenosine deaminase, adenosine kinase, S-adenosylhomocysteine hydrolase, nucleoside transporters, brain, metabolism

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INTRODUCTION

Adenosine regulates multiple physiological and pathophysiological processes, by acting both through G-protein coupled adenosine receptors and intracellularly. It modulates neuronal plasticity (Sebastiao and Ribeiro, 2015), astrocytic activity (Agostinho et al., 2020), learning and memory (Chen, 2014; Simoes et al., 2016; Bannon et al., 2017; Perrier et al., 2019; Temido-Ferreira et al., 2019; Zhang et al., 2020), food intake (Kola, 2008), motor function (Mori, 2020), sleep/wake cycle (Donlea et al., 2017; Lazarus et al., 2019), pain (Vincenzi et al., 2020), immunosupression (Vijayan et al., 2017), proliferation (Jacobson et al., 2019), and aging (Costenla et al., 2011). Adenosine is involved in ischemia and stroke (Williams-Karnesky and Stenzel-Poore, 2009; Melani et al., 2014; Pereira-Figueiredo et al., 2021), epilepsy (Boison and Jarvis, 2020; Tescarollo et al., 2020), and

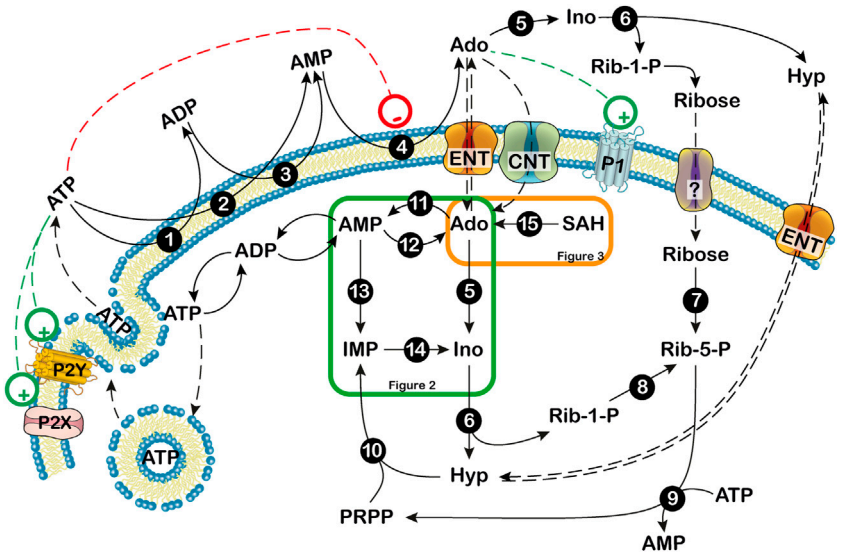

FIGURE 1 | Extra- and intracellular adenosine production. Extracellularly, ATP can be dephosphorylated to AMP by ectonucleoside triphosphate diphosphohydrolase (CD39) or ecto-nucleotide pyrophosphatase/phosphodiesterase. Then, AMP can be dephosphorylated to adenosine by the extracellular 5′-nucleotidase, CD73. Extracellular adenosine can be converted into hypoxanthine (Hyp) and ribose-1 phosphate (Rib-1-P) by the combined action of ectosolic adenosine deaminase and purine nucleoside phosphorylase. Extracellular Rib-1-P might be dephosphorylated by membrane phosphatases and equilibrates with the intracellular ribose through a not yet defined transporter (?). Inside the cell, at low energy charge, adenosine originates mainly from AMP and can be exported or deaminated. When extracellular adenosine generated from ATP breakdown is transported inside the cell, it might be phosphorylated by the low K_M ADK or deaminated by the high K_M ADA if adenosine reaches high levels. 1,3: ecto-nucleoside triphosphate diphosphohydrolase; 2: ecto-nucleotide pyrophosphatase/phosphodiesterase; 4: ecto-5′-nucleotidase; 5: adenosine deaminase; 6: purine nucleoside phosphorylase; 7: ribokinase; 8: phosphoribomutase; 9: 5-phosphoribosyl-1-pyrophosphate synthetase; 10: hypoxanthine guanine phosphoribosyltransferase; 11: adenosine kinase; 12: cytosolic 5′ nucleotidase I; 13: AMP deaminase; 14: cytosolic 5′ nucleotidase II; 15: S-adenosylhomocysteine hydrolase. Ado: adenosine; CNT: concentrative nucleoside transporter; ENT: equilibrative nucleoside transporter; Hyp: hypoxanthine; Ino: inosine; P1: purinergic receptor type 1; P2: purinerigic receptor type 2; Rib-1-P: ribose-1-phosphate; Rib-5-P: ribose-5-phosphate. Green and orange boxes indicate that these pathways are described in more details in **Figures 2**, **3**. +: stimulation; -: inhibition.

neurodegenerative pathologies such as Parkinson's disease (PD) (Fredholm and Svenningsson, 2020; Glaser et al., 2020), Alzheimer's disease (AD) (Rahman, 2009; Cunha and Agostinho, 2010; Cellai et al., 2018), amyotrophic lateral sclerosis (ALS) (Ng et al., 2015; Sebastiao et al., 2018), and Huntington's disease (HD) (Lee and Chern, 2014). Extracellular adenosine, interacting with P1 receptors (A1R, A2AR, A2BR, and A3R) regulates metabolism through different signaling pathways. In fact, the binding of adenosine to A1R and A3R activates the $G_{i/o}$ family of G-proteins, which inhibits adenylate cyclase, while the binding to A2AR and A2BR, through activation of G_s protein, stimulates the production of cyclic AMP (Burnstock, 2017), thus contributing to the fine-tuning of synapses and to the coordination of neuronal circuitry (Agostinho et al., 2020). In neurons and astrocytes, heteromers can be formed by interaction of A1R with A2AR, A2AR with A2BR, and A2AR with dopamine D2, D3 and D4 receptors (Guidolin et al., 2020). The interactions between receptors in these complexes modify the signaling and function of the individual receptors and lead to signal integration in the central nervous system. For example, the heteromer A2AR-D2 receptor is found in the striatum where it transforms the dopamine-facilitated inhibition into adenosine-induced activation of adenylate cyclase (Ferré and Ciruela, 2019). Higher order heteroreceptor complexes of A2AR-D2 receptor with either-metabotropic glutamate receptor 5 or Sigma1 receptor have also been described (Borroto-Escuela et al., 2020). It has been hypothesized that heteroreceptors could be involved in learning and memory, including memory associated drug addition and that heteroreceptor complexes could be a target for the treatment of schizophrenia, drug addition, and PD (Borroto-Escuela et al., 2020, 2021; Glaser et al., 2020).

The concentration of extracellular adenosine depends on both intracellular and extracellular purine nucleotide catabolism and on the traffic of the nucleoside across the membrane through specific transporters (Baldwin et al., 2004; Pastor-Anglada and Pérez-Torras, 2018; Camici et al., 2018b). Extracellular adenosine can arise from the degradation of intracellular ATP (**Figure 1**) (Camici et al., 2018b). At high energy charge, mainly inosine and hypoxanthine are generated by ATP catabolism. In fact, in these conditions, both the cytosolic 5'-nucleotidase II (NT5C2) and AMP deaminase (AMPD) are allosterically activated by ATP (**Figure 2**) (Ashby and Holmsen, 1983; Tozzi et al., 2013), therefore AMP is deaminated by AMPD into IMP, which is dephosphorylated into inosine by NT5C2. Conversely, at low energy charge, the accumulation of AMP inside the cell, leads to the activation of a specific AMP-activated protein kinase (AMPK), the main regulator of cellular energy homeostasis (Hardie et al., 2012; Rosso et al., 2016; Peixoto et al., 2017; Liu et al., 2020) (**Figure 2**). The dephosphorylation of AMP by a high

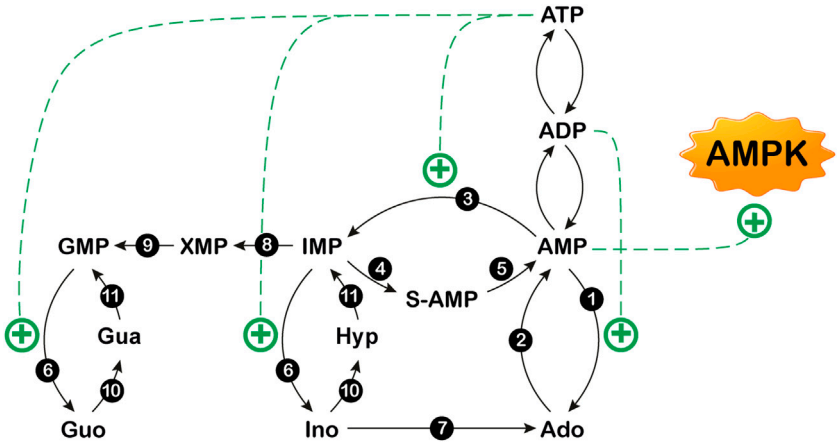

FIGURE 2 | Purine nucleotide cycles. 1: 5′-nucleotidase I; 2: adenosine kinase; 3: AMP deaminase; 4: adenylosuccinate synthase; 5: adenylosuccinate lyase; 6: cytosolic 5′-nucleotidase II; 7: adenosine deaminase; 8: IMP dehydrogenase; 9: GMP synthase; 10: purine nucleoside phosphorylase; 11: hypoxanthine guanine phosphoribosyltransferase. The figure also shows that AMP is an activator of AMP-activated protein kinase (AMPK). Ado: adenosine; Gua: guanine; Guo: guanosine; Hyp: hypoxanthine; Ino: inosine. S-AMP: succinylAMP. +: stimulation.

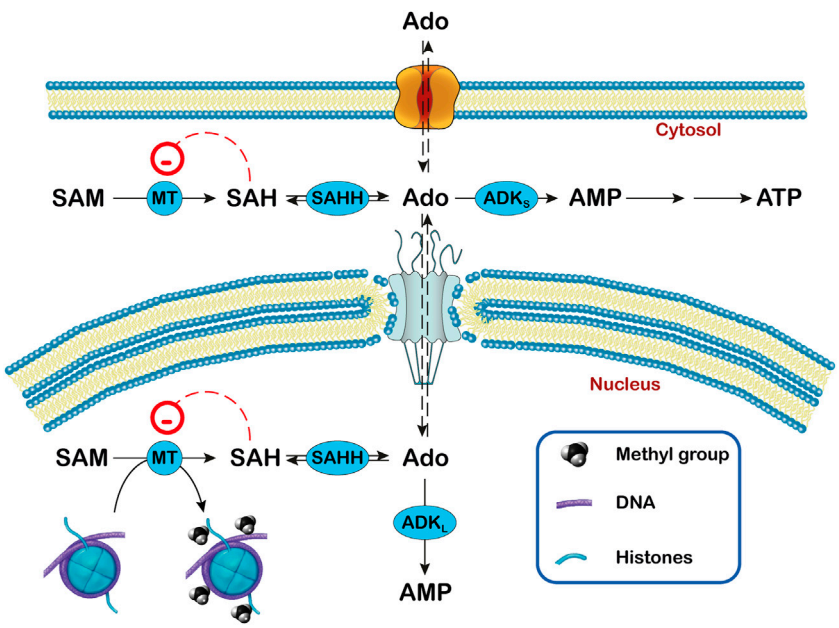

FIGURE 3 | Relationship between adenosine, ADK and transmethylation reactions in subcellular compartments. In the transmethylation reactions catalyzed by methyltransferases (MT), S-adenosylmethionine (SAM) donates the methyl group to various acceptors and is converted to S-adenosylhomocysteine (SAH), which generates adenosine (Ado) by the action of S-adenosylhomocysteine hydrolase (SAHH). In the nucleus, the methyl group can be transferred to DNA and histones. The activity of ADK (ADK_S in the cytoplasm and ADK_L in the nucleus) decreases the concentration of Ado and favors the transmethylation reactions. In this way, ADK_L contributes to the DNA methylation in the nucleus.

K_M AMP-specific 5'-nucleotidase I (NT5C1) which is strongly activated by ADP, leads to increased intracellular adenosine (Skladanowski and Newby, 1990). In such conditions, adenosine might be deaminated, but the K_M of adenosine deaminase (ADA) for adenosine is sufficiently high (25–150 μM) (Ford et al., 2000; Ipata et al., 2011) to favor adenosine increase and exit through nucleoside transporters (Baldwin et al., 2004). Therefore, the generation of intracellular adenosine and its exportation to the external medium occurs when AMP accumulates, giving the nucleoside the possibility to act as a danger signal, both by interacting with specific receptors on the same cell or on the neighboring cells. Intracellular adenosine can be converted into both AMP, by adenosine kinase (ADK) and inosine, by ADA. In turn, AMP,

through the sequential action of AMPD and cytosolic NT5C2 is converted into inosine, which, by the action of purine nucleoside phosphorylase (PNP) is transformed into hypoxanthine (**Figure 1**) (Camici et al., 2018b). Inosine and hypoxanthine can also be transported to the extracellular compartment (Yao et al., 2002). As shown in **Figures 1**, **3**, adenosine can be also generated intracellularly from S-adenosyl homocysteine (SAH) through the action of S-adenosyl homocysteine hydrolase (SAHH) (Garcia-Gil et al., 2018).

ATP can exit the cells through vesicles or through pannexin channels, or can be released by dying cells (Bodin and Burnstock, 2001). The nucleotide can interact with two families of receptors: P2X receptors, which are ion-gated channels displaying neuromodulatory functions, and P2Y receptors, which are G-protein coupled (Burnstock, 2017). This interaction modulates neuronal firing and mediates neuroinflammation (Rodrigues et al., 2015). Extracellularly, ATP can be dephosphorylated to AMP by ectonucleoside triphosphate diphosphohydrolase (CD39). Then, AMP can be dephosphorylated to adenosine by the extracellular 5'-nucleotidase (CD73). Extracellular adenosine can be converted into hypoxanthine and ribose-1 phosphate by the combined action of ectosolic ADA and PNP (**Figure 1**). In fact, there are several indications that both ADA and PNP are present inside the cells and are also released in the extracellular space ensuring a rapid degradation of extracellular adenosine into hypoxanthine, thereby helping to prevent a dangerous accumulation of the nucleoside. This occurrence has also been reported in the brain (Wall et al., 2007; Gracia et al., 2008). Extracellular ribose-1-phosphate might be dephosphorylated by several phosphatases present on the membrane and equilibrates with the intracellular ribose possibly through one member of the family of glucose transporters as demonstrated in *Leishmania* (Naula et al., 2010).

As a result of the regulation of its metabolism, adenosine does not reach very high concentrations in healthy cells because it is readily metabolized. Hypoxanthine can be salvaged by hypoxanthine-guanine phosphoribosyltransferase (HPRT) into inosine monophosphate (IMP) or can be excreted as uric acid. Ribose- 5- phosphate can be utilized for 5-phosphoribosyl-1-pyrophosphate (PRPP) synthesis, and can be used for energy repletion or glucose synthesis (Garcia-Gil et al., 2018). Indeed, adenosine accumulation can impair essential cellular functions. In fact, deficiency of ADA which brings about an accumulation of adenosine and deoxyadenosine is the most common cause of severe combined immunodeficiency (SCID). These purine compounds are detrimental particularly for the immune system and also impair the functionality of the nervous system (Sauer et al., 2017). In this review, we describe the contribution of 5'-nucleotidases, ADK, ADA, AMPD and nucleoside transporters in epilepsy, cognition, and neurodegenerative diseases with a particular attention on serious pathological conditions such as ALS and HD. For a better insight on adenosine receptor expression, function and regulation, the reader is referred to the numerous excellent reviews covering the different aspects of purinergic receptors (Cunha, 2016; Burnstock, 2017).

5'–NUCLEOTIDASES

NT5C1, which has been mainly studied in skeletal muscle, and NT5C2, which is ubiquitously expressed, are the major cytosolic NT5Cs acting on intracellular nucleotides (Camici et al., 2020). Among purine nucleotides, AMP, with a K_M in the millimolar range (Hunsucker et al., 2001; Tkacz-Stachowska et al., 2005) is the preferred substrate for NT5C1, while IMP and GMP are better substrates for NT5C2 (K_M in the micromolar range) (Tozzi et al., 2013; Pesi et al., 2021), but this enzyme catalyzes also the hydrolysis of the phosphoester bond of AMP (with a K_M in the millimolar range) (Tozzi et al., 2013). The intracellular concentrations of IMP, GMP and also AMP depend on the rate of the AMP-IMP-GMP cycles (**Figure 2**) which in turn depends on NT5C2 activity (Barsotti et al., 2003). In fact, at high energy charge, excess IMP, synthesized by *de novo* or salvage pathways, is converted to inosine and therefore directed to catabolic pathways, while at low energy charge IMP and AMP accumulate (Pesi et al., 1994; Allegrini et al., 2004; Wallden and Nordlund, 2011; Camici et al., 2018a). NT5C1 has been associated to some autoimmune diseases (Rietveld et al., 2018), while an association has been reported between NT5C2 expression and psychiatric and psychomotor disorders including schizophrenia (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Duarte et al., 2016; Duarte et al., 2019) and hereditary spastic paraplegias (HSP) (Garcia-Gil et al., 2018; Camici et al., 2020). At early stages of AD, NT5C activity is reduced in membranes and cytosol in distinct cortical regions such as the frontal cortex, and only at advanced stages in cytosol in the temporal cortex (Alonso-Andres et al., 2018). Recently, decrease in NT5C2 activity has also been found in the senescence-accelerated mouse-prone 8, a model of AD (Sanchez-Melgar et al., 2020).

HSPs comprise a group of genetically heterogeneous neurodegenerative disorders presenting progressive spasticity in the lower limbs (Blackstone, 2018). In addition, skin abnormalities, epilepsy, intellectual disability, deafness, optic atrophy, peripheral neuropathy and ataxia, have been reported in association with autosomal recessive inheritance. Several mutations associated with NT5C2 in HSP type 45 have been described (Novarino et al., 2014; Darvish et al., 2017; Elsaid et al., 2017; Straussberg et al., 2017; Naseer et al., 2020). A splice mutation associated with a substantial reduction in NT5C2 level has been found in two children with severe early spasticity, mild cognitive impairment, and dysgenic and thin corpus callosum (Elsaid et al., 2017), whereas corpus callosum with normal white matter was found in their apparently normal heterozygous brother. Therefore, homozygous alteration in NT5C2 appears to be necessary to produce central white matter developmental defects (Elsaid et al., 2017). A 1954-bp homozygous deletion at the *NT5C2* locus involving the entire coding exon 11 was identified in two siblings with HSP and intellectual disability (Darvish et al., 2017). Microcephaly has been found in two cases described by Novarino et al. (2014) and Naseer et al. (2020). The mechanisms underlying the clinical manifestations of NT5C2 mutations are unknown but it has been reported that NT5C2 expression is higher during fetal

development and that, within the adult brain, NT5C2 is enriched in neurons compared to glial cells (Duarte et al., 2019). The same authors have demonstrated that *NT5C2* knockdown in human neural stem cells increased the expression and the phosphorylation of the α−subunit of AMPK (Duarte et al., 2019). Moreover, studies in *Drosophila melanogaster* have shown that knockdown of the *NT5C2* homologue in neurons is associated with climbing impairment suggesting a role for NT5C2 in motility (Duarte et al., 2019). Furthermore, we demonstrated that transitory *NT5C2* silencing in an astrocytoma cell line (ADF) caused apoptosis, while a constitutive silencing increased oxidative metabolism and decreased cell proliferation (Careddu et al., 2008; Pesi et al., 2018). It would be valuable to obtain information on the levels of expression and activity of NT5C2 and the concentration of AMP and ATP in HSP patients, since alterations of their ratio affect AMPK activity, and a continous activation of this enzyme could result in abnormal functioning and development of the nervous system (Pesi et al., 2000; Garcia-Gil et al., 2003; Williams et al., 2011; Domise et al., 2019) (see section AMPK).

In humans, the family of NT5C2 is encoded by five genes (*NT5C2, NT5DC1-4*). The presence of a nucleotidase domain in *NT5DC1-4*, allows to hypothesize that these genes code for proteins having 5'-nucleotidase activity, but the enzymatic activity has not been directly measured. Singgih et al. (2021) have recently reported the expression of orthologues of the NT5C2 family in the brain of *Drosophila melanogaster* and that the neuronal knockdown of two of them resulted in impaired habituation learning. *NT5DC2* has also been associated with cognitive ability, bipolar disorder and schizophrenia (Watanabe et al., 2019) while the expression level of *Nt5dc3*, has been positively correlated with reversal learning performance in mice (Laughlin et al., 2011).

ECTONUCLEOTIDASES

Ectonucleotidases control the levels of ATP and its hydrolysis products ADP, AMP and adenosine in the synaptic cleft. The major families of ectonucleotidases are CD39, ecto-nucleotide pyrophosphatase/phosphodiesterases, alkaline phosphatases, and CD73. Adenosine, the final product of ATP extracellular degradation, can be either degraded by extracellular ADA and PNP into hypoxanthine and ribose-1-phosphate, or enter the cell through equilibrative and/or concentrative nucleoside transporters (see below, nucleoside transporters section) (**Figure 1**). CD73 is inhibited by micromolar concentration of ADP and ATP (Zimmermann, 1992), therefore when ATP is released and accumulates outside the cell, it is hydrolyzed into ADP and AMP and is converted to adenosine only after the disappearance of ATP and ADP. In the basal ganglia, CD73, which generates adenosine from extracellular AMP, colocalizes with A2ARs (Augusto et al., 2013) and the enzyme has been recently demonstrated to act a part in A2AR signaling in both PD models and patients (Carmo et al., 2019; Meng et al., 2019). CD73-derived adenosine-A2AR signaling is able to modulate microglial immunoresponses and the extension of microglial processes and movement (Meng et al., 2019). Moreover, the reduction of adenosine generated from CD73 decreased microglia-mediated neuroinflammation, increased dopaminergic neuron viability and motor function in a model of PD (Meng et al., 2019). In a model of AD, β-amyloid increased ATP release and CD73 activity, leading to adenosine generation, activation of A2AR and impairment of synaptic plasticity and memory (Goncalves et al., 2019). The hippocampal astrogliosis observed in mesial temporal lobe epilepsy patients is associated to an increase in expression of A2AR and CD73 (Barros-Barbosa et al., 2016). Finally, in addition to its role in the generation of adenosine, CD73 might participate in the regulation of cell adhesion, migration and differentiation, since CD73 acts as receptor for extracellular matrix molecules including tenascin C, fibronectin and laminin (Sadej et al., 2008).

In many cases neurodegenerative diseases are the consequence of neuroinflammation. In reactive astrocytes, increased *CD73* gene expression or increased CD73 activity was reported in ALS (Gandelman et al., 2010), epilepsy (Bonan et al., 2000; Bonan, 2012), ischemia (Braun et al., 1998) and traumatic brain injury (Nedeljkovic et al., 2006; Bjelobaba et al., 2011). Upregulation of CD73 has also been found in glioma (Quezada et al., 2013; Xu et al., 2013), while in a model of multiple sclerosis, upregulation of CD73 and CD39 has been reported in reactive astrocytes and in microglia, respectively (Lavrnja et al., 2015; Jakovljevic et al., 2019). The involvement of ectonucleotidases in the regulation of microglial function, the pathogenesis of infectious diseases in the nervous system and the complex regulation of CD73 at the neurovascular unit during neuroinflammation have been recently reviewed by Nedeljkovic (2019), Alves et al. (2020) and Illes et al. (2020). Altogether these observations suggest that reduction of CD73 activity, and therefore, adenosine levels, might be a therapeutical tool to decrease neuroinflammation in PD as well as astrogliosis in mesial temporal lobe epilepsy, and likely, to improve cognition in AD and PD.

ADENOSINE KINASE

ADK catalyzes the transfer of the γ-phosphate from ATP to adenosine, leading to the formation of AMP, and has the capability to regulate both extracellular adenosine and intracellular adenine nucleotide levels. Human ADK consists of two alternatively spliced forms with distinct cellular and subcellular localization. ADK-short (ADK_S) is mainly cytoplasmatic and the longer form (ADK_L) is nuclear (Cui et al., 2009; Kiese et al., 2016; Boison and Jarvis, 2020). ADK is found mainly in neurons at early stages of development but later it becomes more abundant in astrocytes. The function of the two isoforms appears to be different. Indeed, overexpression of ADK_S in the brain resulted in spontaneous seizures and increased brain injury after ischemic stroke (Li et al., 2008; Shen et al., 2011). Overexpression of ADK_L in dorsal forebrain neurons attenuated neural stem cell proliferation (Gebril et al., 2020), while transgenic mice lacking ADK_L in the dental gyrus showed increased neurogenesis. In addition, ADK_L might have a more

prominent role in epigenetic mechanisms requiring transmethylation than ADK_S (Williams-Karnesky et al., 2013). Methyltransferases catalyze the transfer of methyl groups from the donor S-adenosylmethionine (SAM) to proteins such as histones and DNA yielding S-adenosylhomocysteine (SAH) (**Figure 3**), whose cleavage into adenosine and homocysteine is catalyzed by S-adenosylhomocysteine hydrolase (SAHH). The removal of adenosine by ADK favors the transmethylation reactions. When the clearance of adenosine is impaired, the increased levels of SAH inhibit DNA methyltransferases (James et al., 2002) (**Figure 3**).

Both deficiency and excess of ADK are harmful. ADK deficiency is a very rare inborn error of metabolism, and is characterized by defects in transmethylation reactions associated with developmental delay, hepatic encephalopaties as well as seizures in some individuals (Bjursell et al., 2011; Shakiba et al., 2016; Staufner et al., 2016; Alhusani et al., 2019; Becker et al., 2021). Mutations of ADK or modification of ADK levels have been associated with several diseases (Garcia-Gil et al., 2018), such as stroke (Shen et al., 2011), Rasmussen encephalitis (Luan et al., 2013), focal cortical dysplasia (Luan et al., 2015), epilepsy (Boison, 2016) and gliomas (de Groot et al., 2012; Huang et al., 2015), as well as in cognition deficits (Bjursell et al., 2011; Singer et al., 2012; Sandau et al., 2016; Shakiba et al., 2016; Staufner et al., 2016; Osborne et al., 2018; Kuptanon et al., 2019). Notably, genetic variants of ADK are associated with post-traumatic epilepsy in humans (Diamond et al., 2015). Dysfunction of adenosine signaling, which is common in neurological disorders, might explain comorbid phenotypes such as epilepsy, PD, ALS and AD among others (Boison and Aronica, 2015).

Inhibition of ADK strengthens A1AR activation and has a protective effect in ischemia, epilepsy and glutamate excitotoxicity (Boison, 2013). Williams-Karnesky et al. (2013) have demonstrated that epileptogenesis is modulated by intracellular adenosine through the transmethylation pathway. Indeed, seizures in murine models involve alteration of adenosine homeostasis (increased ADK and reduced adenosine), increased DNA methyltransferase activity and increased hippocampal DNA methylation (Williams-Karnesky et al., 2013). Seizure susceptibility was reduced by DNA methyltransferase inhibitors (Williams-Karnesky et al., 2013). Different methods are being tried to increase adenosine in order to reduce seizures. When adenosine-releasing polymers were implanted intraventricularly, methylation reverted to control levels and seizure activity decreased (Williams-Karnesky et al., 2013). Recently, attenuation of epilepsy development in mice has also been obtained after transient application of an ADK inhibitor (Sandau et al., 2019). ADK-deficient stem cells could be a tool to increase adenosine. Poppe et al. (2018) have obtained ADK-deficient epithelial stem cells able to differentiate in neurons and astrocytes with high ability to release adenosine. ADK levels are reduced by ketogenic diet used for the treatment of epilepsy. It is interesting to note that the ketogenic diet suppresses seizures by A1R-dependent and likely also by adenosine-dependent epigenetic mechanisms (Masino et al., 2011; Lusardi et al., 2015; Boison and Rho, 2020).

Transgenic mice with brain-wide or telencephalon ADK hyperexpression showed dysregulation of brain adenosine which resulted in impairment of working memory and of associative memory measured using the conditioned freezing paradigm (Yee et al., 2007; Singer et al., 2012; Singer et al., 2013), while mice with brain-wide deletion of ADK developed spontaneous seizures and profound deficits in hippocampus-dependent learning and memory (Sandau et al., 2016).

Recently, Osborne et al. (2018) have distinguished the neurobehavioral consequences of gestational ADK deletion vs. adult-onset ADK deficiency. Interestingly, gestational depletion of ADK produced deficits in social memory in males, and contextual learning impairments in both sexes, and a hyper-responsiveness to amphetamine in males. In contrast, the tardive astrocyte deficiency of ADK resulted in normal social memory and contextual learning. These results point to a role for adenosine homeostasis during development in the determination of the susceptibility to later neuropsychiatric diseases such as schizophrenia, autism and attention deficit hyperactivity disorder, in which males more frequently express social deficits than females.

Clinical radiation therapy for the treatment of central nervous system tumors leads to impairments in cognition. Adult rats exposed to cranial irradiation showed significant declines in performance of hippocampal-dependent memory tasks such as novel place recognition, novel object recognition and contextual fear conditioning associated to astrogliosis and elevated ADK expression in the hippocampus. The treatment with an ADK inhibitor prior to cranial irradiation improved performance in all cognitive tasks one month post exposure (Acharya et al., 2016).

ADENOSINE DEAMINASE

ADA catalyzes the deamination of adenosine and deoxyadenosine into inosine a deoxyinosine respectively. There are two genes codifying for ADA: *ADA1* and *ADA2*/cat eye syndrome chromosome region, candidate 1 (*CECR1*) which are localized in the chromosomes 20 and 22, respectively. The proteins have 27% identity and differ in structure and probably in functions (for recent reviews see Meyts and Aksentijevich, 2018; Moens et al., 2019).

Adenosine deaminase 1

ADA1 is a 41-kDa monomer protein that is present in all human tissues, with the highest expression in T and B lymphocytes. ADA1 plays a crucial role in adaptive immune system development and exhibits an affinity for its substrates adenosine and deoxyadenosine significantly higher than ADA2. ADA1 not only reduces extracellular adenosine concentration preventing adenosine receptor desensitization, but is also able to directly interact with dipeptidyl peptidase-4 (CD26) (Franco et al., 1997) and, by interacting with A1R and A2R, increases receptor functionality in the striatum (Gracia et al., 2008; Ciruela et al., 2010; Gracia et al., 2011). CD26 is more expressed in microglia and astrocytes than in neurons and is more abundant in astrocytes during inflammation and in

microglia in neuropathy. Spinal application of CD26 inhibitors induces a strong antihyperalgesic effect during inflammatory pain (Kiraly et al., 2018), but the role of ADA1 in CD26 function in the nervous system is unknown.

Moreno et al. (2018) have demonstrated the formation of trimeric complexes CD26-ADA-A2AR and have suggested that ADA could have a role in communication between cells expressing CD26 (such as T cells) and those expressing adenosine receptors (such as neurons and dendritic cells). If this is the case, ADA deficiency could also affect the cell-cell communication in the nervous system.

Mutations in the *ADA1* gene are among the most common causes for severe combined immunodeficiency (SCID). When ADA activity is absent, deoxyadenosine increases both extracellularly and intracellularly. Within cells, it is converted by the action of deoxycytidine kinase and/or adenosine kinase to deoxyadenosine monophosphate and then to deoxyadenosine triphosphate (dATP). Deoxyadenosine and dATP in lymphocytes are considered the main agents of toxicity. The raise of intracellular dATP interferes with DNA synthesis and repair by inhibiting ribonucleotide reductase and terminal deoxynucleotidyl transferase and results in apoptosis of developing thymocytes (for recent reviews Whitmore and Gaspar, 2016; Flinn and Gennery, 2018; Garcia-Gil et al., 2018; Camici et al., 2019) while deoxyadenosine inactivates SAHH, leading to accumulation of SAH and inhibits the transmethylation reactions (**Figure 3**) which are required for lymphocyte activation.

In addition to immunodeficiency, ADA-SCID patients display skeletal, hepatic, renal and lung alterations, as well as neurological and behavioral impairments such as reduced verbal expression, seizures, learning disability, hyperactivity, attention and hearing deficits (Rogers et al., 2001; Nofech-Mozes et al., 2007; Titman et al., 2008; Garcia-Gil et al., 2018). The accumulation of adenosine and deoxyadenosine caused by ADA deficiency might contribute to the alterations in the nervous system. Notably, a polymorphism in the *ADA1* gene in autistic children with mild intellectual disability has been found to be associated with reduced ADA activity in serum (Stubbs et al., 1982; Bottini et al., 2001; Saccucci et al., 2006). Neurological deficits persist after bone marrow transplant or replacement therapy which do improve the immunological and metabolic aspects of the disease (Honig et al., 2007; Booth and Gaspar, 2009; Cicalese et al., 2016). Recently, the degree of neurological impairment of *Ada−/−* mouse treated with PEG-ADA has been compared with untreated controls (Sauer et al., 2017). The knockout mice show undetectable ADA activity, increased adenosine in total brain extracts, slightly reduced brain size, alterations in explorative behavior, increased anxiety, reduced pain sensitivity and normal sensorimotor development. Both untreated and PEG-ADA-treated knockout mice exhibited reduced A2AR level compared to control brains, suggesting that adenosine signaling is affected by ADA deficiency. Adenosine metabolite levels in the brain, ventriculomegaly and pain sensitivity showed a tendency to decrease after PEG-ADA treatment (Sauer et al., 2017) while exploration and anxiety abnormalities remained uncorrected. Therefore, reduction of A2AR appears to contribute to the phenotype of the *Ada−/−* mice but the involvement of other mechanisms, such as epigenetic alterations have not been addressed. Interestingly, it has been recently reported that when the enzyme replacement therapy with PEG-ADA is performed early, it is effective in improving hearing defects in *Ada−/−* mice (Xu et al., 2019). ADA enzyme therapy in these mice normalized cochlear adenosine levels, and prevented demyelination while treatment with an A2BR-antagonist improved hearing loss and myelin compaction (Manalo et al., 2020).

Adenosine deaminase 2

ADA2 has a 100-fold higher K_M for adenosine (2 mM) than ADA1 (Schrader et al., 1978). Therefore, the deaminase activity of ADA2 is low under physiological conditions but could be relevant during inflammation and tumorigenesis, when adenosine levels increase. For recent reviews see Meyts and Aksentijevich (2018) and Moens et al. (2019).

Deficiency of ADA2 arises from mutations affecting catalytic activity, protein dimerization, and secretion of ADA2 and causes vasculopathy and inflammation in many organs and/or hemorrhagic stroke (Navon Elkan et al., 2014; Zhou et al., 2014; Meyts and Aksentijevich, 2018; Gibson et al., 2019; Sahin et al., 2020; Zervou et al., 2020; Sozeri et al., 2021). Less frequent neurological manifestations include spastic diplegia or paraplegia, peripheral polyneuropathy, ataxia, neurosensory deafness, and cerebral atrophy (Meyts and Aksentijevich, 2018). ADA1 activity is not impaired and deoxyadenosine nucleotides do not accumulate in ADA2 deficiency (Meyts and Aksentijevich, 2018).

In addition to its deaminase activity, ADA2 may have a growth factor activity and contrarily to ADA1, does not bind to CD26. ADA2 is a potent regulator of tumor associated microglia/macrophages polarization. It has been found highly expressed by tumor associated microglia/macrophages (M2-type) in high-grade glioma. In these cells, paracrine effects induced by ADA2 include activation of MAPK signaling and stimulation of proliferation and migration of glioma cells (Zhu et al., 2017a). Moreover, ADA2/CECR1 mediates cross-talk between macrophages and pericytes in glioblastoma multiforme resulting in pericyte recruitment and migration, and thus promoting tumor angiogenesis (Zhu et al., 2017b).

AMP DEAMINASE

AMPD converts AMP into IMP by deamination (**Figures 1**, **2**). Together with CD39 and NT5C, it regulates the purine pool of nucleotides. The three genes coding for AMPD, AMPD1, AMPD2, and AMPD3, are differently expressed in various organs and in various types of cells. AMPD1 is highly expressed in skeletal muscle and diaphragm, AMPD2 is mainly expressed in brain, liver, and thymus and AMPD3 is most strongly expressed in erythrocytes (Morisaki et al., 1990; Mahnke-Zizelman and Sabina, 1992). Mutations in AMPD2 result in pontocerebellar hypoplasia due to loss of brainstem and cerebellar parenchyma (Akizu et al., 2013; Marsh et al., 2015;

Accogli et al., 2017; Kortum et al., 2018; Abreu et al., 2020) but a homozygous *AMPD2* frameshift variant has been associated with HSP type 63 (Novarino et al., 2014). AMPD2 plays a role in guanine nucleotide homeostasis by regulating the feedback inhibition of adenosine derivatives on *de novo* purine synthesis; AMPD2 deficiency results in increase of ATP and decrease of GTP levels, which leads to impairment of GTP-dependent initiation of protein synthesis (Akizu et al., 2013). Recently, *AMPD1* polymorphisms have been associated to autism risk in Chinese population (Zhang et al., 2015). Studies performed in lymphoblast cell lines obtained from patients have revealed decreased mitochondrial complex I activity compared to control cells. This result is interesting since reduction of transcription of mitochondrial electron transport complex genes has been found in several regions of autism brains (Anitha et al., 2013). Since AMPD1 is particularly enriched in muscle, it would be interesting to study the effects of these variants on the muscle functionality of the autistic patients.

AMP-ACTIVATED PROTEIN KINASE

AMPK is the principal regulator of cellular energy homeostasis since it is a sensor of the AMP:ATP ratio and mediates the adaptive changes of the cell as a function of the energy charge. In fact, through complex and various signaling pathways, AMPK switches off the anabolic pathways that require ATP and switches on the catabolic pathways that produce ATP (Hardie and Hawley, 2001; Gowans et al., 2013). Increasing evidence supports a relevant role of AMPK in the physiopathology of the central nervous system (Camici et al., 2020). The roles of AMPK in brain and its cross-talk with many hormones in the hypothalamus to mediate their anorexigenic and orexigenic effects as well as thermogenic influences have been thoroughly reviewed (Huynh et al., 2016; Rosso et al., 2016; Peixoto et al., 2017; Liu et al., 2020).

Constant AMPK activation might lead to abnormal functioning and development of the nervous system. We have reported that AMPK activation promotes apoptosis in hippocampal and neuroblastoma cells (Pesi et al., 2000; Garcia-Gil et al., 2003) while other authors have described reduction of axonal growth (Williams et al., 2011) and reduction of synapses (Domise et al., 2019). In addition, it has been reported that dysregulation of the AMPK signaling in motor neurons is an early and common event in ALS (Perera and Turner, 2016) and that psychiatric disorders are also associated with dysregulation of AMPK signaling (Perera and Turner, 2016; Rosso et al., 2016). In particular, it has recently been found that AMPKα1 levels are significantly increased, while AMPKα2 levels are markedly reduced in the hippocampus of AD patients, compared to controls (Zimmermann et al., 2020). These changes in AMPKα expression appeared to be AD specific, since AMPKα1/2 levels were unaffected in either Lewy body dementia or frontotemporal dementia (Zimmermann et al., 2020).

Many research groups have studied the effect of AMPK activation on the development of AD, but the results are controversial. When the effect of metformin, a known activator of AMPK and a drug used for the treatment of type 2 diabetes, has been investigated on cognition deficits, some studies have shown an aggravating effect (Imfeld et al., 2012; Wennberg et al., 2018), whereas others reported a preventive one (Ng et al., 2014; Shi et al., 2019). Metformin has been shown to have a positive effect on AD mouse model (Wang et al., 2020). Recently it has been investigated the possible role of the AMPK isozymes in these conflicting results using mouse models of AD (Zimmermann et al., 2020). *AMPKα2* but not *AMPKα1* knockout mice displayed impaired cognition and hippocampal late long-term potentiation (Yang et al., 2020). In contrast, the brain-specific repression of AMPKα1 attenuated learning and memory deficits and the synaptic failure in mouse models of AD (Zimmermann et al., 2020). Moreover, AD-associated abnormal eEF2 phosphorylation and *de novo* protein synthesis reduction were also alleviated by deletion of the AMPKα1 isoform. This effect is relevant for cognition, since long-term memory requires new protein synthesis (Alberini, 2008). The effects of AMPK on amyloid β peptide accumulation, tau aggregation, and oxidative stress have been recently reviewed (Assefa et al., 2020). The role of AMPK in HD and ALS is discussed later in the appropriate sections.

NUCLEOSIDE TRANSPORTERS IN THE BRAIN

As described in the previous sections, adenosine is produced both extra- and intracellularly and is transported across the cell membrane. Therefore, nucleoside transporters have a major impact on the adenosine level, both inside and outside the cell. The *SLC28* gene family encodes the concentrative Na^+-dependent nucleoside transporters (CNT1-3), while the *SLC29* gene family encodes the Na^+-independent equilibrative nucleoside transporters (ENT1-4) (Cabrita et al., 2002; Engel et al., 2004; Zhou et al., 2010). Among the concentrative transporters, CNT1, which prefers transport of purine nucleosides, and CNT2, which shows a broader substrate specificity, accepting both purine and pyrimidine nucleosides, are expressed in the brain (Anderson et al., 1996). Although all ENTs have been found in the brain (Anderson et al., 1999a; Anderson et al., 1999b; Engel et al., 2004; Baldwin et al., 2005) only ENT1 and ENT2 appear to be relevant in the purinergic signaling.

Concentrative Transporters

CNTs are high-affinity inward transporters for adenosine. CNT1 is the first nucleoside transporter defined as a transceptor (Pérez-Torras et al., 2013), a word deriving from the contraction of **trans**porter and re**ceptor** for the coexistence of these two functions (Pastor-Anglada and Pérez-Torras, 2018). The expression of the highly regulated transporter CNT1 (Valdes et al., 2002; Klein et al., 2009), is reduced in several human tumors (Farre et al., 2004; Bhutia et al., 2011). Pérez-Torras et al. (2013) found that the restoration of human CNT1 in pancreatic cancer cells caused an alteration in cell cycle progression and in the

phosphorylation status of kinases involved in key signaling pathways, promoted polyADPR polymerase activity and non-apoptotic cell death, and decreased cell migration, thus reducing tumor growth. All these effects were mimicked by a translocation-defective human CNT1 variant, indicating that transport is not required for signaling. Although the regulation of CNT1 appears extremely interesting and relevant in tumor biology, its involvement in the effect of adenosine in brain still remains unclear.

CNT2 has been reported to be under the A1R control in hepatocytes (Duflot et al., 2004). A similar cross-talk between CNT2 and A1R appears to operate also in neurons since *in situ* hybridization in rat brain demonstrated that the prominent expression areas of CNT2 are rich in A1R (Guillén-Gomez et al., 2004; Pastor-Anglada and Pérez-Torras, 2018). Nerve growth factor-induced differentiation in phaeochromocytoma PC12 cells increased progressively CNT2 expression and raised A1R mRNA by 15-fold (Medina-Pulido et al., 2013). Although triggering antagonistic signals in target cells, A1R and A2AR significantly up-regulated CNT2 transport activity, both promoting removal of extracellular adenosine in differentiated neuronal PC12 cells (Medina-Pulido et al., 2013). In an intestinal rat epithelial cell line, through a CNT2 and ADK-mediated mechanism, the addition of adenosine rapidly increased AMP intracellular concentration with a consequent activation of AMPK (Aymerich et al., 2006). The same adenosine-dependent AMPK activation has been reported in differentiated neuronal PC12 cells. Interestingly, hypoxia induced a down-regulation of CNT2 and a consequent inactivation of AMPK (Medina-Pulido et al., 2013). Therefore, a functional link between CNT2-mediated adenosine uptake and energy metabolism appears to be present not only in intestinal cells (Aymerich et al., 2006; Huber-Ruano et al., 2010), but also in neuronal cells (Medina-Pulido et al., 2013; Pastor-Anglada and Pérez-Torras, 2018). Brain extracellular adenosine in cats increases during wakefulness and decreases during the spontaneous recovery sleep. The duration and depth of sleep after wakefulness appear to be modulated by adenosine (Porkka-Heiskanen et al., 1997). Guillén-Gomez et al. (2004) demonstrated that total sleep deprivation, which is accompanied by an increase of extracellular adenosine, decreased the amount of CNT2 mRNA in the rat cerebral cortex. Therefore, CNT2 expression appears to be regulated by sleep at transcriptional level. Since CNT2 is far more efficient in the uptake of adenosine than any equilibrative transporter (Parkinson et al., 2011), its specific decrease suggests that this transporter may exert a new physiological role for this transporter in the modulation of extracellular adenosine levels and the sleep/wakefulness cycle.

Equilibrative Transporters

ENTs, contrary to CNTs, are widely distributed in all tissues, including the central nervous system, and are considered important components of the purinergic signaling in brain (Pastor-Anglada and Pérez-Torras, 2018). Most studies focus on ENT1 and ENT2; indeed, ENT3 is distributed in intracellular membranes (Baldwin et al., 2005), while ENT4 cannot be considered a conventional nucleoside transporter, but rather a polyspecific cation transporter (Engel et al., 2004). Alterations in the function of ENTs bring about modified levels of adenosine, thus resulting in aberrant purinergic signaling. Mice lacking ENT1 exhibit dysfunctional behaviors, such as decreased ethanol intoxication and excessive ethanol drinking (Choi et al., 2004; Chen et al., 2010), and reduced anxiety-like behavior (Chen et al., 2007; Ruby et al., 2011; Nam et al., 2013). In the dorsomedial striatum of ENT1 null mice, a correlation between higher ethanol consumption and a decreased adenosine-mediated A2AR signaling has been demonstrated (Nam et al., 2013). On the other hand, the lack of ENT1 or the inhibition of ENT1 in the amygdala, leads to an increased adenosine-mediated A1R signaling, which correlates with a reduced anxiety behavior (Choi et al., 2004; Chen et al., 2007). Indeed, since ENT1 mediates nucleoside transport bidirectionally depending on the concentration gradient across the membrane, Ruby et al. (2011) hypothesize that ENT1 mediates a release of adenosine in the striatum (Ruby et al., 2011; Nam et al., 2013), while in the amygdala, ENT1 primarily mediates uptake of adenosine (Choi et al., 2004; Chen et al., 2010; Ruby et al., 2011). In this regard, an important bias of all these studies is the lack of a reliable technique allowing for the detection of adenosine. In fact, the changes in adenosine concentrations have been inferred, but never directly measured.

A correlation between ENT1 expression and epilepsy severity has been reported in rats (Xu et al., 2015; Zhou et al., 2020). In several epilepsy models, adenosine has been shown to exert an anticonvulsant effect (Boison, 2012; Masino et al., 2014), which appears to be mainly mediated by A1R (Li et al., 2007). ENT1 may be considered a therapeutic target for the control of epileptic seizures (Huang et al., 2017). Indeed, intraperitoneal and hippocampal injections of nitrobenzythioinosine (NBTI), a specific inhibitor of ENT1, decreased the number of seizures in rats (Xu et al., 2015), while blood brain barrier permeable ENT1 inhibitors have shown anti-epileptic effects in different mouse models (Ho et al., 2020). Recently, Zhou et al. (2020) demonstrated that, besides NBTI, also the specific inhibition of the p38 MAPK signaling pathway in the brain tissues of rats with acute status epilepticus, reduced the expression levels of ENT1 as well as A1R, the expression of which was significantly increased after seizure induction, probably as adaptive response to an acute attack. The p38 MAPK signaling inhibition decreased pathological damage of hippocampal neurons and reduced frequency of seizures. However, further investigation is needed to clarify the molecular mechanisms underlying the observed effects (Zhou et al., 2020). The expression of ENT1 in HD models and patients is discussed below.

Increasing adenosine by using an ENT1 inhibitor, improved memory deficits in a mouse model of AD (Lee et al., 2018). Studies in non-mammal models have also suggested a role for adenosine signaling and metabolism in learning. For example, antagonists of adenosine receptors, and inhibitors of ENT and ADA were able to prevent the scopolamine-induced amnesia in zebrafish (Bortolotto et al., 2015).

Adenosine has been reported to aberrantly increase in glioblastoma tumors, and the adenosine signaling has been

associated with increased chemoresistance, migration and invasion, especially in a cell population with an extremely aggressive behavior, called glioblastoma stem-like cells (GSCs) (Niechi et al., 2019; Torres et al., 2019). Recently, Alarcon et al. (2020), using a HPLC technique with fluorescent detection (Torres et al., 2016), demonstrated that extracellular adenosine was significantly increased in GSCs as compared to non-GSCs and that this difference was specifically ascribable to the mesenchymal GSC subtype. A significant reduction in the uptake of adenosine was observed in GSCs as compared to non-GSCs, although mRNA and protein levels of ENT1 were not significantly different (Alarcon et al., 2020). Therefore, post-translational modifications of the transporter may be relevant in the alteration of its transport efficiency. In this regard, ENT1 has been shown to be regulated by kinase-dependent pathways (Bone et al., 2007; Reyes et al., 2011), and, as discussed below, also protein-protein interaction might contribute to the activity of ENT1.

Although mRNA for ENT2 has been reported to be expressed in brain (Anderson et al., 1999a), ENT2 was suggested to play a major role in the regulation of adenosine levels in the gastrointestinal tract (Morote-Garcia et al., 2009; Pastor-Anglada and Pérez-Torras, 2018). Its function for the uptake of purine analogs for chemotherapic treatment of various cancer types and its involvement in the cell cycle progression have been recently reviewed by Naes et al. (2020).

Nucleoside Transporter Interactome

Nucleoside transporters have been considered as independent entities that regulate the traffic of nucleosides across the membrane. However, growing lines of evidence indicate that they may interact with other proteins, therefore belonging to a more complex network (Dos Santos-Rodrigues et al., 2014). For example, glucose-regulated protein 58 (GRP58) and aldolase B were identified as possible partners of CNT2 (Huber-Ruano et al., 2010), therefore a regulatory model dependent on nutrient availability can be postulated for CNT2. Although not yet described, the transceptor role of CNT1 might imply the occurrence of interactions with other proteins, which might explain its function apart from that of nucleoside transporter. Concerning ENT1, the only interactor confirmed so far is calmodulin (Bicket et al., 2016), which could explain, at least in part, the calcium-dependent release of nucleosides in neural cells (Zamzow et al., 2009).

HUNTINGTON'S DISEASE

HD is a neurodegenerative disorder that is caused by expanded CAG repeats within the exon-1 of the gene coding for huntingtin (HTT). CAG repeat lengths of up to 34 are considered to be physiological, while longer CAG repeats are associated to the development of HD. The age of disease onset correlates inversely with CAG repeat length. HD is characterized by involuntary, abnormal movements and postures, psychiatric disturbances, and cognitive alterations (Bates et al., 2015). Research has been performed in cellular and animal models expressing different lengths of CAG repeats (**Table 1**). For example, the R6/2 mouse expresses exon 1 of the human *HTT*; the zQ175 mouse expresses a *HTT* carrying 188 CAG repeats, the Tg51 rat expresses a fragment of the *HTT* gene with 51 CAG repeats, while Hdh 150Q is a knock-in mouse expressing the mouse *htt* gene with 150 CAG repeats (Chou et al., 2005; Li et al., 2015; Lee and Chern, 2014; Guitart et al., 2016; Kao et al., 2017). Downregulation of A2AR has been reported in HD rodent models and also in early stages of the disease in humans (Lee and Chern, 2014). Recent studies suggest that reduced extracellular nucleotide breakdown and reduced glycolysis might contribute to the pathology of HD (Toczek et al., 2018; HD iPSC Consortium, 2020) and that ENT1 could be a therapeutical target for this disease (Kao et al., 2017). Measures of intracellular concentration of ATP and NAD^+, and of the activities of enzymes involved in nucleotide catabolism were performed in the human embryonic kidney cell line HEK 293T transfected with plasmids expressing wild-type or mutant *HTT* gene. Reduction of intracellular ATP, together with increased ADA activity and reduced activities of ectonucleoside triphosphate diphosphohydrolase, ecto-5′-nucleotidase and ectosolic ADA, with no changes of AMPD and PNP activities were observed (Toczek et al., 2018). The authors suggested that mutated HTT could interact with and suppress the activity of the extracellular enzymes involved in nucleotide catabolism thereby contributing to HD pathology (Toczek et al., 2018). More recently, human astrocytes and striatal neurons have been obtained from pluripotent stem cells (iPSCs) derived from unaffected individuals and HD patients with *HTT* gene containing increased number of CAG repeats and used to study the effect of mutant HTT on bioenergetics (HD iPSC Consortium, 2020; Hamilton et al., 2020). While the neurons and astrocytes obtained from iPSCs of control individuals and HD patients had similar levels of ADP and ATP and comparable respiratory and glycolytic activities (Hamilton et al., 2020), the neurons with longer CAG tails, reflecting a more advanced stage of the disease (HD iPSC Consortium, 2020), showed decreased ATP levels and reduced expression of glycolytic enzymes compared to controls. In addition, ATP levels in these HD neurons could be rescued by addition of pyruvate suggesting that the glycolytic deficits play a role in the metabolic disturbance of HD neurons (HD iPSC Consortium, 2020). It would be interesting to test whether the reduced breakdown of ATP takes place in the striatum of HD patients, since this could result in decreased extracellular adenosine and therefore decreased stimulation of the A2AR, which is highly expressed in the striatum and which plays a protective role in this region. Indeed, selective agonists of A2AR reduced DNA damage and oxidative stress-induced apoptosis in HD-iPSC-derived neurons through a cAMP/PKA-dependent pathway (Chiu et al., 2015) and improved motor deficits in mice models of HD (Chou et al., 2005) (**Table 1**). A role of heteromers of adenosine receptors in HD has been hypothesized but experimental evidence has not yet been provided (Glaser et al., 2020). The decline in energy metabolism observed in HD may be caused both by a gain-of-function of the mutated HTT protein and also by the loss of HTT function. The latter has been demonstrated in cardiomyocytes in which *HTT* was knocked out

TABLE 1 | Involvement of adenosine metabolism enzymes, transporters and receptors in HD.

Enzyme/transporter/receptor	Treatment	Model	Expression/activity	Effect	References
ADA		HEK 293T cells expressing HTT with 54 repeats	Increased activity		Toczek et al. (2018)
Ectonucleoside triphosphate diphosphohydrolase, CD73, ectosolic ADA		HEK 293T cells expressing HTT with 54 repeats	Decreased activity		Toczek et al. (2018)
ADA, CD39, CD73		R 6/2 mouse	No change in expression		Kao et al. (2017)
ADK		R 6/2 mouse	Increased expression		Kao et al. (2017)
ADK		Hdh150Q mouse	No change in expression		Kao et al. (2017)
AMPK		Human,Mouse striatal neurons	Increased expression		Ju et al. (2011)
AMPK	Metformin	R 6/2 mouse		Increased survival	Ma et al. (2007)
AMPK	Metformin	Immortalized striatal cells		Increased survival	Jin et al. (2016)
ENT1		zQ175, R6/2 and Hdh150Q	Increased expression		Guitart et al. (2016) Kao et al. (2017)
ENT1	Inhibitor, knockout	R 6/2 mouse		Increased survival, increased motor function	Kao et al. (2017)
ENT2		R6/2 and Hdh150Q mouse	Increased expression		Kao et al. (2017)
A2AR		Human, rat and mouse models	Decreased expression		Lee and Chern (2014)
A2AR		zQ175 mouse	Decreased expression		Guitart et al. (2016)
A2AR		Tg51 rat	No change in expression		Guitart et al. (2016)
A2AR	Agonist	R 6/2 mouse		Decreased motor deficits	Chou et al. (2005)
A2AR	Knockout	R 6/2 mouse		Increased cognitive function	Li et al. (2015)
AR	Caffeine (non selective antagonist)	Human		Decreased disease onset	Simonin et al. (2013)
ARAR gene polymorphisms		Human		Decreased disease onset	Dhaenens et al. (2009); Taherzadeh-Fard et al. (2010)
A1AR		Human	Decreased in symptomatic, increased in presymptomatic		Matusch et al. (2014)
A1AR	Agonist	Rat, 3-nitropropionic acid infusion		Attenuation motor deficit	Blum et al. (2002)
A1AR	Antagonist	R 6/2 mouse	Decreased binding, increased functionality		Ferrante et al. (2014)

by the CRISPR/Cas9 method which showed reduced intracellular ATP and reduced cellular medium concentration of total purine pool (Tomczyk et al., 2020).

Decrease of A2AR has been found in several but not in all HD animal models (Lee and Chern, 2014; Guitart et al., 2016). For example, the number of A2AR antagonist binding sites was not changed in transgenic HD rats with 51 CAG repeats (Tg51 rats). The extracellular level of adenosine measured by *in vivo* microdialysis was lower in rats with 51 CAG repeats and in mice with 175 CAG repeats compared to their wild type littermates (Guitart et al., 2016). Striatal density of ENT1 expression and the expression of ENT1 transcript were significantly increased in mouse expressing HTT with 175 repeats and in postmortem prefrontal cortex from HD patients, respectively (Guitart et al., 2016). Moreover, the ratio of adenosine/ATP in the cerebral spinal fluid was negatively correlated with the disease duration. Chronic inhibition of ENT1 or genetic removal of ENT1 enhanced the survival of mice which express exon 1 of the human *HTT* gene, containing 150 CAG repeats (Kao et al., 2017) (**Table 1**).

Although the lower adenosine levels found in HD models suggest that increasing adenosine could have a protective role in HD, some reports suggest that the adenosine effect could be symptom-specific. Indeed, activation of A2AR has a beneficial effect on the motor impairment, but inactivation of A2AR in some models improves cognitive function (Blum et al., 2018). ADK and ADA contribute to the adenosine tone in the striatum, and some conflicting results obtained in animal models could be due to the specific contribution of these enzymes in compensatory effects among proteins/enzymes that control adenosine homeostasis. For example, ADK transcript and ADK activity are upregulated in the mouse that harbors mutated *HTT* with 187 repeats but not in the one with150 repeats (Kao et al., 2017).

Metformin intake correlates with improved cognitive functions in HD patients suffering from diabetes (Hervas

TABLE 2 | Involvement of adenosine metabolism enzymes and receptors in ALS.

Enzyme/ receptor	Treatment	Model	Expression/activity	Effect	References
ADA		Astrocytes from C9orf72 ALS patients	Decreased expression	Increased cocultured motor neuron cytotoxicity	Allen et al. (2019)
AMPK		ALS patient-derived mesenchymal stem cells	Decreased expression		Yun et al. (2019)
AMPK	Resveratrol (activator)	ALS patient-derived mesenchymal stem cells	Increased expression and activation	Increased differentiation	Yun et al. (2019)
AMPK	latrepirdin (activator)	SOD1(G93A) mouse	Increased activity	Delayed disease progression	Coughlan et al. (2015)
AMPK	calorie restricted diet (activator)	SOD1(G93A) mouse	Increased activity	Decreased neuronal survival and lifespan	Zhao et al. (2015)
A2AR		Human ALS lymphocyte	Increased expression		Vincenzi et al. (2013)
A2AR		Human, spinal cord	Increased expression		Ng et al. (2015)
A2AR		SOD1(G93A) mouse, spinal cord	Increased expression (disease onset)		Ng et al. (2015)
A2AR		SOD1(G93A) mouse, spinal cord	Decreased expression (later disease stage)		Potenza et al. (2013)
A2AR	Knockout inhibitor	SOD1(G93A) mouse		Increased neuronal survival, delayed disease progression	Ng et al. (2015)
A2AR	caffeine (non selective antagonist)	SOD1(G93A) mouse		Decreased survival	Potenza et al. (2013)

et al., 2017). It is known that metformin has a protective effect in HD cell models through activation of AMPK and modulation of mitochondrial dynamics (Jin et al., 2016). However, nuclear localization of AMPKα1 has been reported in the brain tissue of HD patients and it has been demonstrated to potentiate striatal neurodegeneration in HD models (Ju et al., 2011) (**Table 1**). This raises the possibility that there could be a therapeutical window for AMPK activation and that it should be used at early stages since activation during the late stages of HD might be deleterious. However, more research is necessary to understand the role of AMPKα1 and the possible protective effects of metformin on targets different from AMPK.

AMYOTROPHIC LATERAL SCLEROSIS

ALS is characterized by degeneration of both upper and lower motor neurons which leads to progressive weakness and ultimately to death. In addition, there might be extra-motor manifestations including alteration of some cognitive functions, and processing of emotions. In about 10% of cases, frontotemporal dementia has been associated with ALS (Benbrika et al., 2019). Ninety per cent of ALS cases are sporadic and more than 35 genes have been linked to the disease. The main genes involved in the familial forms are those coding for the superoxide dismutase (SOD1), TAR DNA binding protein (TARDBP), chromosome 9 open reading frame 72 (C9orf72) and fused in sarcoma (FUS). Disturbances in RNA metabolism, impaired protein homeostasis, nucleocytoplasmic transport defects, impaired DNA repair, excitotoxicity, mitochondrial dysfunction, oxidative stress, axonal transport disruption, neuroinflammation, oligodendrocyte dysfunction, and vesicular transport defects have been proposed to contribute to ALS pathogenesis (Rothstein, 2009; Mejzini et al., 2019; Floare and Allen, 2020).

It has been hypothesized that, at least in some types of ALS, neurodegeneration could be the result of a higher susceptibility to oxidative stress and experiments designed to test it have pointed to the involvement of AMPK activation. The transgenic mouse SOD1(G93A), that overexpresses a human SOD1 with the substitution of glycine 93 to alanine, is the model of a fifth of the familial cases of ALS (Rosen et al., 1993). Embryonic neural stem cells from these mice exhibited more phosphorylated AMPK and were more susceptible to apoptosis in the presence of oxidative stress compared to those obtained from wild type animals, while treatment with compound C, an inhibitor of AMPKα, attenuated the effects of H_2O_2 (Sui et al., 2014). Strong activation of AMPK was also found in lumbar spinal cords of SOD1(G93A) mice (Coughlan et al., 2015). However, when these mice were treated with the AMPK activator latrepirdine from postnatal day 70 to day 120, they showed a delayed symptom onset and a significant increase in life span compared to untreated mice (Coughlan et al., 2015). These protective effects were not observed when stimulation of AMPK was obtained through a calorie-restricted diet. Indeed, this diet reduced motor neuron survival and reduced lifespan, while lifespan was increased and disease onset was delayed in the SOD1(G93A) mice fed with a high fat diet (Zhao et al., 2015). These experiments are summarized in **Table 2**. Therefore, these results point to inhibition, and not to activation of AMPK as a therapeutic strategy for ALS.

Although many studies have shown that purinergic signaling is altered in ALS, it is not yet clear whether these changes are compensatory or causative. An up-regulation of A2ARs was observed in lymphocytes of ALS patients with

respect to healthy subjects (Vincenzi et al., 2013), and, at the symptomatic onset, in the spinal cords of SOD1(G93A) mice and end-stage human ALS spinal cords (Ng et al., 2015). In addition, adenosine induced embryonic stem cell-derived motor neuron cell death in cultures while pharmacological inhibition and partial genetic ablation of A2AR protected motor neurons from cell death and delayed disease progression of SOD1(G93A) mice (Ng et al., 2015) (**Table 2**). Sebastiao et al. (2018) have suggested that A2AR stimulation or inhibition could play a different role at early and late stages of ALS, since accumulating lines of evidence indicate a beneficial role of both agonists and antagonists of A2AR.

Recent studies have shown that mesenchymal stem cells derived from ALS patients have limited stem cell capacities and exhibit cellular senescence phenotype suggesting that they should be improved before being used in autologous stem cell therapy. Yun et al. (2019) have reported down-regulated AMPK/sirtuin 1 signaling in these cells and have found that resveratrol, which independently stimulates both AMPK and sirtuin 1 (Dasgupta and Milbrandt, 2007; Gertz et al., 2012) restored AMPK/sirtuin 1 and increased differentiation of mesenchymal stem cells into neuron-like cells.

Another recent interesting observation regarding adenosine metabolism has been reported in ALS characterized by a massive hexanucleotide repeat expansion within *C9orf72* (DeJesus-Hernandez et al., 2011; Renton et al., 2011). ADA levels are reduced in fibroblasts, and in both astrocytes and neurons obtained from induced-neuroprogenitor cells from individuals with either C9orf72 mutation or with sporadic ALS (Allen et al., 2019) (**Table 2**). *In vitro* experiments have shown that, when ADA reduction was bypassed by inosine supplementation, the bioenergetic flux and ATP levels increased in induced-astrocytes and that the induced astrocyte-mediated motor neuron cell death was reduced (Allen et al., 2019). Allen et al. (2019) have suggested that inosine supplementation, in combination with modulation of the level of ADA may represent a beneficial therapeutic approach in ALS patients.

CONCLUDING REMARKS

Adenosine and ATP can be released by neurons, astrocytes, oligodendrocytes and microglia and these cells express receptors for both signaling molecules. Adenosine not only acts as neurotransmitter and neuromodulator, but it is also a metabolic sensor in the brain and contributes to the communication between the different cells fine-controlling synaptic circuitry and neuroinflammation. Nucleoside transporters and/or enzymatic activities involved in the metabolism of adenosine, by affecting the levels of both ATP and adenosine, and therefore the activity of adenosine and ATP receptors, impact on many physiological processes. They could have a role in the onset or the development of central nervous system disorders, and be possible targets of drugs for their treatment. The adenosine concentration must be strictly regulated. Indeed, adenosine (and deoxyadenosine) accumulation such as in ADA deficiency, results in severe combined immunodeficiency associated with neurological deficits. Decrease of adenosine is observed in several pathological conditions, as in epilepsy, in which ADK is increased. ADK not only decreases adenosine levels, but also regulates the transmethylation pathway. Both increase in adenosine and DNA methylation inhibition have a protective role in this pathology. Some studies point to the use of ENT inhibition to increase adenosine levels and ENT inhibitors with the ability to cross the brain blood barrier have been used with antiepileptic activity in animal models. The use of ENT1 inhibitors has also been proposed for HD. In this regard, most studies would find a sound support from the setting-up of reliable techniques for the direct determination of adenosine levels in different physiological and pathological conditions.

Many times, the information available from cell and animal models and patients does not clarify whether the changes in adenosine metabolism or signaling are causative or compensative. However, it has to be considered that often compensatory changes occur during the development of the diseases, and there could be temporal windows when a drug could be effective. In other cases, when the energetic state of the cells is involved, modulation of AMPK has been hypothesized to be a target to improve neurodegeneration and/or cognitive impairment. Although stimulation of AMPK improves impairments in stem cell properties derived from ALS patients, the studies in mouse models indicate that different effects could result from distinct activation pathways of the enzyme, and from the AMPK isozyme localization. Notably, in diabetic patients with AD or HD comorbidity, metformin appears to prevent cognitive decline. Increased knowledge on adenosine metabolism in diseases could provide new tools of intervention. As an example, in the C9orf72 type of ALS, reduced ADA activity and *in vitro* models of disease have led to the hypothesis that inosine could have a protective effect. However, it is clear that much research is still necessary to understand the adenosine metabolism and the cross-talk of different cell types in different regions of the brain and also their temporal variations under physiological and pathological conditions in order to design new therapeutical tools.

AUTHOR CONTRIBUTIONS

MG-G conceived the manuscript; MC, MG-G and MGT wrote the draft manuscript. SA and RP collected references and drew the figures. All authors discussed the content.

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GLOSSARY

AD: Alzheimer's disease
ADA: adenosine deaminase
ADF: astrocytoma cell line
ADK: adenosine kinase
ADK_L: ADK-long
ADK_S: ADK-short
ALS: amyotrophic lateral sclerosis
AMPD: AMP deaminase
AMPK: AMP-activated protein kinase
C9orf72: chromosome 9 open reading frame 72
CD26: dipeptidyl peptidase-4
CD39: ectonucleoside triphosphate diphosphohydrolase
CD73: ecto-5′- nucleotidase
CNT1-3: concentrative nucleoside transporter 1-3
dATP: deoxyadenosine triphosphate
ENT1-4: equilibrative nucleoside transporters 1–4
FUS: fused in sarcoma
GRP58: glucose-regulated protein 58
GSCs: glioblastoma stem-like cells
HD: Huntington's disease
HPRT: hypoxanthine-guanine phosphoribosyltransferase
HSP: hereditary spastic paraplegia
HTT: huntingtin
IMP: inosine monophosphate
iPSCs: induced pluripotent stem cells
NBTI: nitrobenzythioinosine
NT5C1: cytosolic 5′-nucleotidase I
NT5C2: cytosolic 5′-nucleotidase II
PD: Parkinson's disease
PNP: purine nucleoside phosphorylase
PRPP: 5-phosphoribosyl-1-pyrophosphate
SAH: S-adenosyl homocysteine
SAHH: S-adenosyl homocysteine hydrolase
SAM: S-adenosylmethionine
SCID: severe combined immunodeficiency
SOD1: superoxide dismutase 1
TARDBP: TAR DNA binding protein

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REVIEW
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A_{2B} Adenosine Receptors and Sphingosine 1-Phosphate Signaling Cross-Talk in Oligodendrogliogenesis

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Oligodendrocyte-formed myelin sheaths allow fast synaptic transmission in the brain. Impairments in the process of myelination, or demyelinating insults, might cause chronic diseases such as multiple sclerosis (MS). Under physiological conditions, remyelination is an ongoing process throughout adult life consisting in the differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes (OLs). During pathological events, this process fails due to unfavorable environment. Adenosine and sphingosine kinase/sphingosine 1-phosphate signaling axes (SphK/S1P) play important roles in remyelination processes. Remarkably, fingolimod (FTY720), a sphingosine analog recently approved for MS treatment, plays important roles in OPC maturation. We recently demonstrated that the selective stimulation of A_{2B} adenosine receptors (A_{2B}Rs) inhibit OPC differentiation *in vitro* and reduce voltage-dependent outward K^+ currents (I_K) necessary to OPC maturation, whereas specific SphK1 or SphK2 inhibition exerts the opposite effect. During OPC differentiation A_{2B}R expression increases, this effect being prevented by SphK1/2 blockade. Furthermore, selective silencing of A_{2B}R in OPC cultures prompts maturation and, intriguingly, enhances the expression of S1P lyase, the enzyme responsible for irreversible S1P catabolism. Finally, the existence of an interplay between SphK1/S1P pathway and A_{2B}Rs in OPCs was confirmed since acute stimulation of A_{2B}Rs activates SphK1 by increasing its phosphorylation. Here the role of A_{2B}R and SphK/S1P signaling during oligodendrogenesis is reviewed in detail, with the purpose to shed new light on the interaction between A_{2B}Rs and S1P signaling, as eventual innovative targets for the treatment of demyelinating disorders.

Keywords: adenosine, sphingosine kinase (SphK), remyelination, K+ channels, oligodendrocyte differentiation, sphingosine-1-phosphate, oligodendrocyte progenitor cells (OPCs), A_{2B} receptors

INTRODUCTION

In the central nervous system (CNS), oligodendrocytes (OLs) are responsible for myelin production, which allows fast signal transmission and provides metabolic support to axons (Nave, 2010; Saab et al., 2016). During development, OLs are generated in the germinal zones of the brain, i.e., the subventricular zone (SVZ) (Yu et al., 1994), from migratory bipolar oligodendrocyte precursor cells (OPCs), which are renowned for the expression of the proteoglycan nerve-glial antigen 2 (NG2) (Grinspan, 2002; Brazel et al., 2004). Thanks to their migratory ability, OPCs spread and populate the embryonic brain and spinal cord (Emery, 2010) to differentiate into myelinating OLs. However, a pool of immature OPCs, comprising the 5-8% of total glial cells (Levine et al., 2001), persists within the adult CNS where they represent the major population of cycling cells (Dawson et al., 2003). This process guarantees, under physiological conditions, myelin turnover and remodeling in response to life experience (Malerba et al., 2015; Bergles and Richardson, 2016) and, in conditions of tissue damage, the generation of new OLs able to remyelinate the brain after a lesion or injury, an ability that might be lost through normal aging or chronic diseases. However, under pathological conditions such those characterized by chronic neuroinflammation and neurodegeneration, this process fails, leading to improper nerve conduction due to discontinuities in the myelin sheet (Nave, 2010; Franklin and Ffrench-Constant, 2017). For this reason, it is of critical relevance to identify innovative targets able to encourage OPC differentiation toward the mature, myelin-producing OL phenotype.

Among the factors influencing oligodendrogliogenesis, interest has been focused in the last years on the neuromodulator adenosine (Coppi et al., 2021), on one hand, and on the pleiotropic signaling molecule sphingosine 1-phosphate (S1P).

This review will focus on S1P and adenosine receptor-mediated effects in oligodendrocyte progenitors relevant for oligodendrogenesis and their possible functional interplay with the purpose to deepen the knowledge of molecular mechanisms involved in A_{2B} receptor- (A_{2B}R-) or S1P-mediated effects and their cross-talk in oligodendrocyte biology.

Oligodendrocyte Differentiation

Before being able to produce myelin, oligodendroglial cells progress through a series of highly regulated steps of differentiation from OPCs to mature OLs (de Castro and Bribian, 2005; Barateiro and Fernandes, 2014). This process is characterized by the loss of proliferative and migratory activity and the acquisition of an elaborate and highly ramified morphology (de Castro and Bribian, 2005). Oligodendrogliogenesis implicates a sequence of distinct maturational stages each of them identified by distinct morphological changes and by the expression of specific antigens (Levi et al., 1986; Gard and Pfeiffer, 1990; Warrington et al., 1992; Jung et al., 1996). On these bases, three major stages of differentiation have been proposed: a proliferating, bipolar OPC phase, characterized by the expression of platelet-derived growth factor (PDGF) receptor alpha (PDGFα), NG2 and the transcription factor Olig2 (Pringle et al., 1992; Ligon et al., 2006), a post-mitotic, moderately ramified pre-oligodendrocyte phase (pre-OL), positive for the markers O4 (Szuchet et al., 2011) and the recently deorphanized (Ciana et al., 2006) P2Y-like GPR17 receptor (Lecca et al., 2008; Emery, 2010; Fumagalli et al., 2011; Coppi et al., 2013b), and a mature myelinating OL phase, characterized by a highly ramified morphology and by the expression of galactocerebroside (GC), a specific marker for the oligodendrocyte membrane, myelin specific structural proteins such as 2',3'-Cyclic-nucleotide-3'-phosphodiesterase (CNPase), myelin associated glycoprotein (MAG) and myelin basic protein (MBP) (Scolding et al., 1989; Zhang, 2001; Szuchet et al., 2011; Coppi et al., 2013a, 2015). Mature OLs synthesize large amounts of myelin, giving rise to multilamellar myelin sheaths that wrap and insulate neuronal axons which allow electrical isolation and saltatory conduction of electric impulses.

It is known that, during their maturation, oligodendroglial cells display functional voltage-gated ion channels whose expression changes during differentiation (Sontheimer et al., 1989; Barres et al., 1990; Williamson et al., 1997; Spitzer et al., 2019) including either inward or outward rectifying K^+ channels (Kir and Kv, respectively), Na^+ channels (Nav) and different subtypes of Ca^{2+} channels (Cav) (Verkhratsky et al., 1990).

During mouse brain development, the first ion channel subtype detected in OPCs is the Kv voltage-dependent, outwardly rectifying K^+ conductance, at embryonic day 18 (E18) (Spitzer et al., 2019). However, a fraction of OPCs are described to express also Na_V currents that sharply increase around birth. This OPC population, with high Na_V and K_V densities, reflect a proliferative and migratory state during myelin generation (Gautier et al., 2015; Spitzer et al., 2019). Intriguingly, not all OPCs express Na_V, but only a fraction, described to be around 60% (Kettenmann et al., 1991). Of note, a subpopulation of electrically excitable, spiking, $NG2^+$ OPCs, able to generate full action potentials when stimulated by depolarizing current injection, have been described in brain slices (Karadottir et al., 2008), but the functional role of this "electrically excitable" OPC subpopulation is still unknown. Of note, single action potentials have also been detected in a minority of cultured OPCs (Barres et al., 1990).

The functional role of action potentials (APs) in OPC is still a matter of debate since, up to now, no functional differences have been detected between firing and not-firing OPCs (Bonetto et al., 2020). As long as OPCs undergo functional maturation, the density of Nav currents decreases as well as the expression of outward Kv conductances. In this phase, OPC differentiation potential declines and thus it can be considered a "quiescent" OPC state.

Among Kv currents, OPCs show outward conductances mainly composed by tetraetylammonium (TEA)-sensitive, delayed rectifying K^+ currents (I_K) (Sontheimer and Kettenmann, 1988) characterized by scarce time- and voltage-dependent inactivation and by a threshold for activation around -40 mV (Gutman et al., 2005). They also express a transient outward K^+ current, or I_A (Gallo et al., 1996; Coppi et al., 2013b; Coppi et al., 2015), characterized by a rapid time-dependent inactivation (approximately 50 ms) and a voltage-dependent inactivation at potentials above -80 mV.

During maturation, outward K^+ conductances (both I_K and I_A), as well as I_{Na}, undergo a strong downregulation up to almost completely disappearance in mature OLs (Sontheimer and Kettenmann, 1988; Sontheimer et al., 1989; Barres et al., 1990; Coppi et al., 2013a). Concomitantly, a gradual increase in the expression of inwardly rectifying K^+ currents (Kir), activated at potentials lower than −100 mV, appears. Indeed, Kir currents are the main conductance observed in mature OLs (Knutson et al., 1997). Among the mentioned currents, TEA-sensitive I_K are crucially linked to cell cycle regulation and hence to myelin formation (Chittajallu et al., 2005), as demonstrated by the fact that when OPC cultures are grown in the presence of TEA, a significant inhibition of their proliferation and differentiation is observed (Gallo et al., 1996; Knutson et al., 1997; Chittajallu et al., 2005; Coppi et al., 2013b). Hence, compounds that modulate these currents may affect oligodendrocyte proliferation and myelination, as well as neurotransmitters, cytokines and growth factors acting on specific metabotropic receptors described to modify K^+ current expression in OPCs (Stellwagen and Malenka, 2006; Zonouzi et al., 2011; Lundgaard et al., 2013; Malerba et al., 2015; Spitzer et al., 2019).

Adenosine as a Neuromodulator

Adenosine is an intermediary metabolite acting as a building molecule for nucleic acids and a component of the biological energy currency ATP. In addition, adenosine is one of the most evolutionarily ancient signaling molecules (Verkhratsky and Burnstock, 2014), acting through the stimulation of four distinct adenosine-sensitive metabotropic P1 receptors denoted as A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs) (Fredholm et al., 2011). These receptors are widely expressed both in the periphery or in the central nervous system (CNS) and have been implicated in a myriad of biological functions (Pedata et al., 2007; Coppi et al., 2012).

High extracellular concentrations of the nucleoside adenosine are found under conditions of tissue damage or when an imbalance in oxygen supply occurs (Latini et al., 1999; Pedata et al., 2014). Adenosine is short-lived in the extracellular space due to enzymatic degradation by adenosine deaminase (ADA) or adenosine kinase (AK) (Chen et al., 2013) or re-uptake operated by the equilibrative nucleoside transporters (ENT) isoforms ENT1 and ENT2 (Inoue, 2017), so its effects in the CNS are mainly described as autocrine and/or paracrine.

Among the four different P1 receptor subtypes, different affinities have been shown for the endogenous ligand. The activation of A_1Rs is achieved as long as extracellular adenosine falls in the low nanomolar range (1-10 nM), a concentration generally present in most tissues and organs throughout the body (Latini and Pedata, 2001). A_1R-mediated signal activates $G_{i/o}$ proteins leading to the inhibition of adenylyl cyclase (AC) and to a decrease in intracellular cAMP levels (Antonioli et al., 2019). A_1Rs are the predominant P1 receptor subtype in the CNS, with high levels of expression reported in the cerebral cortex, hippocampus, cerebellum, thalamus, brainstem and spinal cord. It is well known that A_1R activation inhibits synaptic transmission (Corradetti et al., 1984; Dunwiddie, 1984) leading to sedative, anticonvulsant (Muzzi et al., 2013) and anxiolytic (Vincenzi et al., 2016) effects in the CNS whereas, at cardiovascular levels, they are potent bradycardic agents (Jacobson and Gao, 2006).

The A_{2A}R subtype is known to stimulate AC by G_s protein activation, leading to the production of cAMP which acts as a second messenger by activating protein kinase A (PKA) (Antonioli et al., 2019). Within the brain, this receptor subtype is widely expressed with particularly high levels found in the striatum/caudate-putamen nuclei (Peterfreund et al., 1996). In the periphery, its expression is abundant on blood vessels and inflammatory/immune cells (Yu L. Q. et al., 2004). The functional effect of A_{2A}Rs in the brain is at variance from A_1R subtype, as they enhance glutamate release and promote cell excitability (Goncalves and Ribeiro, 1996; Lopes et al., 2002). In accordance, A_{2A}R activation participates to excitotoxic damage due to extracellular glutamate overload during an ischemic-like insult obtained *in vitro* by oxygen and glucose deprivation (OGD) (Colotta et al., 2012; Maraula et al., 2013; Maraula et al., 2014). Concerning peripheral functions of A_{2A}Rs, it is worth to note that adenosine, thanks to its actions on this receptor subtype, is one of the most powerful endogenous anti-inflammatory agents (Antonioli et al., 2019). Indeed, A_{2A}Rs are highly expressed in inflammatory cells including lymphocytes, granulocytes and monocytes/macrophages, where their activation reduces pro-inflammatory cytokines, i.e., tumor necrosis factor-alpha (TNFα), interleukin-1β (IL-1 β) and interleukin-6 (IL-6) (Varani et al., 2011) and enhances the release of anti-inflammatory mediators, such as interleukin-10 (IL-10) (Bortoluzzi et al., 2016).

The A_{2B}R subtype is somewhat the most enigmatic and less studied among the four P1 receptors as its pharmacological and physiological characterization has long been precluded by the lack of suitable ligands able to discriminate among the other adenosine receptor subtypes (Popoli and Pepponi, 2012; Chandrasekaran et al., 2019). The central distribution of A_{2B}Rs on neurons and glia is scarce but widespread, whereas in the periphery abundant levels of A_{2B}Rs are observed in bronchial epithelia, smooth muscles, inflammatory cells such as mast, neutrophils and monocytes, vasculature and digestive tracts like ileum and colon (Feoktistov et al., 1998; Chandrasekaran et al., 2019). It is reported that A_{2B}R activation stimulates G_s and, in some cases, also $G_{q/11}$ proteins, thus activating either or both cAMP-pathway and IP_3- related pathways. In this second case, the activation of phospholipase C (PLC) leads to the production of inositol-(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG), that increases the intracellular levels of Ca^{2+} and activates protein kinase C (PKC), respectively (Antonioli et al., 2019).

Differently from high affinity A_1Rs and A_{2A}Rs, activated by physiological levels of extracellular adenosine (low nM and high nM, respectively (Coppi et al., 2020b), the A_{2B}R needs much higher adenosine concentrations (in the μM range) reached only in conditions of tissue damage or injury (Chandrasekaran et al., 2019). Such a low affinity of A_{2B}Rs for the endogenous agonist implies that they represent a good therapeutic target, since they are activated only by high adenosine efflux reached under pathological conditions or injury, when a

massive release of adenosine occurs (Popoli and Pepponi, 2012) or that they can be driven to function by selective agonists (Coppi et al., 2020b). All the adenosine receptors mentioned above are also associated with mitogen-activated protein kinase (MAPK) pathways, which involves the activation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), whose action can mediate numerous cellular responses, and of JUN N-terminal kinase (JNK) and p38 MAPK (Melani et al., 2009). In particular, A_1Rs and A_{2B}Rs promote the activation of ERK1, ERK2, p38 MPAK and JNK, instead A_{2A}Rs and A_3Rs are involved only in the stimulation of ERK1 and ERK2 signaling (Antonioli et al., 2013).

As mentioned above for the cognate A_{2A}R subtype, A_{2B}R activation within the CNS is reported to increase glutamate release (Goncalves et al., 2015; Fusco et al., 2019) but the mechanism is at variance from the former. In fact, Cunha and co-workers demonstrated that the A_{2B}R selective agonist BAY60-6583 attenuates the predominant A_1R-mediated inhibitory control of synaptic transmission in the CA1 hippocampus (Goncalves et al., 2015). These data are consistent with the relatively abundant expression of A_{2B}Rs in hippocampal presynaptic sites, demonstrated by means of synaptosome preparation, reported by the same authors (Goncalves et al., 2015). The facilitatory effect of A_{2B}Rs on glutamatergic neurotransmission was confirmed by us in acute hippocampal slices by using the electrophysiological protocol of paired pulse facilitation (PPF) (Fusco et al., 2019). We reported that A_{2B}R activation decreases PPF in the CA1 hippocampus, an effect known to be ascribed to enhanced glutamate release (Zucker and Regehr, 2002). Furthermore, the effect of BAY60-6583 was prevented not only by the A_{2B}R antagonists MRS1754 and PSB-603, but also by the A_1R blocker DPCPX (Fusco et al., 2019), confirming the fact that A_1R activation in necessary for the enhancing effect of A_{2B}Rs on glutamate release. Furthermore, we extended results to a newly synthetized A_{2B}R-selective agonist, the recently described BAY60-6583-analog P453 (Betti et al., 2018) which proved higher affinity for the A_{2B}R (50 nM *vs.* 200 nM, respectively) than the commercially available BAY 60-6583. In addition, as reported for A_{2A}Rs, also A_{2B}R activation participates to OGD-induced synaptic failure in the hippocampus (Fusco et al., 2018).

In the periphery, A_{2B}Rs are present on hematic cells, such as lymphocytes and neutrophils, with the highest expression levels found on macrophages (Gessi et al., 2005; Yang et al., 2006). Here, A_{2B}Rs in most cases are co-expressed with A_{2A}Rs and their activation exert anti-inflammatory effects by inhibiting migration and vascular adhesion (Yang et al., 2006) of inflammatory cells (Wakai et al., 2001; Eckle et al., 2008).

The A_3R subtype is known to couple to $G_{i/o}$ proteins and to inhibit AC but, under particular conditions or in different cell types, activation of $G_{q/11}$ by A_3R agonists has also been reported (Antonioli et al., 2019). This receptor subtype presents large interspecies differences, with only 74% sequence homology between rat and human (Koscso et al., 2011). Its expression is not uniform throughout the body: low levels are found in the brain and spinal cord whereas a predominance of this receptor subtype is described in peculiar regions at the periphery, i.e., in the testis, lung, kidneys, placenta, heart, brain, spleen and liver (Gessiaa et al., 2008). Interestingly, most of the cell types of the immune system express functional A_3Rs on their surface (Hasko et al., 2008) and its activation is one of the most powerful stimuli for mast cells degranulation (Ramkumar et al., 1993).

Adenosine in Oligodendrogliogenesis

Oligodendrocyte turnover is rather slow under physiological conditions and guarantees myelin turnover and remodeling in response to life experience (Bergles and Richardson, 2016). However, a disruption in this process, for example in case of a maturation block, could have devastating consequences during aging and in pathological conditions, such as multiple sclerosis (MS).

Recruitment of OPCs to injured areas is in fact one of the most important events to promote remyelination after CNS injury (Simon et al., 2011; Neumann et al., 2019). Unfortunately, it is still not clear why this process fails, or is insufficient to provide myelin repair, during chronic demyelinating diseases (Romanelli et al., 2016). In fact, most OPCs fail to mature into myelin-producing OLs in MS, indicating that remyelination by adult OPCs is hindered principally due to a failure of OPC differentiation into mature OLs rather than a failure of repopulation or migration of OPCs (Chang et al., 2002; Patel and Klein, 2011).

A deep knowledge of the intricate processes regulating OPC maturation to OLs is mandatory to investigate new therapeutic targets aimed at counteracting demyelinating diseases and repair myelin damage in the long run.

Among the neuromodulators contributing to the balance between proliferating, immature OPCs and myelinating, mature OLs there are purines (Stevens et al., 2002; Fields, 2004; Fields and Burnstock, 2006) and, in particular, adenosine (Coppi et al., 2020b).

All P1 receptors are expressed by maturating oligodendroglial progenitors as well as by mature OLs (Stevens et al., 2002; Coppi et al., 2020a) and exert a key role in cell development (Coppi et al., 2015, 2020b). Furthermore, the expression by oligodendrocytes of the nucleoside transporters ENT1 and ENT2, as well as adenosine degrading enzymes ADA and AK, suggests that these cells are able to sense and finely tune extracellular adenosine levels (Gonzalez-Fernandez et al., 2014), thus supporting the notion that purinergic signaling exerts a prominent role in these cells (Burnstock et al., 2011).

Indeed, our research group contributed to demonstrate that adenosine can affect numerous OPC functions such as migration, proliferation and maturation (Fields and Stevens-Graham, 2002; Stevens et al., 2002; Coppi et al., 2013a,b, 2015; Cherchi et al., 2021), with distinct effects mediated by different receptor subtypes, as summarized in **Figure 1**.

It has been demonstrated that tonic electrical stimulation of co-cultures of OPCs with dorsal root ganglion neurons induces the release of adenosine that inhibits OPC proliferation and promotes their maturation toward pre-myelinating OLs, an effect blocked by a cocktail of A_1R, A_{2A}R and A_3R antagonists (Stevens et al., 2002), suggesting that endogenous adenosine released in response to impulse activity promotes oligodendrocyte development and myelination. Furthermore,

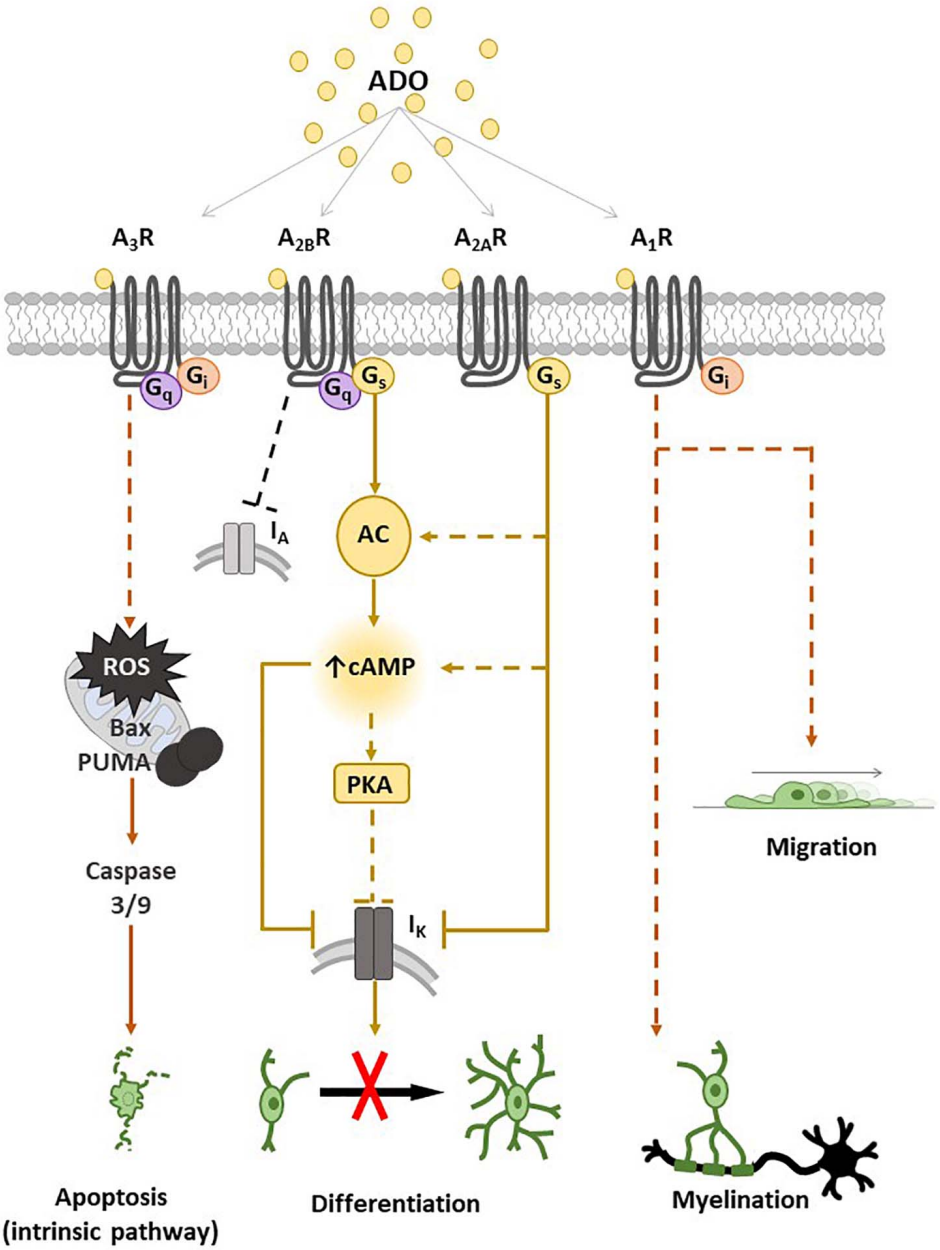

FIGURE 1 | Adenosine receptor expression and main transduction pathways activated in oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs). Schematic representation of A_1, A_{2A}, A_{2B} and A_3 receptor (A_1R, A_{2A}R, A_{2B}R and A_3R) effects on OPCs and main intracellular pathways involved. The activation of A_1R by adenosine (ADO) or other selective receptor agonists promotes myelination and migration. The stimulation of G_s-coupled A_{2A}R and/or A_{2B}R leads to adenylyl cyclase (AC) activation with a consequent increase in intracellular cyclic adenosine monophosphate (cAMP), which closes I_K channels and inhibits OPC differentiation, probably by a mechanism involving protein kinase A (PKA). A_3R stimulation induces OPC apoptosis by the activation of an intrinsic pathway, i.e., through reactive oxygen species (ROS) production and activation of Bcl-2-associated X (Bax), p53-upregulated modulator of apoptosis (PUMA) and caspase 3/9. Dotted lines are used when the intracellular pathway/s have not been described.

A_1R agonists have been reported to stimulate OPC migration (**Figure 1**; Othman et al., 2003).

Concerning the A_{2A}R subtype, our group of research demonstrated that the selective A_{2A}R agonist CGS21680 inhibits TEA- sensitive I_K currents in cultured OPCs and delays *in vitro* cell differentiation without affecting neither cell viability nor proliferation (Coppi et al., 2013a). These effects were completely prevented in the presence of the selective A_{2A}R antagonist SCH58261 (Coppi et al., 2013a). In keeping with data demonstrating that TEA impairs OPC maturation (Attali et al., 1997; Gallo et al., 1996; Coppi et al., 2013b) and blocks myelin deposition in the embryonic spinal cord

(Shrager and Novakovic, 1995) and ovine OPCs (Soliven et al., 1988), it appears that the G_s-coupled $A_{2A}R$ inhibits OPC differentiation by reducing I_K currents. In line with this assumption is the observation that selective activation of GPR17, a G_i-coupled P2Y-like receptor, enhances TEA-sensitive I_K and improves OPC differentiation (Coppi et al., 2013b).

The less known adenosine receptor in OPCs is the A_3R subtype. The only paper available in the literature demonstrates that the A_3R agonist 2-CI-IB-MECA induces apoptosis of cultured oligodendroglial cells isolated from rat optic nerve (Gonzalez-Fernandez et al., 2014) and induces myelin loss in an *ex vivo* preparation of optic nerve, an effect prevented by the A_3R antagonist MRS1220 (Gonzalez-Fernandez et al., 2014). Moreover MRS1220 also prevented optic nerve demyelination induced by *in vitro* ischemic-like conditions, i.e., OGD (Gonzalez-Fernandez et al., 2014). Thus, data suggest that adenosine, via activation of A_3Rs, triggers oligodendrocyte death (**Figure 1**) and contributes to white matter ischemic damage.

Role of A_{2B}Rs in OPCs and Oligodendrogliogenesis

Current research in the field of adenosine is ongoing thanks to the growing interest on this receptor subtype. The pharmacological and physiological characterization of $A_{2B}Rs$ has long been precluded by the lack of suitable ligands (Popoli and Pepponi, 2012).

Very few data are available to date about the role of $A_{2B}Rs$ in oligodendroglial cells, as this adenosine receptor subtype is somewhat the most enigmatic and less studied among the four P1 receptors. We recently found that $A_{2B}Rs$ are crucially involved in OPC maturation by demonstrating that the selective $A_{2B}R$ agonist BAY60-6583, and its recently synthetized analog P453 (Betti et al., 2018), inhibited *in vitro* OPC differentiation, as demonstrated by the reduced expression of myelin-related proteins such as MBP or MAG in primary purified OPC cultures (Coppi et al., 2020a). We also demonstrated that $A_{2B}R$ activation reversibly inhibits TEA- sensitive, sustained I_K, and 4-amynopyridine- (4-AP) sensitive, transient I_A, conductances (Coppi et al., 2020a). As mentioned above, I_K are necessary to OPC maturation (Gallo et al., 1996), so this could be one of the mechanisms by which $A_{2B}Rs$ inhibit myelin production. The AC activator forskolin mimicked BAY60-6583-mediated effect as it decreased I_K currents, in line with previous data (Soliven et al., 1988). Of note, a further application of BAY60-6583 in the presence of forskolin was devoid of effect, thus indicating that $A_{2B}R$ activation inhibits I_K by increasing intracellular cAMP levels (Coppi et al., 2020a).

Data about an inhibitory role of $A_{2B}R$ in myelin formation are consistent with recent findings from Manalo et al. (2020) who demonstrated that elevated cochlear adenosine levels in $ADA^{-/-}$ mice is associated with sensorineural hearing loss (SNHL) due to cochlear nerve fiber demyelination and mild hair cell loss. Intriguingly, $A_{2B}R$-specific antagonists administered in $ADA^{-/-}$ mice significantly restored auditory capacity, nerve fiber density and myelin compaction. The same authors also provided genetic evidence for $A_{2B}R$ upregulation not only in $ADA^{-/-}$ hear-impaired mice but also in age-related SNHL.

Adenosine and Multiple Sclerosis

Multiple sclerosis is a chronic demyelinating disease of the CNS that leads to progressive neurological disability (Courtney et al., 2009). Immune system and inflammatory niche react against the myelin sheath that covers axon fibers altering neuronal transmission leading to the onset of a permanent condition associated with nerve decline. Since the extent and severity of nerve damage is heterogeneous, MS clinical symptoms may differ upon patients depending on the type and amount of the affected nerves. The treatments available for MS are almost limited aiming at reducing relapsing frequency or increasing the remitting speed of the disease with the major aim to manage symptoms.

Adenosine receptors are involved in inflammation and oligodendrogliogenesis, as reported above, and may represent potential therapeutic targets in the treatment of MS (Johnston et al., 2001; Stevens et al., 2002; Coppi et al., 2015; Cherchi et al., 2021). Compounds used in the treatment of MS, such as methotrexate and cladribine, have been shown to act as ligands at adenosine receptors, to exert their anti-inflammatory activities (Montesinos et al., 2000). Therefore, the beneficial actions of these drugs may also involve their ability to activate adenosine A_2R and A_3R in a compensatory manner to regulate cytokine expression (Johnston et al., 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model to study the immunopathogenesis of MS and to test the therapeutic efficacy of novel agents for preclinical study (Constantinescu et al., 2011). EAE can be induced by inoculating the animal with whole myelin or defined myelin protein, as myelin oligodendrocyte glycoprotein (MOG), with adjuvants, which lead to activation of autoreactive peripheral CD4 T-cells and their subsequent trafficking to the CNS by crossing the blood brain barrier (BBB).

The A_1R is expressed on cells of the monocyte/macrophage lineage and a downregulation of A_1R was found in both blood and brain from MS patients (Mayne et al., 1999; Johnston et al., 2001). Accordingly, activation of A_1R is reported to protect from EAE damage (Chen et al., 2010; Wang et al., 2014; Liu G. P. et al., 2018) and A_1R knockout ($A_1R^{-/-}$) exacerbates MOG-induced EAE pathology by increasing demyelination, axonal injury and neuroinflammation (Tsutsui et al., 2004). Finally, A_1R activation improves myelin repair by recruiting OPCs in an experimental model of rat optic nerve demyelination (Asghari et al., 2013).

The role of $A_{2A}Rs$ in MS is controversial. This receptor subtype is upregulated in human lymphocytes (Vincenzi et al., 2013) and in the CNS (Rissanen et al., 2013) of MS patients, thus suggesting an involvement in demyelinating pathologies. However, $A_{2A}R$ overexpression does not correlate with different forms of the disease nor is affected by MS pharmacological treatments (Vincenzi et al., 2013). Surprisingly, $A_{2A}R^{-/-}$ mice developed more severe EAE than wild type animals (Mills et al., 2012; Yao et al., 2012) and the adoptive transfer of peripheral blood cells lacking the $A_{2A}R$ into wild type animals induced more severe EAE than both wild type and

$A_{2A}R^{-/-}$ mice (Mills et al., 2012). This suggests that $A_{2A}R^{-/-}$ mice are susceptible to a severe acute form of EAE due to the lack of A_{2A}R-mediated anti-inflammatory effects. Indeed, in human lymphocytes, A_{2A}R agonist inhibits the release of proinflammatory cytokines, cell proliferation, the expression of the adhesion molecule VLA-4, and the activation of the transcription factor NF-κB; these effects were more evident in lymphocytes from MS patients in comparison to healthy subjects, in line with upregulation of A_{2A}Rs in MS lymphocytes (Vincenzi et al., 2013). Consistent with this, the activation of A_{2A}R signaling by selective agonist inhibits the EAE progression by suppressing the specific lymphocyte proliferation, reducing the infiltration of $CD4^+$ T lymphocytes, increasing intracellular Ca^{2+} levels (Liu et al., 2016), and reducing the effects of Th1 stimulation on the BBB permeability (Liu Y. et al., 2018). Additional evidence demonstrates that A_{2A}R agonism in EAE leads to prevention of the disease when used in early disease stage (Ingwersen et al., 2016) whereas in late-stage EAE the number of foci with marked amount of myelin debris was higher in $A_{2A}R^{-/-}$ mice than wild type (Ingwersen et al., 2016).These findings led to hypothesise that the relevance of A_{2A}R for the pathogenesis of chronic autoimmune neuroinflammation may depend on the time point or the compartment, i.e., the systemic immune response vs. the CNS, an issue that has been elegantly addressed by Rajasundaram in a recent review (Rajasundaram, 2018).

Little is known about the role of A_{2B}R in MS or EAE. Similarly to A_{2A}Rs, A_{2B}R expression increases in peripheral lymphoid tissues of EAE mice. However, up to now, evidences converge on indicating that A_{2B}R blockade is protective in EAE models. Indeed, Wei and colleagues demonstrated that A_{2B}R-specific antagonists or genetic ablation of the receptor attenuated the clinical signs of EAE and protect the CNS from immune damage, probably by eliminating adenosine-mediated IL-6 production (Wei et al., 2013). Furthermore, the A_{2B}R agonist BAY60-6583 reversed mesenchymal stem cells-induced downregulation of AQP4 expression in cultured astrocytes, that is protective for maintaining the integrity of BBB in EAE (Liu et al., 2020).

Sphingosine 1-Phoshate and Its Metabolism

Sphingosine 1-phosphate (S1P) is a natural sphingolipid present in plasma and tissues (Ksiazek et al., 2015) that acts as a modulator of different physiological and pathological processes, such as angiogenesis, vascular stability and permeability, T- and B-cell trafficking, as well as tumorigenesis (Hla et al., 2008). Circulating S1P is mainly produced and released by erythrocytes together with platelets and endothelial cells; it is mainly transported in association with apolipoprotein M (apoM) in high-density lipoprotein (HDL), while a small fraction is transported bound to albumin. ApoM can be considered a S1P chaperone protein (Obinata et al., 2019), which carries the sphingolipid in blood and interstitial fluids, facilitates S1P signaling in tissues and is responsible for physio-pathological effects different from that elicited by albumin-bound S1P. ApoM-bound S1P is able to exert multiple effects such as reduction of vascular inflammation, improvement of endothelial barrier function, inhibition of oxidized low-density lipoprotein- (ox-LDL-) induced inflammation, modification of BBB permeability and protection from bacteria-induced inflammatory responses (Christoffersen et al., 2011; Galvani et al., 2015; Christensen et al., 2016; Zheng et al., 2019). Endothelial cells embracing the BBB are able to secrete S1P into the blood as well as into brain compartments (Hajny and Christoffersen, 2017). S1P concentration is much higher in blood and lymph at μM range than in interstitial tissues due to the activity of S1P degrading enzymes. This concentration gradient, termed the "vascular S1P gradient" causes lymphocyte and hematic cell trafficking from lymph organs or bone marrow to the circulation (Schwab et al., 2005; Pappu et al., 2007; Hla et al., 2008; Cyster and Schwab, 2012). Enhanced vascular permeability, which occurs especially during inflammation, induces a burst of S1P that becomes available in the extravascular milieu, suggesting that the vascular S1P gradient may contribute to physiological and pathological conditions.

The catabolic pathway of plasma membrane-derived complex sphingolipids, mainly sphingomyelin, gives rise to different bioactive molecules, among others S1P. The first event is the sphingomyelinase-dependent hydrolysis of sphingomyelin, that leads to the production of the pro-apoptotic ceramide (Proia and Hla, 2015). Ceramidase deacylates ceramide to sphingosine, which in turn is converted into S1P by ATP-dependent phosphorylation catalyzed by two isoforms of the enzyme sphingosine kinase (SphK), namely SphK1 and 2 (**Figure 2**). These isozymes have been cloned and characterized, and their encoding genes are localized in different chromosomes, 17 and 19 respectively, known to produce multiple splicing variants (Imamura et al., 2001; Alemany et al., 2007). SphK1 and SphK2 differ for subcellular localization, showing partial overlapping but sometimes different biological functions (Liu et al., 2002; Pyne et al., 2016). In this regard, although knockout mouse models for either SphK1 or SphK2 have no gross phenotypic alterations, the double null mutation is lethal to embryos due to alterations in vasculogenesis and hemorrhages (Allende et al., 2004; Mizugishi et al., 2005; Michaud et al., 2006). SphK1 and SphK2 have structural homologies and share five conserved catalytic domains including an ATP-binding motif related to that of the diacylglycerol kinase family (Pitson et al., 2000; Liu et al., 2002). However, the sequence of SphK2 shows additional regions at the N-terminal and central proline-rich sequences which are absent in SphK1 (Igarashi et al., 2003; Gao et al., 2012). A nuclear localization sequence (NLS) and a nuclear exporting sequence (NES) are responsible for SphK2 major localization in the nucleus, since overexpression of SphK2 or SphK1 fused with NLS, but not wild-type SphK1, causes an inhibition of DNA synthesis (Igarashi et al., 2003). Indeed, it has been demonstrated that SphK2 has a role in epigenetic regulation of gene expression being involved in the suppression of histone deacetylase (HDAC) activity (Hait et al., 2009). Moreover, SphK2 is present in five different splicing variants that have been reported to localize into other different intracellular compartments such as the cytosol, mitochondria and the endoplasmic reticulum (ER) (Maceyka et al., 2005; Okada et al., 2005; Strub et al., 2011).

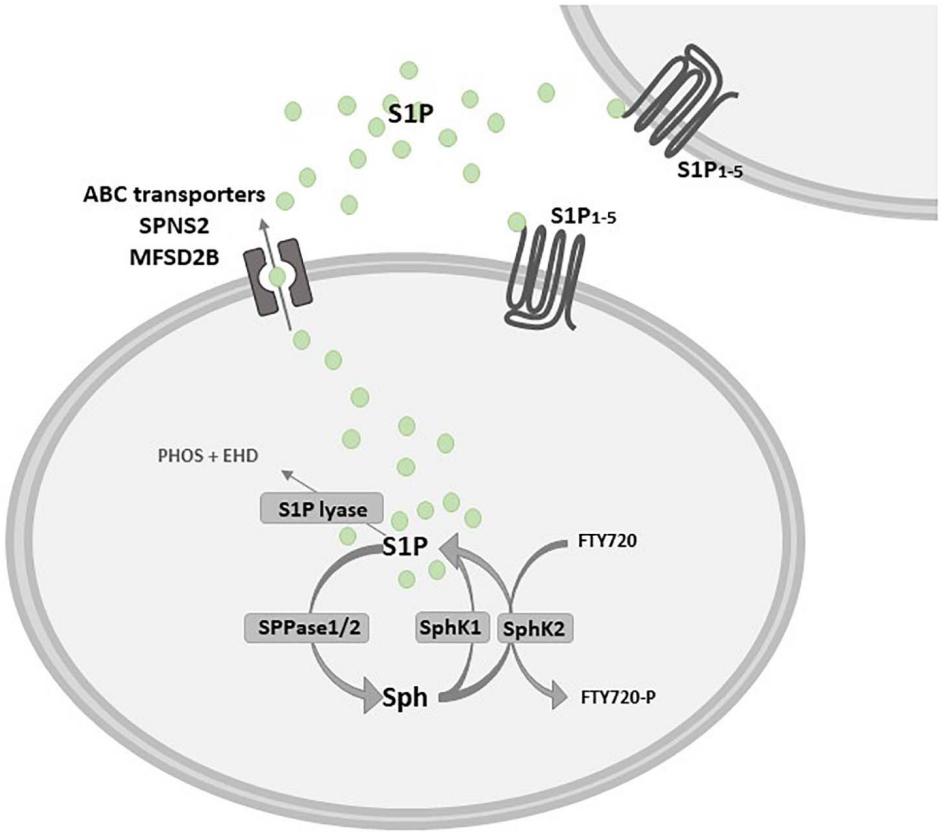

FIGURE 2 | Schematic diagram describing the inside-out signaling of S1P. Sphingosine (Sph) kinase 1 and/or 2 (SphK1/SphK2) activation leads to sphingosine 1-phosphate (S1P) production that can act as an intracellular mediator or is extracellularly released to bind specific S1P receptors ($S1P_{1-5}$). S1P release in the extracellular milieu depends on specific and/or unspecific transporters, such as the S1P transporter spinster homolog 2 (SPNS2) and the major facilitator superfamily transporter 2b (MFSD2B), as well as some ATP-binding cassette (ABC) family members, that are critically implicated in the so called S1P "inside-out signaling". S1P is dephosphorylated by sphingosine 1-phosphate phosphatase 1 or 2 (SPPase1/2) or it is irreversibly degraded by S1P lyase in phosphoethanolamine (PHOS) and hexadecanal (EHD). Fingolimod (FTY720) is a structural analog of Sph that is rapidly phosphorylated by SphK2 in FTY720-P.

Intriguingly, SphK2 displays paradoxical effect on cell survival. Indeed, SphK2 shows a BH3 putative pro-apoptotic domain, although apoptosis is induced via SphK2-Bcl-xL-interaction after overexpression of the kinase (Liu et al., 2003). Moreover, knockdown of SphK2 also affects apoptosis induced by transforming growth factor beta (TGFβ) in C2C12 myoblasts (Cencetti et al., 2013). Alternately, SphK2 is significantly elevated in a broad range of human cancers, including bladder, melanoma, breast, neuroblastoma and leukemia (Neubauer et al., 2016; Bruno et al., 2020); in agreement to SphK2 expression in cancer, SphK2 down-regulation has been demonstrated to decrease the proliferation of cancer cells (Van Brocklyn et al., 2005; Hait et al., 2005), and SphK2-deficient xenografts show a significantly delayed growth (Weigert et al., 2009), pointing at a crucial role of the kinase in carcinogenesis.

Concerning SphK1, its up-regulation is tumorigenic whereas down-regulation results in anti-cancer effects (Sarkar et al., 2005; Taha et al., 2005). The localization of SphK1 is mainly in the cytosol, but upon activation by several stimuli the kinase is recruited at the plasma membrane (Johnson et al., 2002). Moreover, although both SphKs can phosphorylate the sphingosine analog, immunomodulatory drug FTY720 (Fingolimod), SphK2 appears to be more efficient in this activity than SphK1 (Billich et al., 2003; Paugh et al., 2003). Indeed, in mice lacking SphK2, but not SphK1, lymphopenia and lymphocyte retention into lymphoid organs induced by FTY720 are completely lost (Allende et al., 2004; Kharel et al., 2005).

The levels of S1P are tightly regulated by modulation of both anabolic and catabolic pathways. Breakdown of S1P to sphingosine is reversibly triggered by S1P phosphatases (SPP1 and 2), as well as unspecific lipid phosphate phosphatase (LPP), otherwise S1P is irreversibly degraded to hexadecenal and phosphoethanolamine by S1P lyase (Hannun and Obeid, 2008; **Figure 2**). *De novo* sphingolipids pathway alternatively accounts for the production of ceramide, which is the main hub of the sphingolipid metabolism (Hannun and Obeid, 2008): this anabolic pathway is initiated by serine palmitoyl transferase that catalyzes the condensation of serine with palmitoyl-CoA to produce 3-keto-dihydrosphingosine, which is consequently converted into dihydrosphingosine (Hanada, 2003). Ceramide synthases catalyze the acylation of dihydrosphingosine in dihydroceramide and finally dihydroceramide desaturase is

involved in the final step of the *de novo* synthesis by producing ceramide; besides representing a substrate for sphingomyelin synthase giving rise to sphingomyelin and for other complex sphingolipid biosynthetic pathways, also *de novo* synthesized ceramide can fuel the activity of ceramidases and then S1P production. Ceramide is then transported to the Golgi complex, where it serves as substrate for production of complex sphingolipids (Gault et al., 2010).

Sphingosine 1-Phosphate Signaling

Extracellular stimuli including growth factors and cytokines such as epidermal growth factor (EGF), PDGF, vascular endothelial growth factor (VEGF), insulin-like growth factor- 1 (IGF-1), TNFα and TGFβ can induce the activation of SphK1 that translocates from cytosol to the plasma membrane to produce S1P (Donati et al., 2007; Bernacchioni et al., 2012; Bernacchioni et al., 2021). The bioactive sphingolipid can act in an autocrine or paracrine manner after its release in the extracellular milieu (called "inside-out signaling"), depending on specific and/or unspecific transporters, such as the S1P transporter spinster homolog 2 (SPNS2) (Hisano et al., 2012; Spiegel et al., 2019), the major facilitator superfamily transporter 2b (MFSD2B) (Kobayashi et al., 2018) and some, unspecific, ATP-binding cassette (ABC) transporters (Mitra et al., 2006; **Figure 2**).

The bioactive S1P selectively binds to high affinity cell surface G protein-coupled receptors (GPCRs; S1PRs), named $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ (Ishii et al., 2004) that have been involved in the majority of physiological and pathological actions evoked by S1P such as immune response, cardiovascular functions, cancer, atherosclerosis (Cartier and Hla, 2019) as well as MS (Strub et al., 2010; Maceyka et al., 2012). While $S1P_1$, $S1P_2$ and $S1P_3$ show broad tissue expression, $S1P_4$ displays quite selective localization in immune system and $S1P_5$ is primarily expressed in the spleen, on natural killer cells and other lymphocytes, and in CNS, mainly in oligodendrocytes (Mutoh et al., 2012). S1PRs exhibit a distinct capacity to couple to different G proteins thus activating different patterns of intracellular signaling cascades (**Figure 3**). $S1P_1$ exclusively activates members of the G_i family, whereas $S1P_2$ and $S1P_3$ have a broader coupling profile and not only activate G_i but also G_q and $G_{12/13}$. Moreover, activation of Rho, Rac and other small GTPases induces downstream signaling pathways including MAPK, phosphoinositide 3-kinase/Akt, Rho-associated protein kinase (ROCK). Further downstream effectors of S1PRs include AC, PLC, PKC and intracellular Ca^{2+} transients (Windh et al., 1999; Maceyka et al., 2012; **Figure 3**). In addition, several lines of evidence show that S1P can also act as an intracellular messenger (Kohno et al., 2006; Olivera and Spiegel, 2001; Strub et al., 2010; Maceyka et al., 2012) regulating fundamental biological processes, such as gene expression, mitochondrial functions and inflammation by interacting with intracellular targets, including HDACs, E3 ubiquitin ligases and prohibitin 2. Indeed, it has been shown that SphK2 is associated with histone H3, and regulates histone tail acetylation via S1P-medated inhibition of HDAC, thus inducing chromatin remodeling and gene transcription (Hait et al., 2009). Interestingly, TNFα-induced activation of NF-κB requires SphK1 activity and S1P production. In particular, pro-survival TNF signaling requires TRADD-mediated recruitment of TNF receptor-associated factor 2 (TRAF2), which is the prototypical member of E3 ubiquitin ligase (Alvarez et al., 2010). Recruitment of TRAF2 to the TNFα-induced signaling complex results in the polyubiquitination of receptor interacting protein 1 (RIP1) preventing procaspase 8 cleavage that leads to apoptosis. Instead, polyubiquitinated RIP1 is capable of binding and activating IκB kinase (IKK), that phosphorylates the inhibitor of NF-κB (IκB), thus releasing the NF-κB dimer that translocates to the nucleus to exert transcriptional control. The RING domain of E3 ubiquitin ligase TRAF2 binds and activates SphK1, responsible for increased S1P levels essential for NF-κB-dependent p21/cip1 and c-fos gene expression that counteract caspase-8 activation and apoptosis induction. This peculiar molecular mechanism that accounts for S1P pro-survival effects relies exclusively on intracellular effects, since agonists of S1PR (such as dihydro S1P) unable to interact with TRAF2 do not possess anti-apoptotic effects. Moreover, S1P is able to specifically bind to prohibitin 2 (PHB2), regulating mitochondrial respiratory chain assembly. PHB2 is a highly conserved, ubiquitously expressed protein that forms large complexes in the inner mitochondrial membrane composed of heterodimers of PHB2/PHB1. SphK2 located in the mitochondria produces S1P that interacts with PHB2 and affects the assembly of complex IV (COX) respiratory chain activity and oxygen consumption (Strub et al., 2010). Finally, it was reported that both pharmacological inhibition/knockdown of SphK2, and overexpression of S1P lyase/SPP1 in neuroblastoma cells abrogate BACE1-mediated Aβ production (Takasugi et al., 2011) whereas exogenous S1P failed to increase amyloid-beta (Aβ) production. On the other hand, SphK2 overexpression increased the amount of secreted Aβ by producing S1P that interacts with BACE1, suggesting that SphK2-generated S1P regulates Aβ production via BACE1 activation.

Nevertheless, it has been extensively demonstrated that S1PRs have a major role in S1P-dependent biological effects. Importantly depending on the expressed profile of S1PRs, S1P appears capable of differently affecting key cellular events such as proliferation, survival, motility and differentiation in different cell types (Chun et al., 2000; Spiegel and Milstien, 2003; Blaho and Hla, 2011; Soliven et al., 2011). Indeed, S1PR have a wide variety of biological effects in multiple organs and tissues, such as immune, cardiovascular and respiratory systems, as well as in the CNS. Experimental evidence has further highlighted S1P receptors as a potential targets for the regulation of vascular permeability and neuroprotection in different conditions such as pain, stroke and demyelinating diseases (Fyrst and Saba, 2010).

Multiple Sclerosis and Fingolimod

Myriocin-derivative FTY720 (fingolimod), the active substance of Gilenya®, has been authorized by Food and Drug Administration (FDA) and European Medicines Agency (EMA) as the first oral treatment for relapsing-remitting MS, based on extensive clinical trials (Kappos et al., 2010; Cohen et al., 2010). FTY720 is a sphingosine analog prodrug phosphorylated by SphK2 isozyme to produce the active form FTY720-phosphate (FTY720-P) (Paugh et al., 2003; Billich et al.,

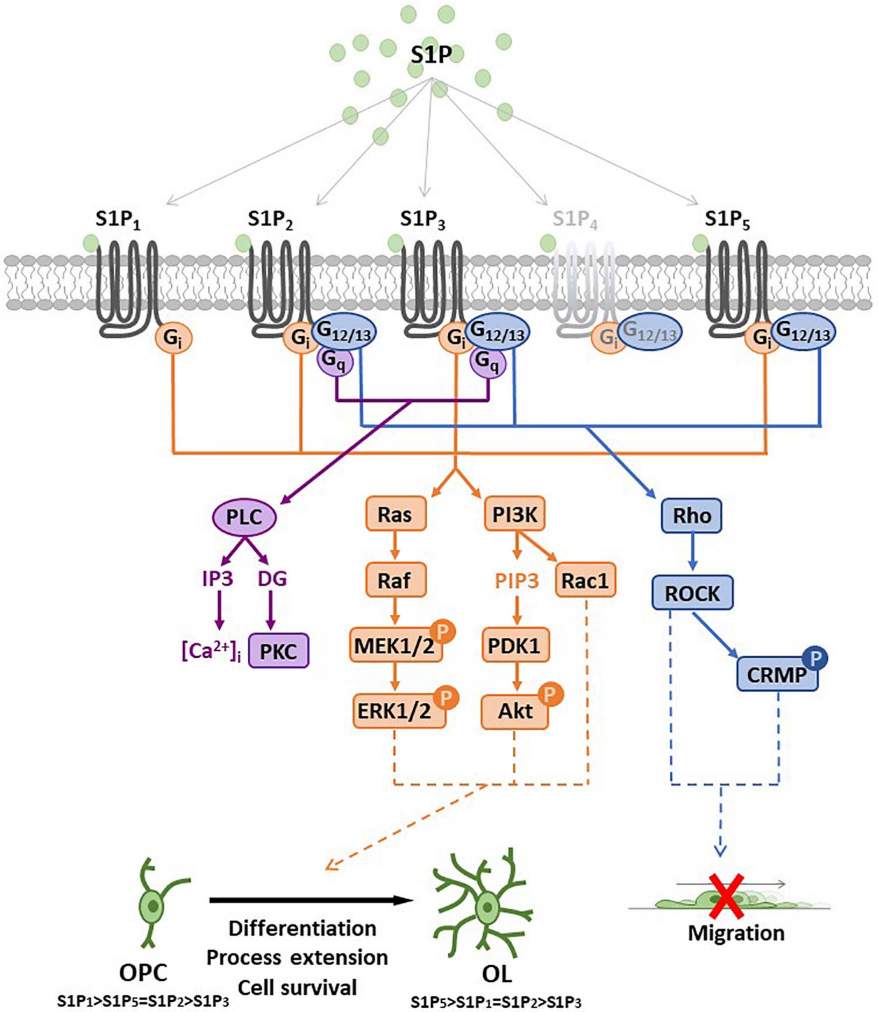

FIGURE 3 | Sphingosine 1-phosphate receptor (S1PR) expression and main signal transduction pathways activated in oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs). Schematic representation of sphingosine 1-phosphate (S1P) G-protein coupled receptors ($S1P_{1-5}$) and main signaling pathways involved after receptor activation. All $S1P_{1-5}$ are coupled to G_i protein that activates Ras/PI3K pathways (in orange) promoting oligodendrocyte differentiation, process extension or cell survival. $S1P_{2/5}$ are also coupled to $G_{12/13}$ protein that activates Rho pathway (in blue) and reduces OPC migration or induces process retraction. $S1P_{2/3}$ are coupled also with G_q protein that activates PLC leading to intracellular calcium increase and PKC activation. Abbreviation: mitogen-activated protein kinase (MEK); extracellular signal-regulated kinases (ERK); phosphatidylinositol 3-kinase (PI3K); phosphatidylinositol (3,4,5)-trisphosphate (PIP3); 3-phosphoinositide dependent protein kinase-1 (PDK1); protein kinase B (Akt); phospholipase C (PLC); protein kinase C (PKC); Rho-associated protein kinase 1 (ROCK); collapsin response mediator protein (CRMP); inositol triphosphate (IP3); diacylglycerol (DG).

2003; Zemann et al., 2006; **Figure 2**), that in turn activates four out of five S1PR receptor subtypes, except $S1P_2$, in the range of sub-nanomolar concentrations (Brinkmann and Lynch, 2002; Mandala et al., 2002). As initially reported, FTY720 was found to act as immunomodulator capable of depleting mature T cells in allograft models (Chiba et al., 2005). Although early studies addressed FTY720 as a low-efficacy suppressor of transplantation rejection (Budde et al., 2006), its successful employment in EAE supported the therapeutic action for MS treatment (Brinkmann and Lynch, 2002; Fujino et al., 2003; Webb et al., 2004; Kataoka et al., 2005; Papadopoulos et al., 2010).

The main recognized mechanism by which FTY720 improves MS disease is by affecting immune responses, specifically regulating lymphocyte trafficking. Circulating T lymphocytes express $S1P_1$ and lower levels of $S1P_{3/4}$ (Graeler and Goetzl, 2002) receptors, and the interaction of exogenous S1P with $S1P_1$ is capable of initiating lymphocyte egress from lymph nodes by overcoming retention signals (Brinkmann et al., 2010). Although acute administration of FTY720-P activates $S1P_1$ (Camm et al., 2014), chronic exposure to the S1P analogue leads to irreversible receptor internalization resulting in 'functional antagonism' of $S1P_1$ signaling (Graler and Goetzl, 2004; Brinkmann, 2009; Gergely et al., 2012). FTY720 has a selective mechanism of action, targeting specific subclasses of lymphocytes: FTY720 treatment negatively modulates $S1P_1$, thus causing a retention of circulating pathogenic lymphocytes (naive and central memory T

cells positive for the chemokine receptor 7, the CCR7) back into the lymph nodes (Matloubian et al., 2004), thereby preventing their infiltration into the CNS, where they exert pathological effects (Cohen and Chun, 2011). However, FTY720 does not significantly affect activation or proliferation of redistributed naïve and central memory T cells and does not block the egress of CCR7-negative effector memory T cells from lymph nodes, preserving immunosurveillance (Mehling et al., 2008).

Intriguingly, differently from FTY720, apoM-S1P is unessential for lymphocyte trafficking although limits lymphopoiesis by activating the $S1P_1$ receptor on lymphocyte progenitors. Indeed, the effect exerted by apoM-bound S1P has been investigated in EAE mice lacking apoM, who developed more severe disease with increased lymphocytes in the CNS and breakdown of the BBB. Moreover, apoM-bound S1P, but not albumin-, inhibits lymphopoiesis *in vitro* and overexpression of apoM in rodents decreases endothelial inflammation and EAE manifestation exerting a protective function against autoimmune inflammation (Blaho et al., 2015; Wang et al., 2019).

In addition to immunological actions, FTY720 can penetrate the BBB acting on different S1P receptors that are expressed in brain resident cells, such as astrocytes and oligodendrocytes, claiming the possibility that FTY720 may elicit direct actions on CNS. Indeed, after the treatment with the pro-drug FTY720, FTY720-P has been detected in the cerebrospinal fluid at sub-nanomolar levels (Foster et al., 2007), which are sufficient to modulate human CNS cell properties *in vitro* (Miron et al., 2008c). This approach is consistent with multiple actions of the lysosphingolipid in the CNS, in line with growing literature describing direct effects of S1P signaling on brain cells. Using S1P receptor specific agonists and antagonists, $S1P_3$ and $S1P_5$ were involved in FTY720-induced effects for the remyelination and astrogliosis, whereas $S1P_1$ and $S1P_5$ affected microgliosis (Miron et al., 2010). Therapeutic interventions that affect oligodendrocyte remyelination processes could be critical factors for long-term functional recovery in MS. In particular, in organotypic cerebellar slices (Miron et al., 2010), the S1P analog was found to enhance remyelination which occurs subsequently to a demyelinating insult, this event being mediated at least in part by OPC differentiating into myelinating OLs.

Although FTY720 treatment has no positive impact on myelin content under basal conditions, FTY720 administration increases both remyelination score subsequent to lysolecithin-induced damage, and the number of endogenous OPCs within the lesion by facilitating migration, recruitment and proliferation of these cells. Furthermore, it enhances OPC process extension and differentiation into mature OLs (Yazdi et al., 2015), as well as amplifies the number of axons with remyelinated myelin sheaths. Despite these findings, in cuprizone model of MS, FTY720 treatment do not enhance remyelination while decreasing severity of demyelination and promoting OPC proliferation (Kim et al., 2011; Alme et al., 2015). However, in this MS model, fingolimod induces higher number of mature OLs near demyelinated areas, indicating a potential effect on differentiation and/or migration without apparent effects on remyelination (Hu et al., 2011; Alme et al., 2015; Nystad et al., 2020). Nevertheless, another research group reported that fingolimod treatment (0.3 mg/kg) causes remyelination in acute cuprizone-induced demyelination model (Slowik et al., 2015). Hypothesis on such inconsistencies can be made regarding the concentration of fingolimod as well as time-dependence that may cause different effects in cuprizone model. Fingolimod (1 mg/kg) treatment after 3 days from cuprizone administration induces survival of mature OLs whereas later administration of the drug (started at 10 days after cuprizone treatment) lacks cytoprotective effect (Kim et al., 2018), claiming the possibility that early intervention appears to be required to prevent demyelination. In cuprizone model of demyelination, FTY720 (0.3 mg/kg), co-administered with transplanted neural progenitor cells derived from induced pluripotent cells (iPS-NPC), increases OPC survival and proliferation. Moreover, the authors assess that differentiation of transplanted iPS-NPC into oligodendroglial lineage may occur (Yazdi et al., 2015).

Treatment with FTY720 at the onset of EAE reduces clinical symptoms and decreases demyelination by blocking the Akt/mTOR signaling pathway (Hou et al., 2016). In later stage of EAE disease, FTY720 treatment increases MBP level and promotes the appearance of newly generated myelinating OLs, via Sonic Hedgehog signaling pathway, decreases EAE clinical manifestation, and improves neurological functions (Zhang et al., 2015). Furthermore, FTY720 administration (1 mg/kg) during EAE induction in female mice reduces demyelinated area, axonal damage, brain atrophy while increasing brain-derived neurotrophic factor (BDNF) level and clinical scores (Fukumoto et al., 2014; Smith et al., 2018), underling the positive effect of FTY720 on regeneration in different types of demyelination models.

Finally, conditional knockout of $S1P_1$ in neural lineages have been used to identify a key role for astrocytes in reducing the severity of pathological changes in EAE. Indeed, the efficacy of fingolimod is lost by astrocytic deletion of $S1P_1$, highlighting that the main protective effect of this compound in EAE comprises the modulation of astrocyte function by $S1P_1$ (Choi et al., 2011).

Sphingosine 1-Phosphate Receptor Signaling in Oligodendrocytes and Interaction With A_{2B}Rs

OPC availability, recruitment and differentiation into mature oligodendrocytes are pivotal aspects involved in remyelination. Experimental evidence demonstrates that S1PRs display different biological effects depending on oligodendrocyte differentiation state. Oligodendroglial cells express four out of five S1PRs, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$, whose expression profiles change and are selectively controlled during oligodendrogenesis. Indeed, the predominant isoform in immature OPC is $S1P_1$ whereas $S1P_2$, $S1P_3$ and $S1P_5$ are present at much lower levels (Im et al., 2000; Yu N. C. et al., 2004; Jaillard et al., 2005; Novgorodov et al., 2007). During differentiation from OPC into mature OLs, $S1P_5$ and $S1P_1$ are reciprocally regulated, the first being augmented while the latter decreases (Jung et al., 2007; Coelho et al., 2007; Miron et al., 2008c; van Doom et al., 2012). Indeed, human mature OLs express S1PR transcripts in relative abundance of $S1P_5 > S1P_3 > S1P_1$ (Miron et al., 2008a). In particular, the

modulation of S1PR expression seems to be mediated by PDGF that is produced by OPCs. Indeed, S1PRs are differentially modulated by PDGF resulting in downregulation of $S1P_5$ and upregulation of $S1P_1$ in OPCs. Downregulation of $S1P_1$ by RNA interference affects PDGF-induced proliferation of OPCs (Jung et al., 2007) whereas, in mature OLs, S1P signaling promotes cell survival, suggesting that S1PRs may exert different functions during oligodendroglial development (Okada et al., 2009). The effects of fingolimod on oligodendrocyte process dynamics depended on both dose and duration of treatment, since S1PR activation by fingolimod induces process retraction in O4-positive pre-OLs, that appears to be transient and restricted to immature cells, not observed at later and mature developmental stages (Miron et al., 2008b). Process retraction is mediated by $S1P_5$ *via* a ROCK/collapsin response-mediated signaling pathway, whereas the effect is abrogated in $S1P_5^{-/-}$ mice derived oligodendrocytes. Prolonged treatment with higher doses of fingolimod induces process extension associated with Rac1-linked cytoskeletal signaling cascades mimicked by a $S1P_1$ agonist SEW2871 (Miron et al., 2008b). Similarly, these results can be corroborated by the fact that, depending on the dose, FTY720-P exerts opposite actions in rat OPC cultures (Jung et al., 2007). Indeed, high concentration of FTY720-P (1 μM) inhibits, OPC differentiation, whereas low concentration (10 nM) enhances both the percentage of mature OLs and MBP expression.

Furthermore, S1P-induced survival of mature OLs is mediated through a pertussis toxin- (PTx) sensitive, Akt-mediated pathway, since the molecular mechanism activated by S1PRs in oligodendrocytes specifically involves the phosphorylation of ERK1/2 and Akt, that subsequently promotes cell survival (Jaillard et al., 2005; van Doom et al., 2012). Noteworthy, the pro-survival effect of S1PR in human mature OLs is mimicked by the administration of $S1P_5$ agonist. Interestingly, S1PR agonists affect oligodendrocytes differentiation stages depending on the molecular mechanisms evoked and in a concentration-dependent manner. In particular, human OPC cultures treated with low nanomolar concentrations of either FTY720-P or S1P present enhanced differentiation into both pre-OLs (O4-positive) and mature (GC-positive) OLs. Of note, ERK1/2 pathway is involved in the differentiation of OPC into O4 positive-cells, since inhibition of MAPK pathway by U0126 prevented pre-OLs generation. However, the transition to mature OLs is mediated by p38MAPK signaling, since PD169316 administration blocks the progression of O4 positive into mature stages of differentiation (Cui et al., 2014).

Notably, experimental evidences highlight that activation of $S1P_5$ induces process retraction whereas activation of $S1P_1$ enhances process extension of OPCs (Miron et al., 2008b), and coherently during early myelination $S1P_1$ abrogation in the oligodendroglial cells delays OPC differentiation into mature OLs (Kim et al., 2011). In agreement, $S1P1^{-/-}$ mice show decreased myelin protein expression, thinner myelin and susceptibility to demyelination induced by cuprizone, that is conceivable with the major expression of $S1P_1$ in OPCs that could play a pivotal role in early OPC differentiation stages. On the contrary, abnormalities in myelination are not evident in $S1P_5^{-/-}$ mice; oligodendrocytes display a higher expression of $S1P_5$ compared to other S1PRs where it may be involved in late myelination processes. Although $S1P_5$ is expressed at lower levels in OPCs compared to OLs and OPCs are devoid of pro-survival effects of $S1P_5$, this receptor subtype is activated by S1P to arrest OPC migration (Novgorodov et al., 2007). Indeed, S1P-induced decrease of chemiotaxis is completely prevented by the specific downregulation of $S1P_5$, but not $S1P_2$, and is insensitive to PTx, suggesting that $S1P_5$-initiated signaling is not mediated by the $G\alpha_i$-protein coupled pathway. The molecular mechanism that is responsible for the impairment of migratory capacity after S1P administration involves $G_{12/13}$ that stimulates the Rho/ROCK signaling pathway (Novgorodov et al., 2007). The authors suggest that glutamate treatment of OPC may increase S1P export and S1P extracellular levels to modulate OPC motility and claim the possibility for S1P to be a part of the neuron-oligodendroglial communication network in developing brain, that could have a role also during remyelination processes. These data are also consistent with the physiological effect of $S1P_5$ in OPCs during differentiation postulating that changes in receptor coupling with heterotrimeric G-proteins may occur, thus leading to the activation of different signaling pathways.

Finally, SphK1 has a protective role against apoptosis in OPC cultures (Saini et al., 2005). In particular, Neurotrophin 3 (NT-3) accounts for SphK1 translocation to plasma membrane and activation to exert its pro-survival effect in these cells. Remarkably, down-regulation of SphK1 negatively affects the capacity of NT-3 to protect oligodendrocyte progenitors from apoptosis. In agreement with these findings, analysis of plaques from MS brains shows reduced levels of S1P and increased sphingosine and C16/18-ceramide (Qin et al., 2010).

In the light of above results, we recently performed a study aimed to merge current knowledge on S1P pathway with A_{2B}R signaling. Namely, by using either or both pharmacological approach and receptor silencing, we demonstrated that an interplay occurs between A_{2B}Rs and SphK/S1P axis in OPCs (**Figure 4**). Indeed, SphK1 enzyme is activated when OPCs are cultured in the presence of the A_{2B}R selective agonist BAY60-6583, thus rising S1P production, whereas its silencing by small interference RNA (siRNA) increases the expression of S1P lyase, promoting S1P removal (Coppi et al., 2020a). This observation led to hypothesize that the anti-differentiating effect exerted by A_{2B}R activation in OPCs is mediated by an increase in S1P intracellular levels (**Figure 4**). This hypothesis was confirmed by findings that the SphK inhibitors VPC96047 or VPC96091 markedly increased MAG and MBP expression in OPC cultures, indicating enhanced cell maturation, and also significantly increased I_K currents, necessary to OPC differentiation (Coppi et al., 2020a). Thus, it appears that an increase in S1P production possibly accounts for the anti-differentiating effect of BAY60-6583 in OPCs, whereas A_{2B}R silencing, by promoting S1P removal through the activation of S1P lyase, facilitates OPC maturation. An additional proof that A_{2B}Rs and S1P signaling are interconnected in OPCs resides in the fact that this receptor subtype is upregulated during cell differentiation, an effect that is completely prevented when cells are differentiated in the presence of the pan SphK inhibitor VPC96047 (Coppi et al., 2020a).

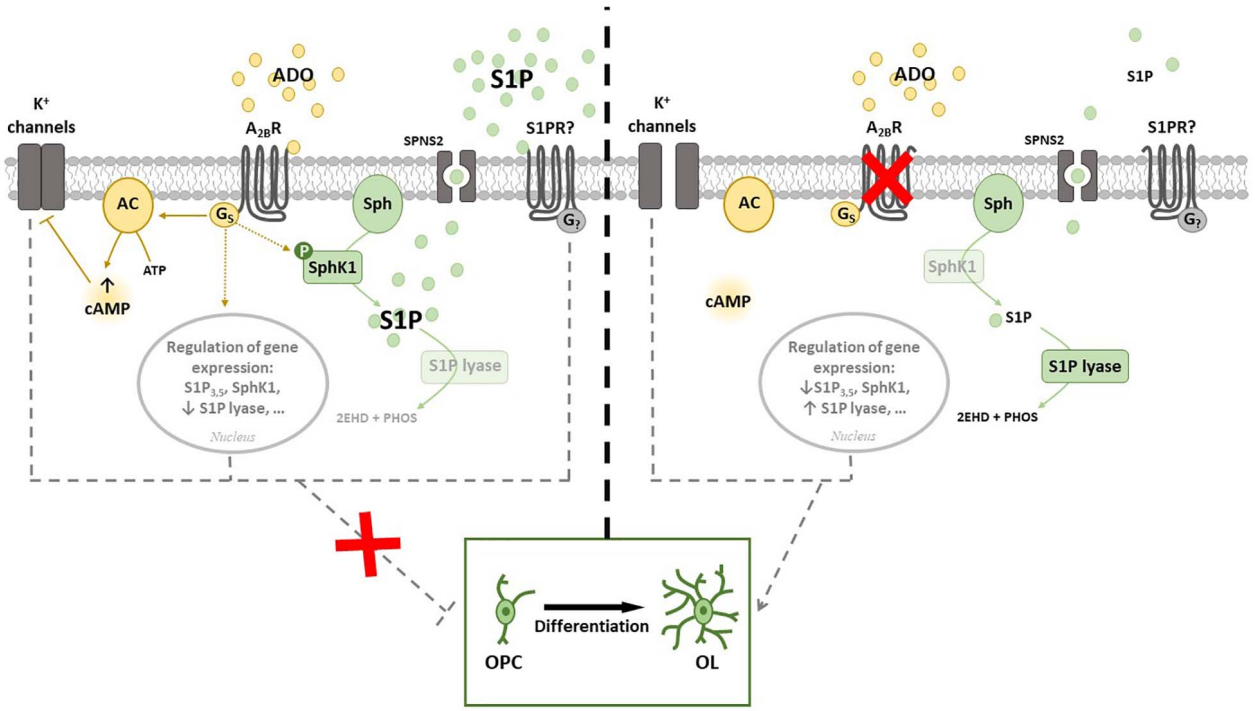

FIGURE 4 | Cross-talk between adenosine A_{2B} receptor (A_{2B}R) and Sphingosine 1-phosphate (S1P). **(Left panel)** A_{2B}R throughout G_s activation leads to an increase of intracellular cAMP that inhibits K^+ channels. The activation of A_{2B}R regulates gene expression of proteins involved in S1P signaling and metabolism and increases the phosphorylation of SphK1. All these events prevent OPC differentiation *in vitro*. **(Right panel)** A_{2B}R silencing allows K^+ channels to be opened and strikingly increases expression of S1P lyase, that irreversible catalyzes S1P and blocks S1P degradation signaling. In this conditions OPC differentiation is facilitated. Abbreviations: Adenosine (ADO); Sphingosine (Sph) kinase 1 (SphK1); specific spinster homolog 2 (SPNS2); adenylyl cyclase (AC) (Coppi et al., 2020a,b).

We also found that FTY720-P differently affects BAY60-6583-mediated K^+ current inhibition depending upon the concentration applied. When administered at 1 μM, it mimicked and partially occluded the effect of a subsequent BAY60-6583 application on voltage-dependent K^+ currents. This confirms, again, that S1P and A_{2B}R pathways converge. On the other hand, the effect of BAY60-6583 on ramp currents was significantly enhanced in the presence of low (10 nM) FTY720-P (Coppi et al., 2020a). Similarly, 10 nM FTY720-P increased, whereas 1 μM decreased, MAG expression after 7 days of OPC maturation. Contrasting effects of this compound depending on the concentration used have been previously reported by others, the effects of fingolimod depending on both concentration and treatment duration (Jung et al., 2007; Miron et al., 2008c).

Differently from FTY720-P, when S1P is used as a ligand, the effect on K^+ currents was not observed (Coppi et al., 2020a). This apparent discrepancy may be ascribed to the fact that receptor ligation by FTY720-P is restricted to all S1P receptors except $S1P_2$, which however is activated by S1P. Moreover, the functional outcome induced by S1PR modulators could differ from one ligand to another since it could differently affect receptor fate. Indeed, it has been shown that FTY720-P can induce $S1P_1$ receptor degradation, whereas S1P affects receptor recycling. Finally the lack of effect exerted by exogenous S1P on I_K is in agreement with previous data in different cellular models, such as skeletal muscle cells, where agonist-induced S1P intracellular production and activity, called inside-out signaling, has different, even sometimes opposite, actions compared to exogenous S1P (Donati et al., 2004; Cencetti et al., 2010; Cencetti et al., 2013). This effect can be explained by a localized release of bioactive lipid in membrane microdomain where the availability of certain receptor subtypes is limited. The spatial regulation of S1P biosynthesis within the cell, together with its localized partitioning into plasma membrane domains, determines the subset of engaged S1PRs and thus the biological outcome (Donati et al., 2013).

CONCLUSION

Multiple evidences indicate that adenosine may interact with S1P signaling in orchestrating the processes concurring to OPC maturation and thus to the remodeling of brain development and/or repair after a demyelinating insult.

In particular, adenosine A_{2B}R appears to play a critical role in oligodendrogliogenesis since its agonism activates SphK1 and reduces OPC differentiation. These data are in agreement with the fact that, on one hand, SphK inhibition decreases A_{2B}R expression leading to an increase in OPC differentiation, and on the other, specific down-regulation of A_{2B}R reduces SphK1 and potently induces S1P lyase expression thus pushing S1P toward catabolism, and thereby facilitating OPC

maturation. On the whole, the available results suggest that A_{2B}R antagonism represents a possible co-adjuvant strategy to improve remyelination promoted by the prodrug sphingosine analog, FTY720 (Fingolimod) for the treatment of MS, as confirmed by previous findings in the literature reporting a protective role of A_{2B}R block in EAE models (Wei et al., 2013; Liu et al., 2020).

AUTHOR CONTRIBUTIONS

EC, FP, PB, AP, and FCe: conceptualization. FP and PB validation. EC, AP, PB, FP, FCe, and CD: resources. EC, FCe, FCh, and AP: writing–original draft preparation. MV, FCh, FCe, and CD writing–review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Purinergic Signaling in the Pathophysiology and Treatment of Huntington's Disease

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Huntington's disease (HD) is a devastating, progressive, and fatal neurodegenerative disorder inherited in an autosomal dominant manner. This condition is characterized by motor dysfunction (chorea in the early stage, followed by bradykinesia, dystonia, and motor incoordination in the late stage), psychiatric disturbance, and cognitive decline. The neuropathological hallmark of HD is the pronounced neuronal loss in the striatum (caudate nucleus and putamen). The striatum is related to the movement control, flexibility, motivation, and learning and the purinergic signaling has an important role in the control of these events. Purinergic signaling involves the actions of purine nucleotides and nucleosides through the activation of P2 and P1 receptors, respectively. Extracellular nucleotide and nucleoside-metabolizing enzymes control the levels of these messengers, modulating the purinergic signaling. The striatum has a high expression of adenosine A_{2A} receptors, which are involved in the neurodegeneration observed in HD. The P2X7 and P2Y2 receptors may also play a role in the pathophysiology of HD. Interestingly, nucleotide and nucleoside levels may be altered in HD animal models and humans with HD. This review presents several studies describing the relationship between purinergic signaling and HD, as well as the use of purinoceptors as pharmacological targets and biomarkers for this neurodegenerative disorder.

Keywords: Huntington's disease, motor dysfunction, A_2A receptors, adenosine, ATP, nucleotide metabolism

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INTRODUCTION

Huntington's disease (HD) is a devastating, progressive, and fatal neurodegenerative disorder inherited in an autosomal dominant manner (Smith-Dijak et al., 2019; Blumenstock and Dudanova, 2020). It is triggered by an expansion of a cytosine-adenine-guanine (CAG) triplet repeat in exon 1 of the huntingtin (*HTT*) gene, located on chromosome 4 (The Huntington's Disease Collaborative Research Group, 1993; Capiluppi et al., 2020). This change leads to an expanded polyglutamine (polyQ) region in the encoded HTT protein (Bailus et al., 2017; Rai et al., 2019). As a result, the expressed HTT protein is a mutant (mHTT; Cybulska et al., 2020). Individuals with up to 35 CAG repeats are usually considered healthy, while people with 36 to 39 CAG repeats may or may not develop the signs and symptoms of HD (Shoulson and Young, 2011; Capiluppi et al., 2020). More than 50 CAG repeats always cause the disease (Capiluppi et al., 2020). There is an inverse correlation

between the number of CAG repeats, age at onset, and the severity of HD symptoms (Bates et al., 2015; Petersén and Weydt, 2019).

It is estimated that the mean HD prevalence is 5 in 100,000 people (Baig et al., 2016; Illarioshkin et al., 2018). HD is characterized by a neurobehavioral progressive triad with motor dysfunction, psychiatric disturbance, and cognitive decline (Stahl and Feigin, 2020). The motor dysfunction is subdivided into two stages: In the early stage, there are abnormal involuntary movements, known as chorea, while in the late stage, the voluntary movements are impaired, causing bradykinesia, dystonia, and motor incoordination. The observed neuropsychiatric symptoms include depression, apathy, irritability, anxiety, and psychosis. The cognitive impairment often precedes the motor abnormalities. The cognitive alterations include impaired attention and visuospatial functions and slow planning processing speed. The cognitive decline progresses to dementia (Stahl and Feigin, 2020), and death becomes imminent 15–20 years after disease onset (Blumenstock and Dudanova, 2020). These dysfunctions can be attributed to multiple brain regions that exhibit neurodegeneration, including the cerebral cortex, thalamus, subthalamic nucleus, globus pallidus, substantia nigra, and hypothalamus. However, the hallmark of the disease is the pronounced neuronal loss in the striatum (caudate nucleus and putamen; Rubinsztein, 2002; Ramaswamy et al., 2007; Coppen and Roos, 2017). Furthermore, HD patients may develop metabolic symptoms including weight loss and cardiac and musculoskeletal dysfunction, among others (Blum et al., 2018; Croce and Yamamoto, 2019; Dufour and McBride, 2019).

The striatum is a region responsible for the control of many behaviors, such as movement, flexibility behavior, motivation, and learning (Koch and Raymond, 2019). Two different striatal pathways express distinct neurotransmitters and neuropeptides (Graybiel, 2000). The indirect pathway contains cholinergic interneurons that express dopamine D_2 receptors (D_2R), adenosine A_{2A} receptors ($A_{2A}R$), and enkephalin; it projects to the globus pallidus external (**Figure 1**; GPe; Albin et al., 1989). This pathway acts by inhibiting voluntary movements; because the neurons are degenerated in the early stage of HD, there is a decrease in D_2R and $A_{2A}R$ and thus uncontrolled voluntary movements, coinciding with chorea symptoms (**Figure 1**; Albin et al., 1989; Graybiel, 2000; Koch and Raymond, 2019). The direct pathway expresses spiny projection neurons (SPNs) that contain gamma-aminobutyric acid (GABA) coexisting with neuropeptide P and dynorphin. Besides, dopamine D_1 receptors (D_1R) project into the substantia nigra pars reticulate (SNpr) and globus pallidus internal (GPi), initiating voluntary movements (**Figure 1**; Albin et al., 1989). In the late stage of HD, besides the damaged indirect pathway, there is the degeneration of direct pathway neurons, a phenomenon that decreases D_1R and cortex stimulation. This phenomenon leads to the hypokinetic symptoms, which are typical of this stage (**Figure 1**; Albin et al., 1989; Graybiel, 2000; Koch and Raymond, 2019).

Furthermore, neurotransmitters, such as dopamine, acetylcholine, glutamate, and GABA, are involved in motor coordination and alterations in their levels induce motor deficits. Evidence has demonstrated alterations in these neurotransmitter levels in early- and late-stage HD (Spokes, 1980; Kish et al., 1987; Jamwal et al., 2015; Jamwal and Kumar, 2019). These changes in neurotransmitter levels might cause important intracellular biochemical changes, such as a decrease in mitochondrial complex II, III, and IV activity and adenosine triphosphate (ATP) levels, calcium (Ca^{2+}) overload, excitotoxicity, oxidative stress, and mitochondrial dysfunction (Johri et al., 2013; Carmo et al., 2018; Jodeiri Farshbaf and Kiani-Esfahani, 2018), triggering cell death (Liot et al., 2017). Thus, there is an imbalance in the activity between the direct and indirect pathways, resulting in an inadequate functioning of different neurotransmitter systems in HD. One of the neurotransmitter systems involved in the pathophysiology of HD is the purinergic signaling (Burnstock, 2015), mediated by the action of nucleotides and nucleosides in the P2 and P1 receptors, respectively. Both ATP and adenosine are the most important messengers in the purinergic system, which participates in the control of several behaviors (Burnstock, 2015). Adenosine acts as a neuromodulator; specifically, it modulates dopaminergic and glutamatergic neurotransmission systems (Ferré et al., 2007; Fuxe et al., 2007; Ciruela et al., 2015). Changes in ATP and adenosine levels have been observed in HD (Seong et al., 2005; Kao et al., 2017). Studies have focused on the impact of purinergic signaling on HD as well as the development of pharmacological strategies related to the purinergic system as therapies for HD (Blum et al., 2002; Chou et al., 2005; Simonin et al., 2013; Villar-Menéndez et al., 2013; Kao et al., 2017). Therefore, this review will discuss the role of purinergic signaling in HD as well as the involvement of purinoceptors in the disease progression and their relevance for application as pharmacological targets and biomarkers for HD.

PURINERGIC SIGNALING

Adenosine triphosphate and adenosine are recognized as the most powerful purinergic signaling messengers (Burnstock, 1972). Purinergic receptors are classified into P1 and P2 according to their biochemical and pharmacological properties (Burnstock, 2018; Cheffer et al., 2018). P2 receptors are activated by purines [ATP, adenosine diphosphate (ADP)] and pyrimidines (uridine triphosphate, uridine diphosphate) and classified as P2X and P2Y receptors (Abbracchio and Burnstock, 1994; Burnstock, 2011). P2X receptors are ATP-gated ion channels permeable to sodium (Na^+) and Ca^{2+} influx and potassium (K^+) efflux, which leads to depolarization of the cell membrane. Seven subunits of these receptors (P2X1–7) are expressed by different cells (Burnstock, 2008). P2Y receptors are metabotropic, activated by purines and pyrimidines, and subdivided into eight receptor subtypes ($P2Y_1$, $P2Y_2$, P2Y4, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$; Burnstock, 2008; Puchałowicz et al., 2014). P1 receptors are metabotropic, selective for adenosine, and exert physiological actions through four subtypes named A_1 (A_1R), A_{2A} ($A_{2A}R$), A_{2B} ($A_{2B}R$), and A_3 (A_3R) (Fredholm et al., 2001; Burnstock, 2018; Ciruela, 2020). Low adenosine levels activate A_1R and A_3R receptors, whereas high adenosine levels activate $A_{2A}R$

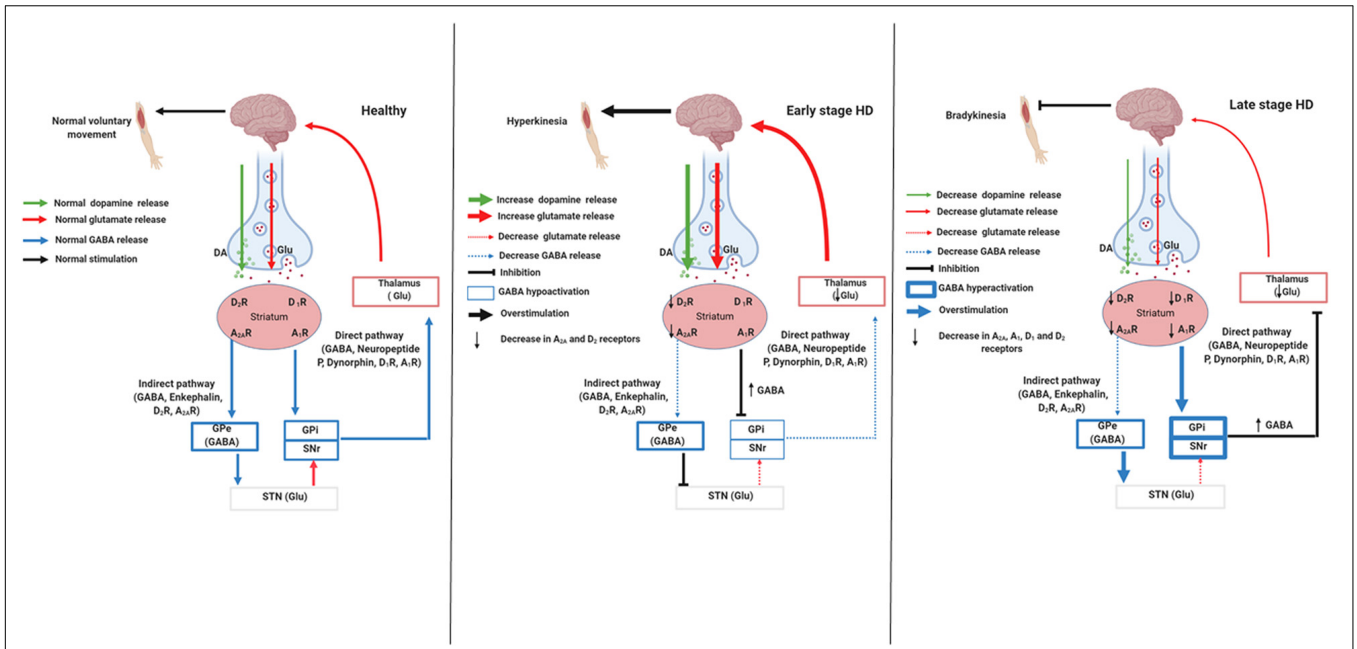

FIGURE 1 | Signaling pathways involved in stages of Huntington's disease. Created with Biorender.com.

and $A_{2B}R$ receptors (Borea et al., 2018). A_1R and A_3R activate $G_{i/o}$ protein, inhibiting the production of cyclic adenosine monophosphate (cAMP), adenylate cyclase (AC), protein kinase A (PKA), and, consequently, GABA uptake. On the other hand, $A_{2A}R$ is activated through Gs protein that stimulates cAMP production, activating AC and PKA, increasing GABA uptake. In addition, $A_{2B}R$ also act through Gs protein (Sheth et al., 2014; Borea et al., 2018).

Nucleotide levels are regulated by ectonucleotidases, a group of enzymes constituted by nucleotide pyrophosphatases/phosphodiesterases (NPPs), nucleoside triphosphate diphosphohydrolases (NTPDases; CD39), alkaline phosphatase, and ecto-5′-nucleotidase (5′-NT, CD73; Bonan, 2012; Zimmermann, 2021). Ectonucleotidases promote the extracellular hydrolysis of ATP, producing ADP, adenosine monophosphate (AMP), and adenosine controlling their extracellular concentrations (Burnstock, 1980; Bonan, 2012; Cheffer et al., 2018; Cieslak et al., 2019).

Adenosine, through the action of adenosine deaminase (ADA), can be subsequently deaminated to inosine (Latini and Pedata, 2001). Inosine is phosphorylated by purine nucleoside phosphorylase (PNP) into hypoxanthine and then degraded to the stable end product uric acid (Yegutkin, 2008; Ribeiro et al., 2016). Adenosine levels are also regulated by unidirectional and bidirectional transporters, which allow nucleosides to move between the intracellular and extracellular compartments (Fredholm et al., 2001; Ribeiro et al., 2016; Stockwell et al., 2017). Finally, the action of nucleosides is then limited either through their conversion to other products of purine catabolism or through re-synthesis into nucleotides (Ribeiro et al., 2016).

THE ROLE OF P1 RECEPTORS IN HUNTINGTON'S DISEASE

As mentioned above, adenosine mediates its effect through four adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3). A_1R and $A_{2A}R$ are mainly involved in the central effects of adenosine (Ciruela et al., 2015). A_1R is the most abundant receptor in the brain–widely expressed in the hippocampus, cerebellum, thalamus, brain stem, and spinal cord–whereas $A_{2A}R$ is concentrated abundantly in the striatum (Burnstock, 2008; Borea et al., 2018).

It is known that A_1R can interact with D_1R to form heterodimers. Pre-synaptic A_1R activation causes depression of excitatory transmission through Ca^{2+} channel inhibition and neuronal hyperpolarization by regulation of potassium channel. This action leads to a reduction in the release of many neurotransmitters, including acetylcholine, glutamate, dopamine, noradrenaline, and serotonin; this reduction may be beneficial in some central nervous system diseases (Yoon and Rothman, 1991; Von Lubitz et al., 1994; Gundlfinger et al., 2007).

A_{2A} receptors can co-localize with D_2R, resulting in $A_{2A}R/D_2R$ heteromers, which have a crucial role in the modulation of motor function. Thus, $A_{2A}R$ activation decreases the affinity and function of D_2R for agonist or antagonist drugs (Borea et al., 2018). In addition, $A_{2A}R$ plays an important role in facilitating glutamate release, potentiating their effects via *N*-methyl-D-aspartate (NMDA) receptors as well as other neurotransmitters, such as GABA, glycine, acetylcholine, noradrenaline, and serotonin (Cunha, 2005; León-Navarro et al., 2018). In addition, there is functional cooperation between $A_{2A}R$ and A_1R (heteromers), leading to antagonist actions on dopamine release–that is, when A_1R is

stimulated it inhibits dopamine release, which would oppose the stimulating effects of $A_{2A}R$ through action on striatal D_2R (León-Navarro et al., 2018). $A_{2A}R$ can also form heterodimers with glutamate receptors (metabotropic 5 subtype [mGlu5]). The $A_{2A}R$/mGlu5 heterodimers exert a synergistic inhibitory effect on dopamine binding to D_2R (León-Navarro et al., 2018). Together, these findings lead to the hypothesis that dysfunction in adenosine receptors may cause an imbalance between dopaminergic, glutamatergic, and GABAergic neurotransmission systems, which would explain pathological processes underlying HD. This hypothesis is supported by several studies that focus on adenosine receptors, mainly $A_{2A}R$, and to a lesser extent A_1R. Here we described the involvement of A_1R and $A_{2A}R$ in the progression of HD.

A_1R in Huntington's Disease

The first study investigating the involvement of P1 receptors in HD was focused on the analysis of the A_1R subtype in the striatum and parietal–frontal culture of post-mortem cerebral samples from HD subjects. Using a selective A_1R agonist–[3N] (N6-cyclohexyl)-adenosine–the authors demonstrated a 60% decrease in A_1R with an increase in the affinity of the binding drug for the receptor in the striatum (Whitehouse et al., 1985). Later, Matusch et al. (2014) quantified, through position emission tomography (PET) imaging, the cerebral binding of A_1R in HD subjects subdivided into four groups: pre-manifest individuals far from (pre-HD-A) or pre-manifest individuals near (pre-HD-B) the predicted symptom onset, manifest HD patients, and controls. The results demonstrated a decrease in caudate and putaminal volumes from pre-HD and HD patients compared with control, more A_1R in the thalamus of pre-HD-A individuals compared with control, and less A_1R in caudate and amygdala in all stages of the disease. There was also a strong direct correlation between A_1R with the years since disease onset–that is, the more advanced the disease, the larger the loss of A_1R (Matusch et al., 2014). This finding indicates that A_1R is involved in the pathogenesis of HD and might be a biomarker in specific brain areas for HD progression.

To shed further light on the possible role of A_1R in HD, pharmacological and genetic mouse models have been used to understand the functionality of these receptors. In a pharmacological model of HD induced by 3-nitropropionic acid (3-NPA)–the main toxin used to induce an HD-like phenotype in an animal model through mitochondrial inhibition–the acute (two or three injections) and chronic (8 days) treatment with A_1R agonist adenosine amine congener (ADAC) provoked different behavioral and neurochemical responses in mice (Blum et al., 2002). In the study, animals that received two injections of ADAC were called $ADAC_{4/5}$, whereas animals that received three injections were called $ADAC_{3/5}$. $ADAC_{4/5}$ and $ADAC_{3/5}$ treatment attenuated dystonia of hindlimbs caused by 3-NPA, while the $ADAC_{4/5}$ group showed increased striatal succinate dehydrogenase (SDH) activity inhibited by 3-NPA and a reduced striatal lesion volume (Blum et al., 2002). Chronic ADAC treatment did not alter the motor symptoms or striatal lesion size, and there was no increase in the SDH activity (Blum et al., 2002). Interestingly, chronic treatment dramatically decreased the A_1R density in the striatum and cortex, whereas acute treatment did not modify the A_1R density (Blum et al., 2002).

Consistently, in R6/2 mice–the most widely used transgenic model of HD–A_1R was significantly reduced in the cortex and striatum compared with the wild type (WT) group (Ferrante et al., 2014). Moreover, corticostriatal slices of R6/2 treated with the selective A_1R agonist cyclopentyladenosine (CPA) showed a marked reduction in synaptic transmission, with consequent inhibition of glutamate release in the pre-synaptic terminal (Ferrante et al., 2014). On the contrary, in a pharmacological model of HD induced by malonate, Alfinito et al. (2003) observed that intraperitoneal and intrastriatal A_1R blockade with the A_1R antagonist CPX potentiated the effect of malonate, reducing striatal dopamine levels, tyrosine hydroxylase (TH) content, and GABA levels as well as GABAergic and dopaminergic neuronal loss (Alfinito et al., 2003). Together, these findings lead to the hypothesis that the pharmacological therapy with A_1R agonists may be a beneficial protective treatment in the early stage of HD (**Figure 2**). However, A_1R antagonist drugs did not seem to be an alternative for the treatment of HD due to the crosstalk with GABAergic neurons, which enhanced the susceptibility to toxic insults, reducing A_1R in these two populations of neurons.

$A_{2A}R$ Alterations in Huntington's Disease Progression

$A_{2A}R$: Focus on Human Studies

Contrary to A_1R, the role of $A_{2A}R$ in HD has been more widely investigated in human and animal models. The first evidence for the involvement of $A_{2A}R$ in HD was provided by autoradiographic mapping using the selective $A_{2A}R$ agonist ligand CGS 21680 in brain sections from HD patients and controls without the pathology. The results showed a dramatic decrease in $A_{2A}R$ in the caudate nucleus, putamen, nucleus accumbens, olfactory tubercle, and globus pallidus lateral in HD compared with control samples (Martinez-Mir et al., 1991). In a subsequent study, analyses of post-mortem neuronal tissue in HD subjects in the early, intermediate, and late stages of the disease showed $A_{2A}R$ bound the selective $A_{2A}R$ agonist ligand CGS 21680 within the caudate nucleus and putamen of the control brain. In the early and intermediate stages of HD, there was a dramatic loss of $A_{2A}R$ binding, whereas in brain tissue with the late stage of the disease there was no detectable $A_{2A}R$ binding (Glass et al., 2000).

On the other hand, higher $A_{2A}R$ levels are detected in peripheral blood cells, such as platelets, lymphocytes, and neutrophils at pre-symptomatic, early, and late stages of HD patients (Varani et al., 2003; Maglione et al., 2005). In addition, the higher $A_{2A}R$ levels in platelets correlated with CAG expansion and with anticipation in years since the onset of symptoms (Maglione et al., 2005, 2006). Thus, these findings indicate that there are $A_{2A}R$ alterations in the peripheral and central nervous systems in HD patients, and $A_{2A}R$ in blood cells might be an easy biomarker to detect and monitor HD progression.

Genetic studies support the relationship between $A_{2A}R$ and HD pathology. Two similar cohort studies performed in France

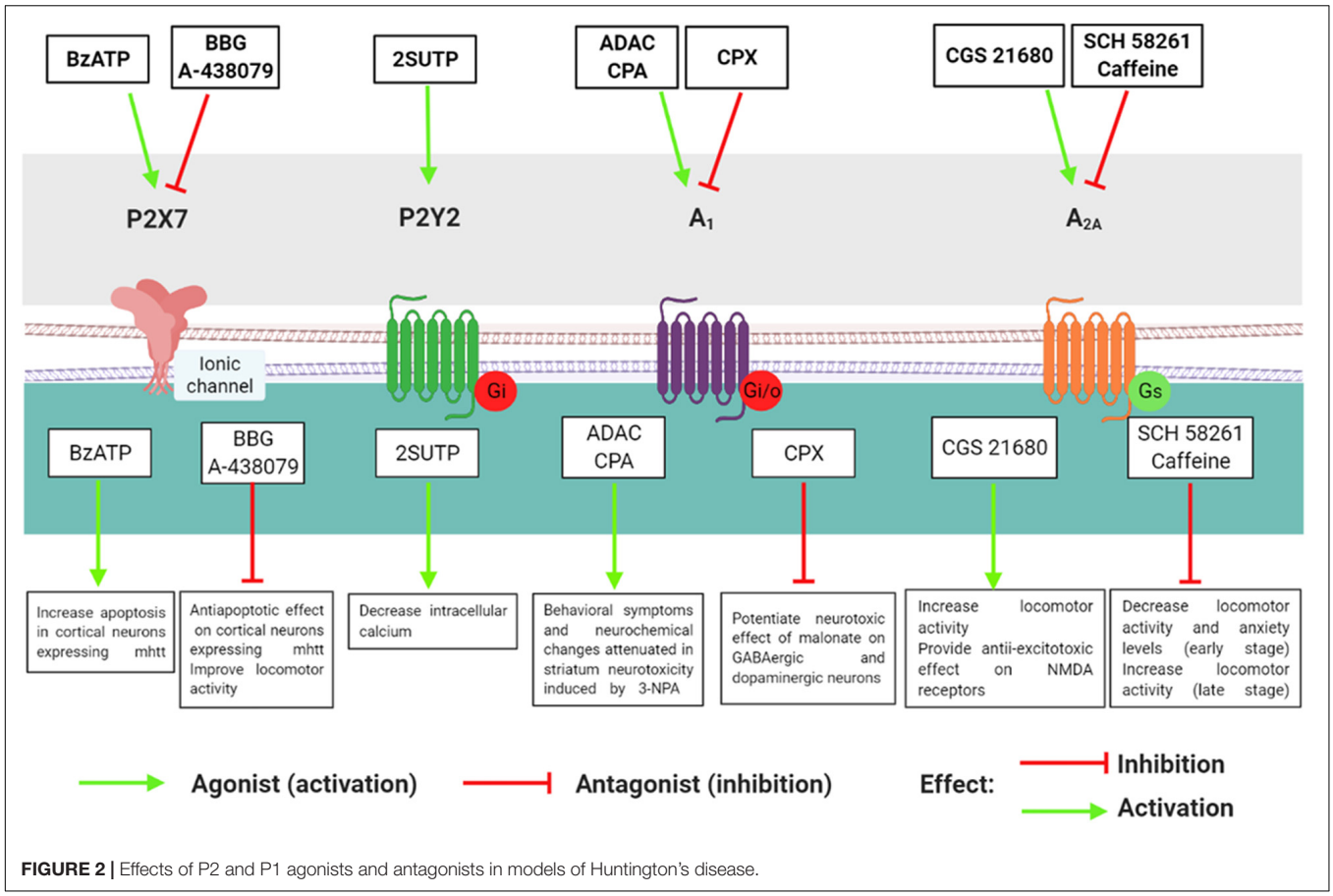

FIGURE 2 | Effects of P2 and P1 agonists and antagonists in models of Huntington's disease.

and Germany with HD subjects showed gene polymorphisms in the $A_{2A}R$ gene (*ADORA2A*), mainly the rs5751876 variant, which was associated with a variation in the age at onset on the disease (Dhaenens et al., 2009; Taherzadeh-Fard et al., 2010). Compelling evidence showed increased methylcytosine and decreased 5-methylhydroxylation (enzymes responsible by DNA methylation) in the 5′ untranslated region (UTR) of *ADORA2A* in the putamen of HD patients compared with their respective controls (Villar-Menéndez et al., 2013). These data suggest that a dysfunction in $A_{2A}R$ gene expression might also contribute to the pathophysiology of HD.

Some habitually consumed drinks and foods–such as coffee, tea, chocolate, and soda–contain caffeine, a non-selective A_1R and $A_{2A}R$ antagonist (Chen and Chern, 2011). Supported by the evidence that adenosine receptor antagonists could exert beneficial effects in neurodegenerative diseases, Simonin et al. (2013) raised the hypothesis that caffeine may be a life-style modifier in HD. For this reason, a retrospective study evaluated a possible relationship between caffeine consumption and age at onset in 80 subjects with HD. The data showed >190 mg/day caffeine consumption was significantly associated with an earlier age at onset of the disease (Simonin et al., 2013). This finding leads to the hypothesis that this association may not be a relationship with food habits but might be related to genetic determinants, such as *ADORA2A* or CYP1A genes, that may cause an earlier age at onset of the disease, influence a higher caffeine intake, and modulate behavioral effects.

$A_{2A}R$: Evidence in Animal Models

Changes in $A_{2A}R$ gene expression and density have been found in transgenic animal models of HD. R6/2, HD100, and tgHD mice at 4 weeks to 24 months of age had a decrease in $A_{2A}R$ levels and density (Cha et al., 1999; Chan et al., 2002; Bauer et al., 2005; Tarditi et al., 2006); R6/2 animals less than 3 weeks of age had an increase in $A_{2A}R$ levels and density (Tarditi et al., 2006). In contrast, Benn et al. (2007) did not find alterations in $A_{2A}R$ gene expression in the YAC128 genetic model of HD (Benn et al., 2007). These results suggest that alterations in gene expression $A_{2A}R$ could have an association with HTT length and age of disease.

Several studies with controversial results have evaluated whether $A_{2A}R$ genetic and pharmacological activation or blockade affects behavioral symptoms and/or striatal degeneration in genetic or pharmacological models of HD. $A_{2A}R$ knockout between 12 and 21 weeks of age in the N171-82-Q transgenic model of HD had a deleterious impact on survival, motor coordination, body weight, striatal volume, and enkephalin and neuropeptide messenger RNA (mRNA) levels

(Mievis et al., 2011). On the other hand, $A_{2A}R$ knockout in two R6/2 transgenic lines of HD (CAG120 and CAG240) did not cause working memory deficits and locomotor impairment compared with HD CAG120 and HD CAG240 WT mice (Li et al., 2015). Moreover, pharmacological blockade of $A_{2A}R$ with 1 mg/kg of the antagonist KW60002 in R6/2 (CAG240) mice at 3 months of age also reversed the working memory deficits compared with R6/2 (CAG240) mice that did not receive KW60002 (Li et al., 2015). Consistent with that finding, Fink et al. (2004) reported that $A_{2A}R$ genetic inactivation is beneficial to the HD animal model. The authors used two HD mouse models on different genetic backgrounds, namely C57BL/6 and 129-Steel; these models had $A_{2A}R$ knockout (KO; C57BL/6-A_{2A}-KO and 129-Steel-A_{2A}-KO). C57BL/6-A_{2A}-KO mice and their respective WT mice at 6 months of age were administered 3-NPA twice daily for 5 days. C57BL/6-WT mice developed bradykinesia with dystonic movements and bilateral striatal lesion compared with C57BL/6-A_{2A}-KO mice (Fink et al., 2004). Furthermore, in the 129-Steel line, 3-NPA administration also resulted in bilateral striatal lesions in WT compared with $A_{2A}R$ KO (Fink et al., 2004).

More recently, Domenici et al. (2018) investigated *in vivo* and *in vitro* the effect of 3-NPA in a transgenic rat strain overexpressing $A_{2A}R$. Surprisingly, there was a reduction in striatal lesion volume induced by 3-NPA in transgenic rats overexpressing $A_{2A}R$ compared with WT animals. There was greater striatal cell viability in transgenic rats overexpressing $A_{2A}R$ than WT rats after exposing corticostriatal slices to 10 mM 3-NPA. In addition, 3-NPA treatment depressed synaptic transmission in corticostriatal slices of WT animals compared with rats overexpressing $A_{2A}R$ (Domenici et al., 2018). These data lead to the idea that $A_{2A}R$ is an important target involved in the pathophysiology of HD and $A_{2A}R$ modulation might be beneficial to attenuate the neurodegenerative progression of the disease.

A_{2A} receptors modulation has demonstrated beneficial effects against neurodegeneration and HD-like behavioral symptoms induced by quinolinic acid and 3-NPA. The behavioral symptoms like hyperkinesia (increased locomotor activity) and the increase in anxiety levels persistent in early stage HD were decreased by blocking $A_{2A}R$ using two $A_{2A}R$ antagonists (SCH 58261 and caffeine; Popoli et al., 2002; Scattoni et al., 2007; Mishra and Kumar, 2014). In addition, the bradykinesia (decrease in locomotor activity) present in late stage of the disease was reverted with the use of SCH 58261 and caffeine (Mishra and Kumar, 2014; Bortolatto et al., 2017). Blocking $A_{2A}R$ using SCH 58261 and caffeine protected against the elevated glutamate extracellular levels, excessive stimulation of NMDA receptors, increased reactive oxygen species, oxidative stress, and mitochondrial dysfunction (Popoli et al., 2002; Mishra and Kumar, 2014; Bortolatto et al., 2017). Furthermore, Fink et al. (2004) showed that striatal neurotoxicity induced by 3-NPA in C57BL/6 mice resulted in bilateral striatal lesions, while mice pre-treated with the $A_{2A}R$ antagonist caffeine before 3-NPA injections did not show striatal lesions.

A_{2A} receptors pharmacological blockade also attenuated the levels of cyclooxygenase (COX-2), prostaglandin E2, and brain-derived neurotrophic factor, a member of the neurotrophin family that participates in synaptic transmission and regulates neuronal proliferation and survival (Minghetti et al., 2007; Potenza et al., 2007). In R6/2 mice, $A_{2A}R$ blockade prevented alterations in anxious responses and abolished the increase in NMDA-receptor-induced striatal toxicity (Domenici et al., 2007). However, $A_{2A}R$ antagonism had a potentially detrimental effect on neurotoxicity induced by quinolinic acid in an animal model of HD; specifically, there was increased striatal glutamate outflow (Gianfriddo et al., 2003).

Considering that a pronounced loss of $A_{2A}R$ occurs in HD, researchers have tested whether A_{2A} agonists could ameliorate HD symptoms. The selective $A_{2A}R$ agonist CGS 21680 reverted the hypolocomotor profile and the increase in the ventricle/brain ratio in R6/2 mice (Chou et al., 2005). CGS 21680 also attenuated the NMDA toxic effects in corticostriatal slices (Martire et al., 2007) and reduced the expression of NMDA receptor subunits NR1 in the striatum of R6/2 and WT mice. In addition, there was a decrease in the NR2A/NR2B ratio in the striatum of WT mice, inducing a pro-excitotoxic effect, whereas the NR2A/NR2B ratio was increased in HD mice (R6/2), and thus the NMDA receptors provided an anti-excitotoxic effect (Ferrante et al., 2010). Finally, in the liver of R6/2 mice, CGS 21680 treatment also had a protective effect, suppressing mHTT aggregates and decreasing the levels of crucial transcription factor and protein chaperones (Hsp27 and Hsp70; Chiang et al., 2009). Taken together, it is possible to suggest that $A_{2A}R$ alterations in HD occur in the central nervous system as well as other tissues. Thus, pharmacological modulation of $A_{2A}R$ through agonist or antagonist administration might exert a neuroprotective effect against HD progression (**Figure 2**), considering the stage of the disease, drug dosage, and period of pharmacological administration.

P2 RECEPTOR INVOLVEMENT IN HUNTINGTON'S DISEASE

Contrary to P1 receptors, there are few studies about P2 receptors in HD and their roles in this neurodegenerative disease remain elusive. Díaz-Hernández et al. (2009) investigated P2X7 receptor involvement in HD using two genetic mouse models of HD (Tet/HD94 and R6/1). First, cortical and striatal neurons showed an increase in P2X7 receptor protein and mRNA levels in Tet/HD94 and R6/1 compared with WT mice (Díaz-Hernández et al., 2009). Moreover, transgenic HD mice had higher P2X7 receptor levels in synaptosomes and were more sensitive to the P2X7 agonist BzATP than WT mice. This increase in sensitivity induced by BzATP led to apoptosis of cultured cortical neurons expressing mHTT, which was prevented by the P2X7 antagonist brilliant blue (BBG) (Díaz-Hernández et al., 2009). Due to the protective effect of BBG in neuronal culture, the authors investigated the efficacy

of P2X7 antagonists BBG and A-438079 in R6/1 mice and their WT on body weight and locomotor parameters. BBG administration fully prevented body weight loss and significantly improved motor coordination and locomotor performance in R6/1 compared with WT mice. The antagonist A-438079 also prevented body weight loss and improved locomotor parameters in R6/1 mice, but the effect was only moderate (Díaz-Hernández et al., 2009). These data support the hypothesis that changes in P2X7 receptor levels and function lead to an increase in Ca^{2+} permeability; this eventuality could induce excitotoxicity and oxidative stress in neurons, changes that are related to HD pathogenesis (**Figure 2**). In the future, studies using P2X7 receptor antagonists should be performed because these compounds may have therapeutic potential for HD treatment.

Martire et al. (2021) investigated the expression and the functioning of P2X7R in two genetic models of HD: ST14A rat striatal cells, expressing full-length wild-type (WT, Q15) or mutant (Q120) htt and R6/2 mice, which resembles to juvenile forms of HD. In the presence of HD mutation, there is an altered P2X7R expression and a larger P2X7R response to the agonist BzATP, inducing cell death and reducing synaptic transmission. BzATP effect observed in the electrophysiology experimental setting are dependent of A_1R activation. These findings may permit a better understanding of the P2X7R mechanisms and evaluate the therapeutic potential of P2X7 antagonists in HD. Certainly, this topic requires a deepen investigation since recent studies demonstrated changes in P2X7R in the brain of HD subjects (Ollà et al., 2020). Ollà et al. (2020) observed that the protein levels of the full-length form of P2X7R, also called P2X7R-A, as well as the exclusively human naturally occurring variant lacking the C-terminus region, named P2X7R-B, are upregulated. These augmented protein levels can be explained by elevated P2X7R mRNA levels. In addition, P2X7R introns 10 and 11 are more retained in HD subjects when compared with controls patients (Ollà et al., 2020). Therefore, further studies are required to evaluate the implications of P2X7R in HD and if this receptor may be a target for the development of new pharmacological therapies for this pathology.

Regarding to other P2 receptors, Glaser et al. (2020) analyzed whether $P2Y_2$ receptors might be involved in the pathogenesis of HD. They investigated the role of the $P2Y_2$ receptor and spontaneous Ca^{2+} concentrations in embryonic stem cells in *in vitro* HD models and controls. In basal state HD, there were higher intracellular Ca^{2+} levels than controls. Besides, there was elevated P2X7 and $P2Y_2$ receptor levels in the HD cell line compared with the controls (Glaser et al., 2020). Moreover, the cells from controls (WT cells) were responsive to the P2X7 receptor agonist BzATP and the $P2Y_2$ receptor agonist 2SUTP, while HD cells (expressing mHTT) showed a decrease in the 2SUTP response, with impaired $P2Y_2$ receptor activation (Glaser et al., 2020). The administration of ATP or 2SUTP in HD cells decreased the concentration of intracellular Ca^{2+} transients (Glaser et al., 2020). Therefore, these results indicate that P2 receptors contribute to the pathogenesis of HD (**Figure 2**) and provided the first evidence for the involvement of the $P2Y_2$ receptor in this neurodegenerative disease.

Together, the above-mentioned studies shed new light on the mechanism underlying HD, leading to the idea that pharmacological therapies based on P2X7 and $P2Y_2$ receptors should be better investigated for HD treatment.

FOCUSING ON THE RELATIONSHIP BETWEEN NUCLEOTIDE AND NUCLEOSIDE METABOLISM AND HUNTINGTON'S DISEASE

Reduced mitochondrial ATP levels and ATP/ADP ratios have been found in striatal cells containing mHTT (Seong et al., 2005). It is known that ATP depletion occurs in HD, so researchers have investigated nucleotide and nucleoside metabolism in this pathological condition. Researchers have demonstrated reduced mitochondrial ATP levels in cortical cells from an animal model of HD induced by 3-NPA, in cardiac tissue from two genetic models of HD (R6/2 and HdhQ150), and in a HEK293T cell line containing mHTT (Riepe et al., 1994; Toczek et al., 2016a, 2018). There was a decrease in the ATP/ADP ratio in cardiac tissue from R6/2 and HdhQ150 mice (Toczek et al., 2016a). There was also a reduction in ATP and AMP degradation in the HEK293T cell line containing mHTT (Toczek et al., 2018). Furthermore, studies in cardiac and cerebral tissue have shown no alteration in ADP levels in R6/2 and HdhQ150 mice. However, AMP levels were increased in R6/2 mice (Toczek et al., 2016a; Kao et al., 2017).

A genetic study of HD using different lines, including Tg51, zQ175, and R6/2, and a pharmacological model of HD induced by quinolinic acid and 3-NPA demonstrated a decrease in adenosine levels in the striatum of Tg51, zQ175, R6/2 lines, and HD animals induced by quinolinic acid and 3-NPA (Gianfriddo et al., 2003; Guitart et al., 2016; Jamwal and Kumar, 2016; Kao et al., 2017). Importantly, researchers have also found a decrease in adenosine levels in the cerebrospinal fluid of patients with HD compared with controls (Kao et al., 2017). Kao et al. (2017) observed that ATP was indirectly correlated with the number of CAG repeats and had a direct correlation with the age at onset of the disease while the adenosine/ATP ratio was negatively correlated with the disease duration of patients with HD (Kao et al., 2017).

Consistently, there was a decrease in ecto-5′-nucleotidase (an enzyme that converts AMP to adenosine) activity in HEK 293T cell line containing mHTT and cardiac tissue from R6/2 mice (Toczek et al., 2016b, 2018), while there was an increase in ADA activity in cardiac tissue from R6/2 mice (Toczek et al., 2016b). Furthermore, in the striatum of rats treated with 3-NPA, there was a decrease in inosine and hypoxanthine levels compared with the control group (Jamwal and Kumar, 2016). On the other hand, Toczek et al. (2016a) found an increase in inosine, hypoxanthine, xanthine, uric acid, and uridine levels in cardiac tissue in R6/2 and HdhQ150 mice (Toczek et al., 2016a).

Genes involved in purine metabolism (*Entpd2*, *Ampd3*, *Pnp*, and *Xdh*), adenosine metabolism (Ada), conversion of adenine nucleotides (adenylate kinase 1 [*Ak1*] and inosine

monophosphatase dehydrogenase 2 [*Impdh2*]), and equilibrative nucleoside transporter-like ENT1 and ENT2 were altered in cardiac tissue, striatum, and skeletal muscle of R6/2 mice of HD (Toczek et al., 2016a; Kao et al., 2017; Mielcarek et al., 2017). Moreover, in HD patients at the early stage of the disease, there was an upregulation of the gene that encodes ENT1 (Guitart et al., 2016). Finally, nitrobenzylthioinosine (NBTI), a selective inhibitor of ENT1, and dipyridamole (DPR), a non-specific inhibitor of ENT1 and ENT2, were intrastriatally perfused in R6/2 mice. Administration of NBTI alone increased adenosine levels 1-h post-treatment, whereas both NBTI and DPR administration increased adenosine levels 1-h post-treatment, sustaining the effects up to 5 h post-treatment (Kao et al., 2017).

Regarding to studies in HD patients, it has been observed differences in the metabolism of nucleoside and its derivates. In plasma samples from HD patients, there was a significant increase in hypoxanthine and uridine levels, but there were not changes in uric acid levels compared with healthy subjects (Toczek et al., 2016a). Interestingly, hypoxanthine and uridine levels were directly correlated with HD duration and indirectly correlated with motor scores and chorea intensity (Toczek et al., 2016a). In contrast, Corey-Bloom et al. (2020) recently investigated uric acid levels in plasma and saliva samples from HD subjects and normal controls, as well as in post-mortem prefrontal cortical samples from HD subjects and controls. The authors surprisingly revealed that plasma and salivary uric acid levels were significantly lower in female pre-manifest HD and manifest HD subjects compared with normal controls, whereas salivary levels of uric acid were also significantly lower in male manifest HD subjects than controls. In male HD patients, plasma and salivary uric acid levels were negatively correlated with total functional capacity, while there were direct correlations with the total motor score. Female HD patients showed a direct correlation between plasma uric acid levels and total functional capacity, while salivary uric acid levels were significantly correlated with disease burden. Finally, in post-mortem prefrontal cortical samples from HD subjects, there was a decrease in uric acid levels (Corey-Bloom et al., 2020).

Overall, these findings indicate that purinergic signaling plays an important role in HD by inhibiting the cascade of ATP hydrolysis, modulating the concentration of adenine nucleotides and nucleosides.

CONCLUSION

This review sheds light on the important regulatory role of purinergic signaling in HD pathophysiology. Notably, ATP, adenosine, and $A_{2A}R$ are the main actors in HD. Although some results in animal models and HD patients are controversial–depending on the model tested, type of pharmacological treatment, and period of drug administration–pharmacological modulation of $A_{2A}R$, through agonist and antagonist drugs, has shown a neuroprotective effect by attenuating the behavioral symptoms and improving neurochemical parameters during HD progression. Besides, $A_{2A}R$ in the central and peripheral nervous system might be considered a powerful biomarker for HD progression and ought to be used in clinical practice. $A_{2A}R$ heterodimerizes with several other G-protein coupled receptors involved in striatal dysfunction and degeneration in HD; thus, $A_{2A}R$ could be considered a target for the development of pharmacological therapies for HD patients.

Several small molecules acting as $A_{2A}R$ antagonists have already been developed and tested in patients several neurological diseases, such as Parkinson's Disease. Although the efficacy of these agents in Parkinson's disease was not proved, the use of antagonists targeting $A_{2A}R$ in cancer immunotherapy has been also investigated. The treatment with small molecules or mAbs aiming to block adenosine signaling, either by limiting its production or its binding to adenosine receptors, has yielded important tumor control in pre-clinical studies. Moreover, simultaneous blockade of adenosine production and receptor binding, achieved by an anti-CD73 mAb co-administered with an $A_{2A}R$ antagonist, for example, have demonstrated synergy. Therefore, it is important to deepen the investigation of $A_{2A}R$ as a target for the development of existing or new agents targeting this axis, along with further testing of combinatorial strategies, which may be relevant in the search for pharmacological therapies for HD patients. Moreover, the role of A_1R and P2 receptors in HD pathogenesis needs to be reconsidered; there should be more specific investigation on these receptors, because they could provide a powerful contribution to understanding the mechanism underlying HD. In addition, nucleoside metabolism and the control of hypoxanthine, xanthine, and uric acid levels should also be deeply investigated, because these nucleosides might be important candidates for future drugs or biomarkers for HD. In summary, purinergic signaling represents a promising research area, and the main players, such as ATP, adenosine, and $A_{2A}R$, as well as the respective coadjutants, such as A_1R, P2 receptors, and other components of nucleotide and nucleoside metabolism, should be considered possible targets for drug development for HD treatment.

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Both authors contributed for the conceptualization, performed the literature review, read and approved the submitted version. MW wrote the original draft of the manuscript. CB wrote and review and editing the final version of the manuscript.

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frontiers in Neuroscience

REVIEW
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The Good, the Bad, and the Deadly: Adenosinergic Mechanisms Underlying Sudden Unexpected Death in Epilepsy

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Adenosine is an inhibitory modulator of neuronal excitability. Neuronal activity results in increased adenosine release, thereby constraining excessive excitation. The exceptionally high neuronal activity of a seizure results in a surge in extracellular adenosine to concentrations many-fold higher than would be observed under normal conditions. In this review, we discuss the multifarious effects of adenosine signaling in the context of epilepsy, with emphasis on sudden unexpected death in epilepsy (SUDEP). We describe and categorize the beneficial, detrimental, and potentially deadly aspects of adenosine signaling. The good or beneficial characteristics of adenosine signaling in the context of seizures include: (1) its direct effect on seizure termination and the prevention of status epilepticus; (2) the vasodilatory effect of adenosine, potentially counteracting postictal vasoconstriction; (3) its neuroprotective effects under hypoxic conditions; and (4) its disease modifying antiepileptogenic effect. The bad or detrimental effects of adenosine signaling include: (1) its capacity to suppress breathing and contribute to peri-ictal respiratory dysfunction; (2) its contribution to postictal generalized EEG suppression (PGES); (3) the prolonged increase in extracellular adenosine following spreading depolarization waves may contribute to postictal neuronal dysfunction; (4) the excitatory effects of A_{2A} receptor activation is thought to exacerbate seizures in some instances; and (5) its potential contributions to sleep alterations in epilepsy. Finally, the adverse effects of adenosine signaling may potentiate a deadly outcome in the form of SUDEP by suppressing breathing and arousal in the postictal period. Evidence from animal models suggests that excessive postictal adenosine signaling contributes to the pathophysiology of SUDEP. The goal of this review is to discuss the beneficial, harmful, and potentially deadly roles that adenosine plays in the context of epilepsy and to identify crucial gaps in knowledge where further investigation is necessary. By better understanding adenosine dynamics, we may gain insights into the treatment of epilepsy and the prevention of SUDEP.

Keywords: adenosine, epilepsy, SUDEP, status epilepticus, seizure-induced respiratory arrest, adenosine kinase, adenosine receptors, epileptogenesis

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INTRODUCTION

The purine ribonucleoside adenosine is found ubiquitously in living tissues. In the central nervous system, adenosine is an essential inhibitory modulator of neuronal excitability (Dunwiddie, 1980; Dunwiddie and Masino, 2001; Boison, 2008). Neuronal activity increases adenosine signaling thereby providing negative feedback on excessive excitation (Mitchell et al., 1993; Brager and Thompson, 2003; Pajski and Venton, 2010). The exceptionally high neuronal activity of a seizure results in a surge in extracellular adenosine to concentrations many fold higher than would be observed under normal conditions (During and Spencer, 1992; Berman et al., 2000; Van Gompel et al., 2014). The neuronal inhibition provided by activity-dependent adenosine surging is critical to the prevention and termination of seizures (Dragunow et al., 1985; Murray et al., 1985; Kochanek et al., 2006). Deficits in adenosine signaling can facilitate status epilepticus, a life-threatening event defined by inordinately protracted seizure activity (Young and Dragunow, 1994; Kochanek et al., 2006). In this sense, seizure-induced adenosine surging is highly beneficial. Seizures can also cause periods of profound cerebral hypoxia through postictal vasoconstriction, increased oxygen demand, and respiratory dysfunction (Posner et al., 1969; Farrell et al., 2017; Lacuey et al., 2018). Cerebral hypoxia likely contributes to the pathophysiology of a number of adverse seizure outcomes such as neurodegeneration, memory loss, postictal generalized EEG suppression (PGES), and the postictal state (Seyal et al., 2012; Farrell et al., 2016; Leal-Campanario et al., 2017; Rheims et al., 2019). Adenosine acts as a cerebral vasodilator and may alleviate the deleterious effects of seizure-induced vasoconstriction (Morii et al., 1986; Arrigoni et al., 2005). Furthermore, adenosine signaling is neuroprotective under hypoxic conditions (Bjorklund et al., 2008; Phillips et al., 2019). As a result, seizure-induced adenosine surging and the increased tissue tone of adenosine are "good" for patient health.

On the other hand, large surges in extracellular adenosine can have detrimental effects. Excessive increases in extracellular adenosine suppress neuronal activity and may contribute to PGES and the postictal state (Rosen and Berman, 1985; During and Spencer, 1992). Though acute seizures cause an increase in extracellular adenosine, chronic epilepsy is associated with a reduction of baseline adenosine levels, which could be a precipitating factor in epileptogenesis (Gouder et al., 2004; Li et al., 2008) and associated co-morbid conditions including cognitive, psychiatric, and sleep disorders (Yee et al., 2007; Boison et al., 2012; Shen et al., 2012; Boison, 2016; Warren et al., 2018). Spreading depolarization waves, which can occur during seizures, result in a prolonged increase in extracellular adenosine (Lindquist and Shuttleworth, 2014; Loonen et al., 2019). This increase in adenosine contributes to the neuronal dysfunction that persists in the wake of a spreading depolarization wave (Lindquist and Shuttleworth, 2017). Additionally, under certain circumstances, A_{2A} receptor activation may have proconvulsant effects (Zeraati et al., 2006; Fukuda et al., 2011); however, anticonvulsant effects of A_{2A} receptor activation have also been described (De Sarro et al., 1999; Huber et al., 2002). Of concern, adenosine suppresses breathing and attenuates the hypercapnic ventilatory response through inhibition of brainstem respiratory sites (Gettys et al., 2013; Falquetto et al., 2018). Seizure-induced increases in brainstem adenosine levels may make seizures more dangerous by preventing an adequate respiratory response to postictal blood gas derangement. For these reasons, seizure-induced adenosine surging is "bad" for patient health.

The adverse effects of seizure-induced adenosine surging may play a critical role in seizure-induced death. The leading cause of epilepsy-related death in patients with refractory epilepsy is sudden unexpected death in epilepsy (SUDEP; Hesdorffer et al., 2011). More years of potential life are lost due to SUDEP than any other neurological condition with the exception of stroke (Thurman et al., 2014). Currently, there are no reliable means of preventing SUDEP or identifying those who are at the highest risk (Massey et al., 2014; Devinsky et al., 2016; Dlouhy et al., 2016). Convergent lines of evidence from epilepsy patients and animal models suggests that SUDEP is the result of some combination of respiratory, cardiac, and electrocerebral dysfunction in the postictal period (Jehi and Najm, 2008; Massey et al., 2014; Aiba and Noebels, 2015; Dlouhy et al., 2016). The precise pathophysiology of SUDEP is the subject of a vibrant ongoing debate (Auerbach et al., 2013; Aiba and Noebels, 2015; Budde et al., 2018; Vega, 2018; Vilella et al., 2019). Currently, the most reliable information on the terminal cascade which precedes SUDEP comes from a case series of SUDEP events occurring in epilepsy monitoring units in which video, electrocardiogram (EKG), and electroencephalogram (EEG) were simultaneously recorded (Ryvlin et al., 2013). In all cases in which breathing and cardiac function could be assessed, terminal apnea preceded terminal asystole indicating that respiratory failure was the primary cause of death (Ryvlin et al., 2013). In the forebrain, seizure-induced adenosine surging has the beneficial effect of stopping seizures; however, excessive adenosine signaling may potentiate SUDEP through respiratory suppression, PGES exacerbation, and attenuation of the hypercapnic ventilatory response (Shen et al., 2010; Ashraf et al., 2020). Experimental evidence from animal models of seizure-induced death suggests that excessive adenosinergic signaling contributes to SUDEP pathophysiology (Shen et al., 2010; Fukuda et al., 2011; Faingold et al., 2016; Kommajosyula et al., 2016). Hence, seizure-induced adenosine surging must be tightly controlled to prevent the potentially disastrous outcomes of status epilepticus and SUDEP. In this review, we summarize the evidence pertinent to the adenosine hypothesis of SUDEP and describe the multifarious effects of adenosine signaling in the context of epilepsy: the good, the bad, and the deadly.

Abbreviations: 5-ITU, 5-iodotubercidin; $A_{1/2A}$, adenosine receptor; ADK, adenosine kinase; ATP, adenosine triphosphate; DPCPX, A_1 receptor antagonist; dnSNARE, N-ethylmaleimide-sensitive factor attachment protein receptor; EEG, electroencephalogram; EKG, electrocardiogram; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; GEPR-9s, genetically epilepsy-prone rats; $GRAB_{Ado}$, GPCR-activation based adenosine sensor; PGES, postictal generalized EEG suppression; SCH 442416, A_{2A} receptor antagonist; SUDEP, sudden unexpected death in epilepsy.

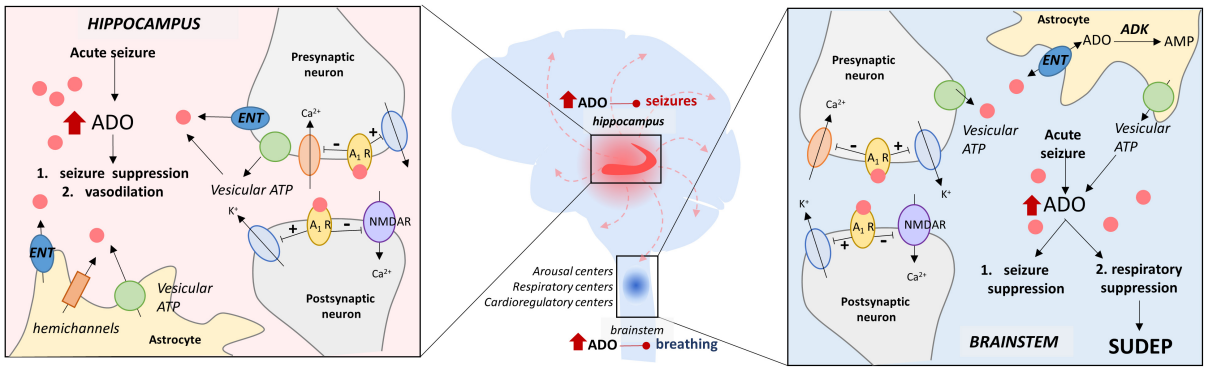

FIGURE 1 | The adenosine hypothesis of SUDEP. This schematic illustrates the hypothesized similarities and differences in periictal adenosine surging and its effects in the hippocampus and the brainstem. In the hippocampus, the ADO surge is important for seizure termination and vasodilation, whereas, in the brainstem, the ADO surge is double-edged and can suppress respiration/breathing in addition to seizure suppression. The key elements of ADO signaling depicted here include (i) vesicular ATP, (ii) equilibrative nucleoside transporters (ENT), (iii) adenosine kinase (ADK), (iv) adenosine receptor (A_1R), and (v) N-methyl D-aspartate receptors (NMDAR).

THE GOOD

Adenosine and Seizure Cessation

In the central nervous system, adenosine is released in neural tissue in response to endogenously generated activity (Mitchell et al., 1993; Nguyen and Venton, 2015). Exogenously evoked neuronal activity also triggers an increase in extracellular adenosine (Sulakhe and Phillis, 1975; Lloyd et al., 1993; Pajski and Venton, 2010; Tawfik et al., 2010). The magnitude of the activity-dependent adenosine release increases with the intensity of stimulation (Mitchell et al., 1993; Sciotti et al., 1993; Pajski and Venton, 2010). The high amplitude and high frequency firing of epileptiform discharges results in more neuronal activity than would occur under normal conditions (Merricks et al., 2015). Therefore, it is unsurprising that seizures result in a surge in extracellular adenosine to concentrations much higher than those seen under normal conditions (**Figure 1**; During and Spencer, 1992; Berman et al., 2000; Aden et al., 2004). Adenosine is an inhibitory modulator of presynaptic neurotransmission and activity dependent adenosine release is critical to keeping neural excitability in check (Dunwiddie and Masino, 2001).

Interestingly, the source of activity-dependent adenosine release remains elusive. There are a number of potential sources of extracellular adenosine (Latini and Pedata, 2001; Wall and Dale, 2008). The mechanism responsible for activity dependent adenosine release varies depending on the brain region (Pajski and Venton, 2013) and the parameters of neural activity (Cunha et al., 1996). Furthermore, activity-dependent adenosine release can occur through several mechanisms simultaneously. A study with transgenic mice with an inducible astrocyte-selective mutation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (dnSNARE-mice) suggests that astrocytic vesicular release of adenosine triphosphate (ATP) is the major source of synaptic adenosine (Pascual et al., 2005). On the other hand, hippocampal neuronal activity results in increased extracellular adenosine *via* a combination of astrocytic ATP release, and neuronal adenosine release through equilibrative nucleoside transporters (Wall and Dale, 2013). Contrary to this, another study showed that blocking the conversion of ATP to adenosine did not alter the inhibition of neuronal activity associated with high frequency stimulation, suggesting that neuronal adenosine release, and not astrocytic ATP release mediated feedback inhibition of excitatory activity (Lovatt et al., 2012).

Whatever the source of extracellular adenosine, the breakdown is controlled by intracellular astrocytic adenosine kinase (ADK); thus, the tone of ambient adenosine is maintained by an astrocyte-based adenosine-cycle (Fredholm et al., 2005). It is noteworthy, that during chronic epileptic conditions ADK expression is upregulated and consequently the tissue tone of adenosine is drastically reduced (Boison, 2012). Hence, the seizure-induced surge of adenosine combined with a low basal level of adenosine creates a complex reperfusion scenario that needs to be investigated particularly in the context of SUDEP.

Adenosine receptors are G-protein-coupled and exert their effects on neuronal excitability through several transduction pathways. A_1 receptors, which are coupled to $G_{i/o}$ proteins (Fredholm et al., 2011), hyperpolarize neurons by activating potassium channels (**Figure 1**; Trussell and Jackson, 1985) and inhibiting voltage dependent calcium channels (**Figure 1**; MacDonald et al., 1986). On the other hand, A_{2A} receptors, which are coupled to $G_{s/olf}$ proteins (Fredholm et al., 2011), are linked to adenylyl cyclase activation and are thought to have an excitatory effect on neurons upon activation (Corvol et al., 2001).

Activity-dependent adenosine release alters seizure dynamics largely *via* interactions with the A_1R. Reducing the influence of activity dependent adenosine release *via* A_1R antagonists prolongs seizures in animal models (Dragunow and Goddard, 1984). Likewise, the genetic deletion of the A_1R increases vulnerability to status epilepticus and traumatic brain injury (Fedele et al., 2006; Kochanek et al., 2006). Conversely, upregulating adenosinergic tone by inhibiting adenosine reuptake or degradation is protective against seizure activity

(Dragunow and Goddard, 1984; Gouder et al., 2004). Stem cell derived implants with deficient adenosine metabolism suppressed seizures in rats (Guttinger et al., 2005). Those findings from rodent models are relevant for the human brain as upregulation of adenosine signaling in excised human epileptic tissue attenuated spontaneous and evoked epileptiform activity (Kostopoulos et al., 1989). Conversely, the adenosine receptor antagonist caffeine is used clinically for the purpose of prolonging seizure duration following electroconvulsive therapy, ostensibly improving treatment outcomes (Bozymski et al., 2018). In summary, the inhibitory influence of adenosine is critical to seizure termination (**Figure 2** and **Table 1**).

Adenosine Is Neuroprotective Under Hypoxic Conditions

The metabolic demand associated with seizures increases O_2 consumption and CO_2 production (Posner et al., 1969). Insufficient blood gas exchange due to cerebral vasoconstriction, increased metabolism, and seizure-induced respiratory dysfunction contribute to cerebral hypoxia (Posner et al., 1969; Farrell et al., 2017; Lacuey et al., 2018). Reduction in cerebral O_2 to less than half of baseline levels can occur within seconds of seizure termination and can last more than an hour (Farrell et al., 2016). Cerebral hypoxia is associated with PGES and the postictal state (Farrell et al., 2016; Kuo et al., 2016; Rheims et al., 2019). Seizure-induced cerebral hypoxia can also contribute to memory disruption (Farrell et al., 2020).

Convergent lines of evidence indicate that adenosine is neuroprotective under hypoxic conditions (**Figure 2** and **Table 1**). Adenosine and A_1 receptor agonists alleviate hypoxic/ischemic damage in cultured cells (Goldberg et al., 1988; Daval and Nicolas, 1994), in isolated slice preparations (Dux et al., 1992; Mori et al., 1992), and *in vivo* (Evans et al., 1987; von Lubitz et al., 1988; Von Lubitz et al., 1994). Likewise, non-selective and A_1 receptor specific adenosine antagonists exacerbate hypoxic/ischemic damage in cultured cells (Daval and Nicolas, 1994; Lynch et al., 1998), in isolated slice preparations (Dux et al., 1992), and *in vivo* (Von Lubitz et al., 1994). The neuroprotective effect of A_1R mediated adenosine signaling has also been demonstrated in the retina (Larsen and Osborne, 1996). Furthermore, increasing adenosine signaling by reducing its metabolic clearance through inhibition of adenosine deaminase (Phillis and O'Regan, 1989; Lin and Phillis, 1992) or ADK (Miller et al., 1996; Jiang et al., 1997) protects against hypoxic/ischemic damage. Lastly, increasing adenosine signaling by inhibiting its reuptake is neuroprotective under hypoxic/ischemic conditions (DeLeo et al., 1988; Matsumoto et al., 1996).

Though A_1 receptor activation is neuroprotective under hypoxic conditions, the opposite appears to be true of the A_{2A} receptor. A_1 receptor knockout mice are more vulnerable to hypoxic/ischemic damage (Johansson et al., 2001), whereas the converse is true in A_{2A} receptor knockout mice (Chen et al., 1999). A_{2A} receptor antagonists alleviate hypoxic/ischemic damage *in vivo* (Gao and Phillis, 1994; Phillis, 1995; Von Lubitz et al., 1995) and the A_{2A} receptor antagonist KW-6002 is now FDA approved as an adjunct treatment for Parkinson's disease (Berger et al., 2020; Chen and Cunha, 2020).

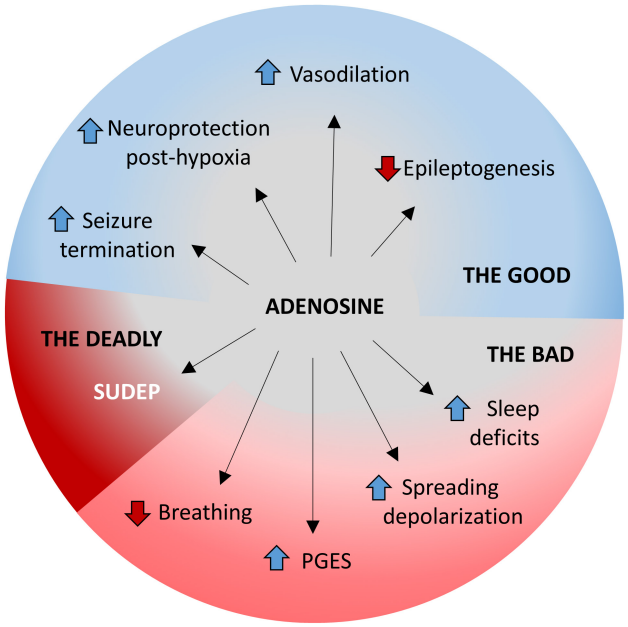

FIGURE 2 | The good, the bad, and the deadly effects of adenosine during seizures. The multifaceted roles of adenosine homeostasis during seizures is highlighted. The beneficial/good aspects include (i) seizure termination, (ii) neuroprotection against hypoxia, (iii) increased vasodilation, and (iv) prevention of epileptogenesis. The detrimental/bad aspects include (i) suppression of breathing, (ii) PGES, (iii) worsened electrographic dysfunction following spreading depolarization, and (iv) development of sleep deficits. Excessive adenosine release or insufficient adenosine clearance may result in a deadly outcome in the form of SUDEP.

TABLE 1 | The beneficial effects of adenosine in the context of seizures and epilepsy.

The Good: Adenosine increases seizure threshold, is critical for seizure termination, and may alleviate some of the adverse effects of seizures.	
Seizure cessation	The inhibitory influence of adenosine makes seizures less likely and is critical for preventing status epilepticus when seizures do occur.
Neuroprotection during hypoxia	Convergent lines of evidence indicate that adenosine is neuroprotective under hypoxic conditions such as those observed during seizures.
Vasodilation	The vasodilating effect of seizure-induced adenosine surging may attenuate the postictal dysfunction elicited by cerebral vasoconstriction.

TABLE 2 | The potentially harmful effects of adenosine in the context of seizures and epilepsy.

The Bad: Adenosine surging due to seizures or to secondary depolarization events may adversely affect breathing and EEG activity acutely and sleep chronically.	
Respiratory suppression	Adenosine suppresses breathing and seizure-induced adenosine surging has been implicated in periictal respiratory dysfunction.
The postictal state and PGES	Excessive increases in extracellular adenosine suppress neuronal activity and may contribute to PGES and the postictal state.
Spreading depolarization	The increase in extracellular adenosine caused by periictal spreading depolarization may contribute to postictal electrocerebral dysfunction.
Proconvulsant effects	Though adenosine is generally inhibitory, there is mixed evidence that under certain circumstances A_{2A} receptor activation can have proconvulsant effects.
Sleep deficits	Adenosinergic dysfunction in chronic epilepsy may contribute to comorbid sleep disorders.

Adenosine-Induced Vasodilation

Cerebral vasoconstriction has been documented in the postictal period in epilepsy patients and in animal models (Newton et al., 1992; Steinhoff et al., 1996; Farrell et al., 2016, 2017). There are several ways in which postictal vasoconstriction might contribute to adverse seizure sequelae. Firstly, vasoconstriction contributes to postictal cerebral hypoxia (Farrell et al., 2016, 2017). As mentioned in the preceding section, postictal cerebral hypoxia has been associated with PGES, the postictal state, and seizure-induced memory impairments (Farrell et al., 2016; Kuo et al., 2016). Secondly, the hypoperfusion caused by repeated seizures may contribute to progressive neurodegeneration (Leal-Campanario et al., 2017). Fortunately, adenosine is known to act as a vasodilator in the central nervous system (Morii et al., 1986; Arrigoni et al., 2005) and is released in large quantities during seizures (During and Spencer, 1992; Berman et al., 2000; Van Gompel et al., 2014). The vasodilating effect of seizure-induced adenosine surging may attenuate the postictal dysfunction elicited by cerebral vasoconstriction (**Figure 2** and **Table 1**). This hypothesis is supported by the finding that adenosine antagonism worsens the cerebral hypoxia caused by postictal hypoperfusion (Phillips et al., 2019). Taken together, the seizure-induced adenosine surge is beneficial in its effects on seizure termination, prevention of epileptogenesis, vasodilation, and neuroprotection under hypoxic conditions (**Figure 2** and **Table 1**).

THE BAD

The inhibitory influence of activity dependent adenosine surging is essential to the regulation of neuronal activity and to the prevention and cessation of seizures (Dragunow et al., 1985; During and Spencer, 1992; Young and Dragunow, 1994; Kochanek et al., 2006); however, excessive adenosine signaling can also have detrimental effects including suppression of breathing, PGES, neuronal dysfunction following spreading depolarization, and may even play a contributing role in the development of comorbid conditions (**Figure 2** and **Table 2**).

Adenosine and Breathing

Adenosine suppresses breathing by a reduction of both respiratory rate and volume (**Figure 2** and **Table 2**; Eldridge et al., 1984, 1985; Lagercrantz et al., 1984). Interestingly, in mechanically ventilated cats, intracerebroventricular administration of an adenosine analog suppressed respiratory drive while causing the medulla to become acidified (Eldridge et al., 1984), which would normally be expected to increase respiratory drive (Hodges et al., 2004). Because the animals in this study were mechanically ventilated, the medullary acidosis appears to be metabolic in origin and is not explicable by changes in breathing (Eldridge et al., 1984). In adults, adenosine suppresses breathing *via* its action on the A_1 receptor (Herlenius et al., 1997; Gettys et al., 2013), but A_{2A}-mediated suppression of breathing has been documented in neonates (Koos et al., 2001; Mayer et al., 2006). Increases in adenosine signaling in the nucleus tractus solitarius, the pontine reticular formation, and the pre-Bötzinger complex have all been demonstrated to suppress breathing (Douglas et al., 1982; Yan et al., 1995; Gettys et al., 2013).

Respiratory disruption can result in potentially dangerous derangement of blood gasses. The hypercapnic ventilatory response is a life-saving reflex that increases breathing in response to rising CO_2 levels (Douglas et al., 1982; Ainslie and Duffin, 2009). In addition to its effect on baseline breathing, adenosine attenuates the hypercapnic ventilatory response (Falquetto et al., 2018). Conversely, adenosine receptor antagonists improve the hypercapnic ventilatory response (Pianosi et al., 1994). Serotonergic neurons in the raphe nuclei are chemosensitive and contribute to the hypercapnic ventilatory response (Hodges et al., 2008; Teran et al., 2014). Serotonin release is inhibited by adenosine agonists and enhanced by adenosine antagonists (Feuerstein et al., 1988; Okada et al., 2001; Arnold et al., 2019). Similarly, the retrotrapezoid nucleus is chemosensitive and may contribute to the hypercapnic ventilatory response (Guyenet et al., 2016). Chemosensitive neurons in the retrotrapezoid nucleus are inhibited by A_1 receptor activation (James et al., 2018). Increased inhibition of brain areas relevant to respiratory chemoreception, such as the raphe and retrotrapezoid nuclei, may be responsible for the effect of adenosine on the hypercapnic ventilatory response. Sustained hypoxia has a biphasic effect on breathing, initially causing tachypnea but later giving way to bradypnea (Lawson and Long, 1983; Vizek et al., 1987). Rising adenosine levels have been implicated in hypoxia induced hypoventilation (Lopes et al., 1994; Yan et al., 1995), a potential contributor to SUDEP (Tao et al., 2010).

Adenosine and PGES

Postictal generalized EEG suppression refers to the period of time immediately following a seizure in which the frequency and

amplitude of EEG activity across the cortex is decreased (Lhatoo et al., 2010; Theeranaew et al., 2018). PGES has been associated with respiratory disturbances, decreased oxygen saturation, and increased postictal immobility (Seyal et al., 2013; Kuo et al., 2016; Rheims et al., 2019). Evidence from animal models indicates that adenosine plays a causal role in PGES (**Figure 2** and **Table 2**). In amygdala kindled rats, systemic pretreatment with an adenosine analog prolongs PGES (Rosen and Berman, 1985; Whitcomb et al., 1990). Pretreatment with the adenosine receptor antagonist caffeine reduces the duration of PGES following amygdala kindled seizures (Whitcomb et al., 1990). Caffeine pretreatment does not alter electrocerebral suppression following electroconvulsive therapy; however, it is not clear whether this would be true in epilepsy patients with spontaneous seizures (Rosenquist et al., 1994). Further evidence is needed to determine whether PGES in epilepsy patients is tractable to adenosinergic manipulation.

Adenosine and Spreading Depolarization

Spreading depolarization is a slow-moving wave which temporarily silences neuronal activity in the affected tissue (Leo, 1944; Pietrobon and Moskowitz, 2014). Spreading depolarization occurs in a number of diseases but is typically studied in the context of migraines (Lauritzen, 1994; Lauritzen et al., 2011). Seizures have the capacity to generate spreading depolarization waves similar to those seen in migraine patients (Kramer et al., 2017; Ssentongo et al., 2017). Adenosine levels are increased in the wake of spreading depolarization waves (Kaku et al., 1994; Lindquist and Shuttleworth, 2012, 2014; Seidel et al., 2016). The increase in extracellular adenosine brought on by spreading depolarization contributes to the suppression of neuronal activity which persists after the wave of depolarization has passed (Canals et al., 2008; Lindquist and Shuttleworth, 2012, 2017). Unlike in migraines, seizure-induced spreading depolarization can spread to the brainstem (Aiba and Noebels, 2015; Loonen et al., 2019). The propagation of spreading depolarization waves into the brainstem has been implicated as a causal factor in seizure-induced respiratory arrest and death (Aiba and Noebels, 2015; Aiba et al., 2016; Loonen et al., 2019). Whether increases in adenosine associated with seizure-induced spreading depolarization exacerbate postictal neuronal dysfunction or contribute to seizure-induced respiratory arrest has not been empirically investigated (**Figure 2** and **Table 2**).

Potential Proconvulsant Effects of A_{2A} Receptor Activation

Though increases in extracellular adenosine are generally anticonvulsant through A_1 receptor activation, there are some findings that suggest that under certain circumstances A_{2A} receptor activation can be proconvulsant, but evidence for this is mixed. Hippocampal microinjection of an A_{2A} receptor agonist increased afterdischarge duration following piriform cortex kindled seizures in rats (Zeraati et al., 2006; Hosseinmardi et al., 2007); however, in this same model, microinjection of an A_{2A} receptor antagonist did not decrease afterdischarge duration (Zeraati et al., 2006; Hosseinmardi et al., 2007). The use of caffeine, a non-selective adenosine receptor blocker, reveals a more nuanced balance between the proconvulsant effect of A_{2A} receptor activation and the opposing effects of A1 receptor activation (Fredholm et al., 1999; El Yacoubi et al., 2008). Indeed, chronic caffeine administration decreased the susceptibility to chemoconvulsants in mice, an effect that involved A_{2A} receptor blockade (El Yacoubi et al., 2008). The neuroprotection from preventing A_{2A} receptor activation was further confirmed using transgenic mice lacking A_{2A} receptors, which were more resistant to pentylenetetrazol-induced seizures (El Yacoubi et al., 2008).

In a hyperthermia model of seizure induction, the threshold for seizure development in young rats was decreased by pretreatment with an A_{2A} receptor agonist and increased by A_{2A} receptor antagonist pretreatment (Fukuda et al., 2011). These findings indicate a proconvulsant effect of A_{2A} receptor activation; however, data collected in audiogenic seizure models indicated a primarily anticonvulsant effect of A_{2A} receptor activation (De Sarro et al., 1999; Huber et al., 2002). In contrast, other studies did not find any effects of A_{2A} receptor activation on seizure activity (Young and Dragunow, 1994; Rezvani et al., 2007; Uzbay et al., 2007; Hargus et al., 2012; Akula and Kulkarni, 2014). Additional evidence is necessary to clarify the conditions under which the A_{2A} receptor has proconvulsant effects and whether these effects are significant to SUDEP (**Figure 2** and **Table 2**).

Sleep Deficits

Epilepsy and sleep are interconnected, with one affecting the other (Kotagal and Yardi, 2008; Lanigar and Bandyopadhyay, 2017). Poor sleep is known to act as a trigger for certain forms of epilepsy such as nocturnal frontal lobe epilepsy, benign epilepsy with centrotemporal spikes, and Panayiotopoulos syndrome. On the other hand, having epilepsy can contribute to sleep disturbances and disorders such as insomnia and obstructive sleep apnea (Bazil, 2003; Staniszewska et al., 2017). In this section, we highlight the role of acute seizure-induced surges in adenosine in sleep/wake regulation (Bjorness and Greene, 2009). High adenosine levels promote sleep by inhibiting cholinergic neurons in the basal forebrain (Porkka-Heiskanen et al., 1997). Consistent with this notion, manipulation of ADK affected sleep regulation in mice (Palchykova et al., 2010). Using Kv1.1 knockout mice, a model of temporal lobe epilepsy with comorbid sleep disorders, Warren et al. (2018) demonstrated that surges in adenosine in the dorsal hippocampus and lateral hypothalamus contributed to lower seizure threshold and chronic partial sleep deprivation, respectively. Taken together, these studies suggest that adenosine dysregulation in chronic epilepsy may be responsible for the sleep disruption and sleep-related co-morbidities observed in epilepsy patients (**Figure 2** and **Table 2**; Boison and Aronica, 2015). It is interesting to note that there is a strong association of SUDEP with sleep, with $\sim$ 70% of SUDEP-related deaths occurring during sleep (Ryvlin et al., 2013; Ali et al., 2017). Hence, investigating the relationship between sleep, adenosine, and epilepsy, particularly in the context of SUDEP, may yield significant insights into the pathophysiology of SUDEP.

THE DEADLY

The Adenosine Hypothesis of SUDEP

In 2010, it was observed in a kainic acid rodent seizure model that upregulating adenosine tone by inhibiting its metabolism had the seemingly paradoxical effect of initially preventing seizure activity, but then causing death when a seizure did occur (Shen et al., 2010). To explain this counterintuitive finding and, hopefully, to gain insights into the pathophysiology of SUDEP the adenosine hypothesis of SUDEP was formulated. The adenosine hypothesis of SUDEP suggests that seizure-induced increases in extracellular adenosine result in excessive inhibition of brain areas that are necessary for breathing which precipitates terminal respiratory arrest (**Figure 1** and **Table 3**; Shen et al., 2010). This adenosine based explanation of SUDEP causally links the well-known increase in adenosine during and after seizures to respiratory failure.

The adenosine hypothesis of SUDEP has significant explanatory power regarding the timing of respiratory arrest seen in SUDEP cases. As mentioned in the introduction, the best data currently available on the sequence of events which trigger SUDEP comes from a case series of SUDEP occurring in epilepsy monitoring units (Ryvlin et al., 2013). A consistent, but perplexing observation in these instances of SUDEP is that terminal respiratory failure began in the postictal period. In other words, the seizure ended, the patient was breathing for a period of 1–10 min, then the patient stopped breathing (Ryvlin et al., 2013). In this investigation, breathing was assessed by chest excursions observed by video along with the EEG artifacts associated with breathing (Ryvlin et al., 2013). This is not the most reliable method of respiratory measurement, particularly in situations where the view of the camera might be obstructed with bedding. Furthermore, quantification of tidal volume is not possible using video and EEG artifacts leaving open the possibility of severe hypoventilation prior to the terminal apnea. Nevertheless, the fact remains that the patients were breathing in the postictal period prior to the onset of fatal respiratory arrest (Ryvlin et al., 2013). This observation raises the following question: why do terminal apneas that are caused by seizures emerge when the seizure is over instead of during the seizure or at the end of the seizure? One possible clue that might be useful in answering this question is that the peak of seizure-induced adenosine surging occurs during the postictal period, not during the seizure itself (Van Gompel et al., 2014). If rising adenosine levels were responsible for the seizure-induced respiratory arrest seen in SUDEP one would expect terminal apnea to appear during the postictal period, when adenosine levels are at their highest. This prediction is borne out by clinical observations of SUDEP (Ryvlin et al., 2013).

Evidence Concerning the Role of Adenosine in SUDEP

In a kainic acid mouse seizure model, the effects of impaired adenosine clearance were investigated by inhibition of the enzymes responsible for adenosine degradation, adenosine deaminase and ADK (Shen et al., 2010). Mice were pretreated with the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (EHNA) and the ADK inhibitor 5-iodotubercidin (5-ITU) prior to seizure induction *via* kainic acid. All mice with pharmacologically impaired adenosine clearance underwent seizure-induced death, whereas there was no mortality in the animals that received a saline injection prior to seizure induction. To ascertain whether the observed mortality was due to excessive adenosine signaling, the adenosine receptor antagonist caffeine was administered after the onset of seizure activity in animals treated with kainic acid and inhibitors of adenosine clearance. It was observed that caffeine treatment delayed death, supporting the hypothesis that excessive adenosine surging may play a causal role in the seizure-induced death phenotype (Shen et al., 2010). A limitation of this investigation is the lack of respiratory, cardiac, and electrocerebral quantification which preclude any conclusions regarding the cause of death.

In a more recent study, a similar approach of pharmacological suppression of metabolic adenosine clearance prior to kainic acid seizure induction was taken in rats (Ashraf et al., 2020); however, in this investigation concomitant measurements of EEG, heart rate, blood pressure, and phrenic nerve activity were made to clarify the cause of death. Furthermore, the rats in this study were tracheostomized prior to seizure-induction to rule out the possibility of laryngospasm. Seizure-induced laryngospasm is difficult to differentiate from seizure-induced central apnea and has been hypothesized to contribute to SUDEP pathophysiology (Nakase et al., 2016; Stewart et al., 2017; Budde et al., 2018; Irizarry et al., 2020). Seizure-induced death was observed in animals treated with 5-ITU and kainic acid, but not in animals treated with 5-ITU or kainic acid alone. Suppression of phrenic nerve activity preceded EEG flattening, cardiovascular failure, and death indicating a primarily respiratory cause of death. Unexpectedly, 5-ITU and kainic acid administration resulted in abnormal partial phrenic nerve bursts. These partial bursts were reduced by treatment with caffeine suggesting that they were related to excessive adenosine signaling. The precise cause of these partial phrenic nerve bursts and whether they occur in other seizure models has yet to be determined (Ashraf et al., 2020).

Audiogenic seizures in DBA/2 mice are a frequently used and well characterized model of seizure-induced death (Tupal and Faingold, 2006; Faingold et al., 2011; Irizarry et al., 2020). When subjected to a high intensity broadband acoustic stimulus, susceptible DBA/2 mice experience seizures that can evolve into seizure-induced respiratory arrest and death. Inhibition of adenosine metabolism by 5-ITU pretreatment was associated with an increased incidence of seizure-induced respiratory arrest. Conversely, caffeine pretreatment reduced the incidence of seizure-induced respiratory arrest. These findings indicate that excessive adenosine signaling may contribute to seizure-induced death in this model. Pretreatment with SCH 442416, an A_{2A} receptor antagonist, reduced the incidence of seizure-induced death. On the other hand, the A_1 receptor antagonist DPCPX did not alter the probability of seizure-induced death suggesting that excessive A_{2A} receptor activation is the driving force in the effect of adenosine on vulnerability to seizure-induced death (Faingold et al., 2016).

TABLE 3 | Experimental evidence which directly supports the adenosine hypothesis of SUDEP.

The Deadly: Seizure-induced increases in extracellular adenosine may precipitate SUDEP by excessive inhibition of brain areas that are necessary for breathing.

Reference	Seizure model	Core findings
Shen et al., 2010	Kainic acid in unanesthetized mice	Increasing adenosinergic tone by inhibiting adenosine metabolism initially prevented seizure activity, but later precipitated seizure-induced death. This mortality was delayed by an adenosine receptor antagonist.
Ashraf et al., 2020	Kainic acid in anesthetized and tracheostomized rats	Seizure-induced death was only observed in rats with inhibited adenosine metabolism. Death was the result of central respiratory arrest as opposed to cardiac failure or laryngospasm. Impaired adenosine metabolism during seizures resulted in abnormal partial phrenic nerve bursts which were reduced by treatment with an adenosine receptor antagonist.
Faingold et al., 2016	DBA/2 audiogenic seizures	Pharmacological inhibition of adenosine metabolism increased the likelihood of seizure-induced death. Non-selective and A_{2A} specific adenosine receptor antagonism decreased the likelihood of seizure-induced death.
Kommajosyula et al., 2016	GEPR-9 audiogenic seizures	Inhibition of adenosine metabolism prolonged postictal motor impairment, exacerbated respiratory dysfunction, and increased the probability of death.

Like DBA/2 mice, genetically epilepsy-prone rats (GEPR-9s) experience seizures and seizure-induced respiratory disruption following exposure to a high intensity broadband acoustic stimulus (Faingold, 1988). Seizures in GEPR-9s result in a period of postictal immobility, as indicated by a loss of the righting reflex, and respiratory disruption (Jobe et al., 1995; Kommajosyula et al., 2016). Seizures in GEPR-9s sometimes result in death; however, unlike the DBA/2 mouse, death is uncommon and does not immediately occur after the seizure (Kommajosyula et al., 2016). In this model, pharmacological inhibition of adenosine clearance by co-administration of EHNA and 5-ITU prolonged postictal motor impairment, exacerbated respiratory dysfunction, and increased the probability of death (Kommajosyula et al., 2016).

DISCUSSION

In summary, the influence of adenosine signaling in the context of epilepsy is nuanced and cannot be indiscriminately categorized as either beneficial or harmful (**Figure 2** and **Tables 1–3**). Insufficient adenosine signaling results in inadequate neuronal inhibition, increased vulnerability to seizures, and the potentially fatal outcome of status epilepticus (Dragunow et al., 1985; Murray et al., 1985; Kochanek et al., 2006); however, paradoxically, excessive adenosine signaling may worsen periictal breathing, exacerbate PGES, and potentiate SUDEP (Rosen and Berman, 1985; Shen et al., 2010; Faingold et al., 2016; Kommajosyula et al., 2016). Though our understanding of the role of adenosine in epilepsy is rapidly improving there are still many unresolved questions and weaknesses in the existing literature that impede the development of adenosine-based therapeutic strategies to prevent SUDEP.

The adenosine hypothesis of SUDEP is largely predicated on the assumption that seizure-induced adenosine surging occurs in the brainstem, where adenosine is known to suppress breathing (**Figure 1**; Douglas et al., 1982; Yan et al., 1995; Gettys et al., 2013). Seizure-induced adenosine surging has primarily been studied in the context of seizure termination as opposed to seizure-induced death. As a result, seizure-induced adenosine surging has been identified in the hippocampus (**Figure 1**; During and Spencer, 1992; Berman et al., 2000; Aden et al., 2004; Etherington et al., 2009) and the cortex (Schrader et al., 1980; Van Gompel et al., 2014), but never directly measured in the brainstem. Characterizing the peri-ictal changes in adenosine levels in brainstem respiratory nuclei and nuclei previously implicated in SUDEP pathophysiology will be crucial to validating the adenosine hypothesis of SUDEP (**Figure 1**).

As discussed in the main body of this review, there are a number of mechanisms responsible for activity-dependent adenosine release. The relative contributions of these mechanisms appear to be regionally dependent. The mechanisms of activity dependent changes in adenosine signaling have primarily been studied using spontaneously occurring or electrically evoked neuronal activity. Insights gained regarding the mechanisms of activity dependent adenosine release in the context of evoked or naturally occurring neural activity may, or may not, be generalizable to seizures. Future investigations should examine the mechanisms underlying seizure-induced adenosine release and elucidate the spread of adenosine wave to the brainstem. Spreading depolarization waves result in an increase in extracellular adenosine which prolongs neuronal dysfunction (Canals et al., 2008; Lindquist and Shuttleworth, 2012, 2017); however, whether seizure-induced spreading depolarization waves elevate extracellular adenosine levels or whether such and increase might contribute to SUDEP is unknown.

Anatomically, where adenosine acts to potentiate SUDEP is unknown. Adenosine suppresses breathing in a variety of brainstem sites (Douglas et al., 1982; Yan et al., 1995; Gettys et al., 2013); however, direct evidence on seizure-induced alteration in adenosine levels in these brain areas is lacking. Deficits in serotonergic neurotransmission have been consistently implicated in SUDEP pathophysiology (Richerson and Buchanan, 2011; Faingold et al., 2014; Massey et al., 2014; Zhan et al., 2016; Petrucci et al., 2019). The mechanisms responsible for periictal suppression of serotonergic activity are unknown. The inhibition of serotonin neurons during and after

seizures may be the result of adenosine surging; however, this has not been empirically investigated.

In addition to the inhibitory effect of adenosine on serotonergic neurotransmission, which is discussed in more detail in the "*Adenosine and breathing*" subsection, adenosine modulates the signaling of a number of other neurotransmitter systems which are relevant to epilepsy and seizure-induced death. Presynaptic A_1 receptor activation inhibits the synaptic release of glutamate in brain areas notable for seizure activity, such as the hippocampus (Burke and Nadler, 1988). In contrast, A_{2A} receptor activation facilitates hippocampal and striatal glutamate release ostensibly by diminishing the inhibitory influence of A_1 receptor activation (Popoli et al., 1995; Lopes et al., 2002). Correspondingly, A_1 receptor antagonism increases glutamate release (Di Iorio et al., 1996; Quarta et al., 2004), whereas A_{2A} receptor antagonism decreases it (Popoli et al., 2003; Quarta et al., 2004).

Similar to glutamate, GABA release is decreased by A_1 receptor activation and increased by A_{2A} receptor activation (Jeong et al., 2003; Hong et al., 2005; Yum et al., 2008). A_{2A} receptor mediated excitation of GABA releasing neurons in the respiratory brainstem has been used to explain the observation that both A_1 and A_{2A} receptor agonists suppress breathing, despite their divergent effects on neuronal excitability (Wilson et al., 2004; Mayer et al., 2006).

Like serotonin, the monoaminergic transmitter norepinephrine may be protective against seizure-induced death. The norepinephrine reuptake inhibitor, atomoxetine, reduces the likelihood of seizure-induced death following maximal electroshock and audiogenic seizures (Zhang et al., 2017; Zhao et al., 2017; Kruse et al., 2019). Adenosine suppresses neuronal activity in the locus coeruleus (Shefner and Chiu, 1986) and focally inhibits norepinephrine release in the cortex (Harms et al., 1978; Taylor and Stone, 1980). Whether adenosinergic inhibition of norepinephrine neurons alters vulnerability to seizure-induced death has yet to be empirically investigated.

Arousal promoting cholinergic structures in the basal forebrain and brainstem are inhibited by adenosine (Rainnie et al., 1994; Porkka-Heiskanen et al., 1997; Peng et al., 2020). Seizures suppress the activity of cholinergic neurons in the basal forebrain and pedunculopontine tegmental nucleus (Motelow et al., 2015). Given the hypothesized role of the ascending arousal system in the prevention of SUDEP (Massey et al., 2014), the role of adenosinergic inhibition of the cholinergic system during seizures should be investigated.

Most of the experimentation pertinent to the role of adenosine in seizure-induced death has been conducted in acute seizure models, often in seizure-naïve animals. Epileptogenesis and the occurrence of repeated seizures alters the brain in ways that might be meaningful to SUDEP; for instance, an altered expression/function of ADK and adenosine receptors has been noted during epileptogenesis (Patodia et al., 2020). Therefore, it would be beneficial to use models of epilepsy which feature spontaneous seizures and spontaneous seizure-induced death for investigating SUDEP. To this end, *Kcna1*$^{-/-}$ mice, which lack voltage-gated Kv1.1 channels, experience spontaneous seizures and undergo seizure-induced death at approximately postnatal day 50 (Moore et al., 2014). Dravet syndrome is a severe infantile onset epilepsy with a high rate of SUDEP (Dravet, 1978; Genton et al., 2011). Dravet syndrome is the result of mutations in the *Scn1a* gene which encodes the voltage-gated sodium channel Nav1.1. Mice with similar mutations display phenotypes comparable to those seen in Dravet syndrome, including seizure-induced death which might prove to be an effective tool for SUDEP investigations (Ito et al., 2013; Kalume et al., 2013; Kim et al., 2018).

A growing body of evidence implicates adenosine signaling in a variety of adverse seizure outcomes such as respiratory suppression, PGES, and SUDEP (**Figure 2** and **Tables 2**, **3**); however, it is not yet clear how this information can be leveraged to inform clinical preventative strategies. Systemic adenosine receptor antagonism may reduce vulnerability to SUDEP, but there are a number of reasons why this might not be a viable clinical option. Most cases of SUDEP occur during the night, presumably while the patient is asleep (Lamberts et al., 2012; Ali et al., 2017; Purnell et al., 2018). An adenosine antagonist, such as caffeine, taken before bed would be likely to disrupt the patients sleep (Drake et al., 2013). Sleep disruption can result in a variety of adverse health outcomes including an increased likelihood of seizures (Bennett, 1963; Mendez and Radtke, 2001; Medic et al., 2017). Because it is generally agreed that SUDEP occurs consequent to a seizure (Ryvlin et al., 2013; Massey et al., 2014), anything that might impair a patients seizure control should be avoided. Furthermore, adenosine receptor antagonists can prolong seizures potentially increasing the amplitude of the seizure-induced adenosine surge (Dragunow and Goddard, 1984; Bozymski et al., 2018). The beneficial effect of antagonizing adenosine receptors might be counteracted by a higher surge in extracellular adenosine following seizure termination. Lastly, chronic administration of adenosine receptor antagonists can result in increased adenosine receptor expression (Fredholm, 1982). An increase in adenosine receptor expression, particularly in the brainstem, might increase vulnerability to seizure-induced respiratory arrest.

Sudden unexpected death in epilepsy typically occurs during sleep (Ali et al., 2017). Given the role of changing adenosine concentrations in sleep/wake regulation (Basheer et al., 2004) and the adenosine hypothesis of SUDEP outlined in this review, the reader may arrive at the conclusion that differential adenosine concentrations during sleep might be in some way related to the increased incidence of SUDEP during sleep. It should be noted that the increase in adenosine over the course of wakefulness is primarily localized to the basal forebrain and is absent in the dorsal raphe, an arousal promoting brainstem nucleus which has been implicated in SUDEP (Porkka-Heiskanen et al., 2000; Zhang et al., 2018; Petrucci et al., 2020). Furthermore, extracellular adenosine concentrations quickly fall during sleep to levels lower than those seen during wakefulness (Porkka-Heiskanen et al., 1997). Thus, it is not clear whether fluctuations in adenosine are related to the increased rate of SUDEP during sleep.

By improving our understanding of periictal adenosine dynamics and developing novel strategies for influencing adenosinergic signaling we may gain insights into

how seizures and their most tragic sequelae can be prevented. Therapeutically, adenosine augmentation strategies are some of the most effective strategies for seizure control (Gouder et al., 2003; Boison, 2012), however, SUDEP risk needs to be considered and local adenosine augmentation strategies might be the most effective (**Figure 1**; Boison, 2009). In addition to the benefits noted here, adenosine augmentation might also help improve affective, psychiatric, and cognitive impairments (Boison and Aronica, 2015); co-morbidities that are prevalent among patients with epilepsy (Gaitatzis et al., 2004; LaFrance et al., 2008). Most importantly, novel findings show that adenosine therapy can prevent epilepsy development through an epigenetic mechanism (Williams-Karnesky et al., 2013; Lusardi et al., 2015; Sandau et al., 2019). Those strategies employed only transiently in a pre-epileptic brain are not expected to be associated with increased SUDEP risk, rather it is intended to avert epilepsy, and thereby prevent the primary antecedent to seizure-induced death.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Adenosine A_{2A} Receptors as Biomarkers of Brain Diseases

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Extracellular adenosine is produced with increased metabolic activity or stress, acting as a paracrine signal of cellular effort. Adenosine receptors are most abundant in the brain, where adenosine acts through inhibitory A_1 receptors to decrease activity/noise and through facilitatory A_{2A} receptors ($A_{2A}R$) to promote plastic changes in physiological conditions. By bolstering glutamate excitotoxicity and neuroinflammation, $A_{2A}R$ also contribute to synaptic and neuronal damage, as heralded by the neuroprotection afforded by the genetic or pharmacological blockade of $A_{2A}R$ in animal models of ischemia, traumatic brain injury, convulsions/epilepsy, repeated stress or Alzheimer's or Parkinson's diseases. $A_{2A}R$ overfunction is not only necessary for the expression of brain damage but is actually sufficient to trigger brain dysfunction in the absence of brain insults or other disease triggers. Furthermore, $A_{2A}R$ overfunction seems to be an early event in the demise of brain diseases, which involves an increased formation of ATP-derived adenosine and an up-regulation of $A_{2A}R$. This prompts the novel hypothesis that the evaluation of $A_{2A}R$ density in afflicted brain circuits may become an important biomarker of susceptibility and evolution of brain diseases once faithful PET ligands are optimized. Additional relevant biomarkers would be measuring the extracellular ATP and/or adenosine levels with selective dyes, to identify stressed regions in the brain. $A_{2A}R$ display several polymorphisms in humans and preliminary studies have associated different $A_{2A}R$ polymorphisms with altered morphofunctional brain endpoints associated with neuropsychiatric diseases. This further prompts the interest in exploiting $A_{2A}R$ polymorphic analysis as an ancillary biomarker of susceptibility/evolution of brain diseases.

Keywords: adenosine A_{2A} receptors, central nervous system, antagonism, caffeine, biomarkers, polymorphisms

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INTRODUCTION

The increased use of intracellular ATP, either because of increased workload or need to cope with stressful conditions, is a main source of increased extracellular levels of adenosine, which generally acts as a paracrine allostatic regulator by locally decreasing metabolism through inhibitory A_1 receptors (A_1R) and increasing metabolic supply through $A_{2A}R$ (Agostinho et al., 2020). Adenosine receptors are most abundant in the brain, where adenosine fulfills a role as neuromodulator apart from its general paracrine allostatic role: post-synaptic as well as astrocytic integrative activity are major contributors of an adenosine tone acting through inhibitory A_1 receptors to decrease activity/noise in excitatory synapses; ATP release, characteristic of increased firing rate conditions associated with synaptic plasticity, is the major source of a second pool of synaptic extracellular adenosine selectively activating facilitatory A_{2A} receptors ($A_{2A}R$) to promote synaptic plastic

changes in physiological conditions (Cunha, 2016). However, ATP is also a general danger signal in the brain (Rodrigues et al., 2015), acting through a variety of ATP/ADP-activated P_2 receptors to re-shape the function of astrocytes and microglia to cope with potential threats (Agostinho et al., 2020). Such threats also require adaptive plastic changes in neuronal circuits, which may explain the increased extracellular formation of ATP-derived adenosine by ecto-nucleotidases, with a burst of its rate-limiting step—ecto-5′-nucleotidase or CD73 (Cunha, 2001)—under noxious brain conditions to sustain an overfunction of $A_{2A}R$ that contributes to synaptotoxicity and neurotoxicity in different brain diseases (Cunha, 2016).

ADENOSINE A_{2A} RECEPTORS IN BRAIN DISEASES

Upon acute brain injury, probably best exemplified by an ischemic brain stroke, concurrent pharmacological and genetic evidence show that $A_{2A}R$ blockade affords a robust neuroprotection (reviewed in Chen and Pedata, 2008). In parallel, ischemia is accompanied by ATP release (Melani et al., 2005) and up-regulation of CD73 (Braun et al., 1997), thus increasing the formation of extracellular ATP-derived adenosine (Koos et al., 1997; Chu et al., 2014). Likewise, seizure-like activity characteristic of epileptic conditions triggers a neurodegeneration that is critically controlled by pharmacological or genetic $A_{2A}R$ blockade (Canas et al., 2018). Seizure activity also increases ATP release (Wieraszko and Seyfried, 1989) and up-regulates CD73 (e.g., Schoen et al., 1999; Rebola et al., 2003), increasing the contribution of extracellular ATP-derived adenosine formation to overactivate $A_{2A}R$ (reviewed in Tescarollo et al., 2020). $A_{2A}R$ blockade also attenuates brain damage following traumatic brain injury (TBI) (e.g., Li et al., 2009); TBI also bolsters the release of ATP (Faroqi et al., 2021) and CD73 levels (Zheng et al., 2020), although the contribution of extracellular ATP-derived adenosine has not yet been tested in TBI.

Overall, this evidence is compatible with an increase of extracellular adenosine, namely extracellular ATP-derived adenosine, leading to an overactivation of $A_{2A}R$ that contributes for brain dysfunction upon acute brain injury. A similar scenario seems to occur in chronic brain conditions. Thus, the pharmacological or genetic blockade of $A_{2A}R$ affords a consistent neuroprotection in animal models of Alzheimer's disease (AD) (e.g., Canas et al., 2009; Laurent et al., 2016; Viana da Silva et al., 2016), Parkinson's disease (PD) (reviewed in Schwarzschild et al., 2006)—where $A_{2A}R$ antagonists were approved by the US-FDA as novel anti-Parkinsonian drugs (Chen and Cunha, 2020), repeated stress/depression (Batalha et al., 2013; Kaster et al., 2015; Padilla et al., 2018), Machado-Joseph disease (Gonçalves et al., 2013), amyotrophic lateral sclerosis (ALS) (Ng et al., 2015; Rei et al., 2020; Seven et al., 2020), Angelman syndrome (Moreira-de-Sá et al., 2020, 2021), or glaucoma-like disorders (Madeira et al., 2015). Most of these chronic neuropsychiatric conditions are also associated with increased release of ATP, as occurs in animal models of AD (Gonçalves et al., 2019), PD (Carmo et al., 2019; Meng et al., 2019) or as concluded by the anti-depressant effects of P2 receptor antagonists (Ribeiro et al., 2019; but see Cao et al., 2013). Moreover, there is an increased contribution of extracellular ATP-derived adenosine for $A_{2A}R$ overactivation in chronic brain diseases, as best heralded by the observation that CD73 knockout mice phenocopy $A_{2A}R$ knockout mice (Augusto et al., 2013; Carmo et al., 2019; Gonçalves et al., 2019).

$A_{2A}R$ overactivation is not only necessary, but actually sufficient to trigger brain dysfunction, as concluded from the observation that the pharmacological overactivation of $A_{2A}R$ (Pagnussat et al., 2015), the optogenetic activation of $A_{2A}R$ transducing system (Li et al., 2015) or the over-expression of $A_{2A}R$ in the hippocampus (Coelho et al., 2014; Carvalho et al., 2019; Temido-Ferreira et al., 2020) are sufficient to trigger or aggravate brain dysfunction. Notably, $A_{2A}R$ overfunction seems to be an early event in different brain disorders (reviewed in Cunha, 2016), although $A_{2A}R$ antagonists seem to maintain their neuroprotective profile after the establishment of symptoms (e.g., Kaster et al., 2015; Faivre et al., 2018; Orr et al., 2018; Silva et al., 2018).

The tight association between increased release of ATP and its extracellular catabolism to overactivate $A_{2A}R$ as part of the expression of neuronal dysfunction at the onset and throughout the evolution of several brain diseases prompts exploiting this danger signaling pathway as new biomarkers to identify dysfunctional brain circuits in brain diseases. Although the tools are yet to developed, it may be promising to devise soluble sensors to detect altered levels of extracellular ATP to allow an *in vivo* estimate of brain circuits undergoing a particular purinergic pressure and, consequently, are at risk of undergoing dysfunction. An alternative could be the development of PET ligands (not yet available) to assess the density of CD73, which is paramount to link ATP upsurge with the selective overactivation of $A_{2A}R$; CD73 seems to be consistently up-regulated upon brain stressful conditions and may be a selective biomarker of glia and synapses undergoing adaptive processes (Schoen and Kreutzberg, 1997).

UP-REGULATION OF ADENOSINE A_{2A} RECEPTORS IN BRAIN DISEASES

The $A_{2A}R$ overactivation associated with brain dysfunction and disease is not only sustained by an increased bioavailability of the trigger of $A_{2A}R$—ATP-derived extracellular adenosine—but also involves an up-regulation of $A_{2A}R$ in the afflicted brain areas (reviewed in Cunha, 2016). Indeed, an increased density of cortical $A_{2A}R$ has been reported in animal models of epilepsy (Rebola et al., 2005; Cognato et al., 2010; Canas et al., 2018; Crespo et al., 2018), Rasmussen's encephalopathy (He et al., 2020), TBI (Zhao et al., 2017), AD (Espinosa et al., 2013; Viana da Silva et al., 2016; Silva et al., 2018), Lyme neuroborreliosis (Smith et al., 2014), ALS (Seven et al., 2020), or chronic stress/depression (Kaster et al., 2015; Machado et al., 2017), as well as in the diseased human brain (Albasanz et al., 2008; Temido-Ferreira et al., 2020). Likewise, $A_{2A}R$ levels are also increased in the cerebellum of Machado-Joseph's ataxic mice (Gonçalves et al., 2013) and in the

amygdala or fear-conditioned mice (Simões et al., 2016). $A_{2A}R$ up-regulation is in fact an upsurge since it occurs shortly (within hours) after abnormal neuronal function (i.e., convulsions; Canas et al., 2018), but it gradually increases with aggravation of brain dysfunction (Temido-Ferreira et al., 2020). $A_{2A}R$ up-regulation mostly occurs in synapses, in accordance with the involvement of synaptic alterations at the onset of most brain diseases (e.g., Rebola et al., 2005; Kaster et al., 2015; Viana da Silva et al., 2016; Canas et al., 2018), but is also observed in glia cells in the progression of chronic brain diseases (Matos et al., 2012; Orr et al., 2015; Barros-Barbosa et al., 2016; Patodia et al., 2020). It is still unclear if this $A_{2A}R$ up-regulation only involves an increased readout of $A_{2A}R$ mRNAs (Canas et al., 2018) or also involves an overexpression of $A_{2A}R$ mRNA, which has been reported in the dysfunctional or diseased brain (e.g., Costenla et al., 2011; Espinosa et al., 2013; Hu et al., 2016; Dias et al., 2021). In fact, the triggers and mechanisms of this $A_{2A}R$ up-regulation in the diseased brain are essentially unknown. The $A_{2A}R$ gene in both

TABLE 1 | Summary of the reported associations between known polymorphic variants of the human adenosine A_{2A} receptor gene (*ADORA2A*) and different response susceptibility to either pathological threats **(A)** or distinct physiological responses to external stimulus **(B)**.

(A) SNP	Type/Position	Risk allele/Genotype	Associated CNS Disorder	References
rs5751876	Exon 2	TT	Huntington's disease (significant variability in age of onset)	Taherzadeh-Fard et al., 2010
		T	Susceptibility locus for Panic Disorder and Agoraphobia	Deckert et al., 1998; Hamilton et al., 2004; Domschke et al., 2012
		T	Prevalent in Gilles de la Tourette syndrome (GTS) patients	Janik et al., 2015
rs2298383	5′ UTR	TT	Huntington's disease (significant variability in age of onset)	Taherzadeh-Fard et al., 2010
		CT	Higher predisposition for Childhood Epilepsy (CE)	Fan et al., 2020
		CC	Greater risk for CE patients to develop comorbid neurologic disorders	Fan et al., 2020
		CC/CT	Increased risk of Depression, Attention Deficits and Sleep-disturbances	Oliveira et al., 2019
		CC	Possible risk factor for Schizophrenia	Miao et al., 2019
rs2236624	Intron 4	CC	Associated with Autism Spectrum Disorders symptom severity	Freitag et al., 2010
rs71651683	5′ UTR	T	Inverse association with the likelihood to develop Parkinson's disease (∼49%)	Popat et al., 2011
rs5996696	Promoter variant region	C	Inverse association with the likelihood to develop Parkinson's disease (∼30%)	Popat et al., 2011
rs2298383, rs5751876, rs35320474, and rs4822492	5′ UTR, Exon 2, Exon 4, and 3′ UTR	C, T, deletion and C, respectively, (Haplotype A)	Predisposition of children to develop Acute Encephalopathy with biphasic seizures and late reduced diffusion (AESD)	Shinohara et al., 2013
(B) SNP	**Type/Position**	**Risk allele/Genotype**	**Physiological Response to Stimuli**	**References**
rs5751876	Exon 2	TT	Significant enhancement of caffeine-induced anxiety	Alsene et al., 2003; Childs et al., 2008
		TT	Associated with an overall lower caffeine intake and a prospective lesser vulnerability to caffeine dependence	Cornelis et al., 2007
		TT	Highest startle magnitudes upon caffeine administration in response to unpleasant pictures (maladaptive emotional processing)	Domschke et al., 2012
		TT	Associated with an ergogenic beneficial effect upon caffeine consumption	Loy et al., 2015
		TT	Higher anxiogenic response susceptibility to caffeine ingestion in usual non-consumers or low consumers (<40 mg per day), but no significant correlation with habitual caffeine intake	Rogers et al., 2010
		C	Sleep disturbances (and insomnia) triggered by caffeine intake, higher β-activity in non-REM sleep	Rétey et al., 2007
		T	Lower total sleep time in habitual low caffeine consumers	Erblang et al., 2019
		T	Increased sleep latency associated with caffeine consumption, lower percentage of N3 sleep stage	Nunes et al., 2017
rs2298383	5′ UTR	CC	Significant enhancement of caffeine-induced anxiety	Childs et al., 2008
		C	Lower total sleep time in habitual low caffeine consumers	Erblang et al., 2019
rs4822492	3′ UTR	CC	Significant enhancement of caffeine-induced anxiety	Childs et al., 2008
		CC	Lower total sleep time in habitual low caffeine consumers	Erblang et al., 2019
rs3761422	5′ UTR	TT	Greater increase in anxiety upon caffeine ingestion in habitual non-consumers or low consumers (<40 mg per day)	Rogers et al., 2010
		T	Lower total sleep time in habitual low caffeine consumers	Erblang et al., 2019

rodents and humans has a complex promoter region and can give raise to multiple transcripts (Peterfreund et al., 1996; Lee et al., 2003a; Yu et al., 2004; Kreth et al., 2008; Huin et al., 2019). Although multiple controllers of the $A_{2A}R$ gene have been proposed, such as methylation patterns of the promoter (Falconi et al., 2019; Micioni Di Bonaventura et al., 2019), transcription factors ZBP-89 and Yin Yang-1 (Buira et al., 2010), microRNAs (e.g., Heyn et al., 2012; Villar-Menéndez et al., 2014; Zhao et al., 2015; Tian et al., 2016), NFk-B (Morello et al., 2006), cAMP-response element-binding protein (Chiang et al., 2005), hypoxia inducible factor-2α (Ahmad et al., 2009; Brown et al., 2011), AP1 transcription factor (Kobayashi and Millhorn, 1999; Lee et al., 2014), or nuclear factor 1 (Lee et al., 2003b), the regulation of the relative expression of these transcripts is largely unknown (Yu et al., 2004; Huin et al., 2019) and little is also known about the relative stability of the different mRNA transcripts. This is certainly an area of research that might open new avenues to design neuroprotective strategies linked to $A_{2A}R$.

The association of $A_{2A}R$ up-regulation with brain diseases offers another promising opportunity to develop informative biomarkers of the susceptibility and/or evolution of different brain diseases once PET ligands are optimized to detect extra-striatal $A_{2A}R$. In fact, $A_{2A}R$ throughout the brain are most abundant in the striatum (reviewed in Svenningsson et al., 1999) and the available PET ligands have been optimized to detect striatal $A_{2A}R$ (e.g., Mishina et al., 2011; Ishibashi et al., 2018); however, this population of $A_{2A}R$ has a different pharmacology (Orrú et al., 2011; Cunha, 2016), a different adaptive profile (Cunha et al., 1995) and a different role in most brain conditions (Shen et al., 2008, 2013; Yu et al., 2008; Wei et al., 2014). Thus, it is likely that the currently available PET ligands might not be useful to assess modifications of extra-striatal $A_{2A}R$. New cortical $A_{2A}R$-directed PET ligands need to be designed based on the particular properties and interacting partners of cortical $A_{2A}R$ (reviewed in Franco et al., 2020) to allow an *in vivo* detection of $A_{2A}R$ upsurge as potential general biomarkers of brain dysfunction (Sun et al., 2020).

$A_{2A}R$ are not only located in the brain, but are also present in several peripheral tissues, namely in different blood cells such as leukocytes and platelets (reviewed in Gessi et al., 2000). Based on the association of brain diseases with $A_{2A}R$ up-regulation in afflicted brain regions, several studies explored if $A_{2A}R$ in blood cells could be biomarkers of brain diseases, such as AD (Arosio et al., 2010, 2016; Merighi et al., 2021), PD (Falconi et al., 2019), or ALS (Vincenzi et al., 2013). However, only the understanding of the mechanisms underlying $A_{2A}R$ up-regulation in brain diseases will allow providing a rationale (or lack of thereof) to consider alterations of the density of peripheral $A_{2A}R$ as valid readouts of altered $A_{2A}R$ density that occurs selectively in afflicted brain circuits in the diseased brain.

POLYMORPHISMS OF ADENOSINE A_{2A} RECEPTORS AND BRAIN DISEASES

The gene encoding human $A_{2A}R$ (ADORA2A gene) harbors several single nucleotide polymorphisms (SPNs), which have been associated to an altered susceptibility to several neuropsychiatric and neurodegenerative disorders (Huin et al., 2019). In fact, as listed in **Tables 1A,B**, naturally occurring variabilities in the ADORA2A gene collectively influence predisposition risk and even age of onset for several CNS disorders as well as individual susceptibility to the anxiogenic and sleep-related consequences of caffeine. Although, it is still unknown if the different $A_{2A}R$ polymorphisms are associated with a different expression, subcellular location, trafficking, heteromerization or pharmacological properties of $A_{2A}R$, the relation between $A_{2A}R$ polymorphisms and the susceptibility and age of onset of brain dysfunction prompts the interest in exploiting $A_{2A}R$ polymorphic analysis as an ancillary biomarker of susceptibility/evolution of brain diseases.

DISCUSSION

$A_{2A}R$ overfunction is necessary and actually sufficient for the expression of neuronal dysfunction upon brain diseases. In particular, $A_{2A}R$ overfunction associated with aberrant synaptic plasticity and synaptotoxicity seems to be associated with the onset of symptoms of brain diseases. However, some of these symptoms are comorbidities of other brain diseases, associated with their aggravation, which often involves a spreading of neuroinflammation, also known to be controlled by $A_{2A}R$. Thus, it is also likely that $A_{2A}R$ overfunction might be also associated with the evolution of brain diseases. These neuropathological roles of $A_{2A}R$ prompts considering the exploitation of this system as candidate biomarkers of the susceptibility and evolution of brain diseases. The development of PET ligand with adequate signal-to-noise ratio and selectivity to detect the relevant extra-striatal $A_{2A}R$ may allow a minimally invasive assessment of $A_{2A}R$ in different brain regions. This may be complemented by the definition of $A_{2A}R$ polymorphisms as an ancillary biomarker for the susceptibility and evolution of brain diseases, which still requires a firm establishment of structural-functional relationships between $A_{2A}R$ polymorphisms and brain dysfunction. Finally, the future development of PET-based sensors of extracellular ATP and/or adenosine may well be of additional interest as a biomarker of the status of brain diseases to be used in complement of other available methods.

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All authors contribute to the organization and writing of the review.

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REVIEW
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Inosine as a Tool to Understand and Treat Central Nervous System Disorders: A Neglected Actor?

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Since the 1970s, when ATP was identified as a co-transmitter in sympathetic and parasympathetic nerves, it and its active metabolite adenosine have been considered relevant signaling molecules in biological and pathological processes in the central nervous system (CNS). Meanwhile, inosine, a naturally occurring purine nucleoside formed by adenosine breakdown, was considered an inert adenosine metabolite and remained a neglected actor on the purinergic signaling scene in the CNS. However, this scenario began to change in the 1980s. In the last four decades, an extensive group of shreds of evidence has supported the importance of mediated effects by inosine in the CNS. Also, inosine was identified as a natural trigger of adenosine receptors. This evidence has shed light on the therapeutic potential of inosine on disease processes involved in neurological and psychiatric disorders. Here, we highlight the clinical and preclinical studies investigating the involvement of inosine in chronic pain, schizophrenia, epilepsy, depression, anxiety, and in neural regeneration and neurodegenerative diseases, such as Parkinson and Alzheimer. Thus, we hope that this review will strengthen the knowledge and stimulate more studies about the effects promoted by inosine in neurological and psychiatric disorders.

Keywords: adenosine, uric acid, pain, depression, Alzheimer, Parkinson, memory, neural regeneration

INTRODUCTION

The history of purine nucleosides began and gained therapeutic importance in 1929 with a paper published by Drury and Szent-Gyorgyi, who described the potent actions of purine on the heart and blood vessels, nucleotides and nucleosides, ATP, and adenosine (Burnstock, 2006). In the 1970s, the hypothesis that adenosine 5′-triphosphate (ATP) was a transmitter in non-adrenergic, non-cholinergic (NANC) inhibitory nerves was confirmed, and ATP was identified as a co-transmitter in sympathetic and parasympathetic nerves. The aforementioned supported the concept of purinergic neurotransmission and enhanced interest in the role of purine nucleosides in the brain and spinal cord, which was reported in various studies (North and Verkhratsky, 2006; Burnstock, 2007, 2020). In the subsequent decades, a number of studies demonstrated that adenosine can act as a signaling molecule essential for biological and pathological processes of the central nervous system. At

the same time, evidence showed that adenosine metabolism generates inosine, a metabolite with possible biological and pharmacological effects in the peripheral and central nervous systems (see **Figure 1**). Inosine, which was first considered an inert adenosine metabolite after being used as a nutritional supplement to improve muscle function in trained endurance runners (Williams et al., 1990; McNaughton et al., 1999), has gained special attention due to a few studies in the 1990s that demonstrated that inosine is a signaling molecule that can modulate the immune system when produced under stressful conditions, such as those that occur during injury, ischemia, and inflammation. In these situations, an elevation in extracellular inosine concentrations due to high adenosine metabolism levels caused by an increase in adenosine deaminase expression was demonstrated (Haskó et al., 2000; Garcia Soriano et al., 2001; Liaudet et al., 2002; Eltzschig et al., 2006; Eltzschig, 2009; Fredholm et al., 2011).

The theory that inosine contributes to, or mediates adenosine effects is reinforced by studies that consider inosine as a natural trigger of adenosine receptors (Jin et al., 1997; Tilley et al., 2000; Gomez and Sitkovsky, 2003; Haskó et al., 2004; Eltzschig et al., 2006). These receptors were first formally recognized by Burnstock in 1978 and are subdivided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3. The adenosine A_1 and A_3 receptors preferentially interact with members of the Gi/o family of G proteins, lowering the intracellular levels of cyclic adenosine monophosphate (cAMP), whereas the adenosine A_{2A} and A_{2B} receptors interact with members of the Gs family of G proteins, elevating intracellular cAMP (Ralevic and Burnstock, 1998; Eltzschig et al., 2006). Inosine binds to the adenosine receptors with affinities that are typically lower than that of adenosine. Although direct binding of inosine to adenosine A_{2B} receptor has not been demonstrated, there is convincing evidence suggesting that inosine interacts functionally with all four adenosine receptor subtypes to elicit various effects depending on the species' biological context (Nascimento et al., 2010; Kaster et al., 2013; Lapa et al., 2013; Muto et al., 2014; Doyle et al., 2017, 2018; Junqueira et al., 2017).

In the last four decades, a number of studies have shown that receptor-mediated inosine effects involve decreased

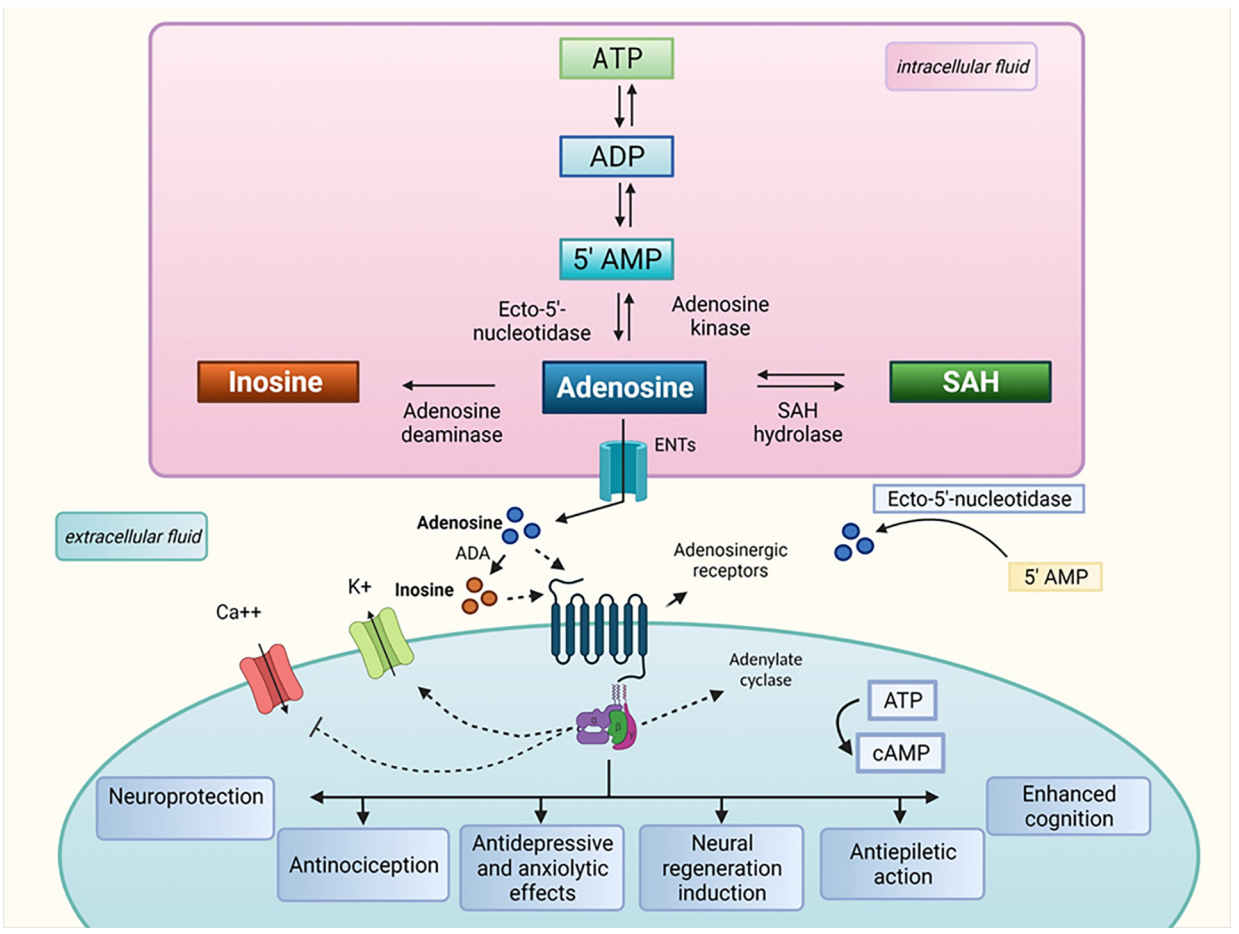

FIGURE 1 | Inosine metabolism and proposed therapeutic properties. ATP, adenosine triphosphate; ADP, adenosine diphosphate; ADA, adenosine deaminase; AMP, adenosine monophosphate; Ca++, calcium; cAMP, cyclic adenosine monophosphate; ENTs, equilibrative nucleoside transporters; K+, potassium; SAH, *S*-adenosylhomocysteine.

inflammation, neuroprotection/neuroregeneration, and axon outgrowth in the central nervous system, which elicits scavenger activities and immunomodulatory effects. These effects directly reflect the therapeutic potential observed in pathological processes involved in chronic pain, schizophrenia, depression, anxiety, and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (for a summary see **Table 1**). Given the increasing interest in the pharmacological potential of inosine as a new alternative therapy for CNS diseases, this review will focus on inosine effects in preclinical and clinical studies and describe the "state of the art" of the scientific research panorama regarding this promising molecule.

INOSINE AND PAIN

Adenosinergic and purinergic ligands are well-known modulators of acute and chronic pain of different etiologies in animals and humans both centrally and at the periphery, displaying beneficial and sometimes deleterious roles in pain pathology (Sawynok, 1998; Sawynok and Liu, 2003; Adebiyi et al., 2019). Most studies evaluating the analgesic effect of adenosine or adenosine receptor agonists have shown that these substances are able to enhance the pain threshold. However, in some cases these drugs can facilitate pain transmission. These controversial findings are probably due to the distinct signaling and distribution of adenosine receptors. Adenosine A_1 and A_3

TABLE 1 | Summary of *in vitro*, preclinical and clinical studies that have demonstrated the therapeutic potential of inosine to treat neurological and psychiatric diseases.

CNS disorder	Specie	Agent	Dose/Concentration/ Route	Effect	References
Pain	Mice	Inosine	10–300 mg/kg/i.p. and p.o. 10 μg/i.c.v./i.t.	Inosine mediates reduction time of licking in the formalin test, inhibition of glutamate-induced nociception, reduction mechanical allodynia and hyperalgesia	Nascimento et al., 2010
Pain	Mice	Inosine	10 mg/kg/i.p.	Inosine promotes antinociception through pertussis toxin sensitive G-protein coupled receptors and voltage gated K+ channel, large conductance Ca2+-activated and ATP-sensitive K+ channels or inactivation of voltage-gated Ca2+ channels	Macedo-Junior et al., 2013
Pain	Mice	Inosine	10 and 100 mg/kg/i.p	Inosine reduces flinching behavior induced by formalin and systemic and central nociception in A1-wild type mice	Nascimento et al., 2015
Pain	Mice	Inosine	200–600 nmol/i.t.	Inosine inhibits nociceptive response induced by injection of capsaicin and increases ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine, and uric acid CSF levels	de Oliveira et al., 2016
Learning and memory	Rat	Inosine	100 and 200 mg/kg/i.p.	Learning and memory improvement, levels of TNF-alpha reduction, antioxidant properties increment	Ruhal and Dhingra, 2018
Epilepsy	Rat	Inosine	500 and 1000 mg/kg, i.p.	Inosine increases number and total time of spike-waves discharges	Kovács et al., 2015a,b
Epilepsy	Mice	Inosine	400 nmol/4 μl, i.c.v.	Inosine protect against quinolinic-acid induced seizures	Ganzella et al., 2011
Alzheimer	Rat	Inosine	100 mg/kg, i.c.v.	Inosine induces memory deficit prevention and AChE activity reduction	Teixeira et al., 2020
Parkinson	Dopaminergic cells culture	Inosine	—	H_2O_2-toxic effect on dopaminergic cells reduction, indicators of free radical generation and oxidative damage in MES 23.5-astrocytes co-cultures were reduced	Cipriani et al., 2010
Parkinson	Human	Inosine	500 mg; p.o.	Average serum urate increment (more pronounced in woman), reduction of motor symptoms in UPDRS scale	Schwarzschild et al., 2019
Neural regeneration	Goldfish	Inosine	50 and 100 μM	Inosine increases retinal ganglion cells neuronal outgrowth and GAP-43 levels	Petrausch et al., 2000
Neural regeneration	PC12 cells and neuronal embryonic cells Sprague-Dawley rat fetuses	Inosine	100 μM – 1 mM	Inosine activates Mst3b kinase activity	Irwin et al., 2006
Anxiety	Mice	Inosine	10 – 300 mg/kg, i.p.	Inosine antagonizes anxiety-reducing actions of benzodiazepines	Crawley et al., 1981
Anxiety	Mice	Inosine	10 – 60 mg/kg, s.c.	Inosine presents anxiolytic effect in the Vogel-type anti-conflict test	Okuyama et al., 1998
Depression	Mice	Inosine	10 mg/kg, i.p.	Inosine reduces mice immobility time in the forced swim test and in the tale suspension test	Kaster et al., 2013
Depression	Human	Inosine plasma levels	—	Inosine plasma levels are significantly higher in individuals treated with antidepressant drugs when compared to healthy individuals	Zhou et al., 2019

receptors are coupled to inhibitory G proteins while the A_{2A} and A_{2B} are coupled to stimulatory G proteins. Further, the distribution of adenosine receptors in the periphery and central nervous system also are implicated in how these drugs act on pain modulation (Sawynok, 1998; Fredholm et al., 2011).

Reports detailing the antinociception – analgesia in pain animal models – exhibited by adenosine are not a novelty; however, until a few years ago, there were no studies evaluating the inosine antinociceptive effects. The first study that aimed to investigate the potential analgesic effects of inosine was published by our research group (Nascimento et al., 2010). This study used several acute pain animal models and demonstrated the antinociceptive action of this nucleoside in mice and rats. Inosine induced pain behavior reduction when given by oral, intraperitoneal, intratecal and intracerebroventricular, suggesting its systemic and central activity. Further, these findings suggest that inosine crosses the brain blood barrier (Nascimento et al., 2010). In addition, Nascimento and colleagues demonstrated that adenosine A_1 and A_{2A} receptors are involved in the inosine antinociceptive effects. Also, this same study evaluated that inosine was able to reduce nociception in chronic neuropathic and inflammatory pain models. It suggests that inosine acts on neural and inflammatory mechanisms to reduce pain threshold. Some years later our group using pharmacological, biochemical and genetic approaches confirmed that inosine antinociceptive action was dependent on the adenosine A_1 receptor. Finally, this study proved that inosine is an endogenous agonist of adenosine A_1 receptor (Nascimento et al., 2015).

In addition to the involvement of adenosine receptors in the antinociceptive effects of inosine, Macedo-Junior et al. (2013) showed that the inosine effects involves the activation of voltage gated K^+ channels, large conductance $Ca2^+$ and ATP-sensitive channels and the blockade of voltage-gated $Ca2^+$ channels. The involvement of these ion channels could occur through adenosine A_1 or A_{2A} receptor activation. Peripherally, the inosine antinociception is mediated only by adenosine A_1 receptors but not by A_{2A} (Macedo-Júnior et al., 2021).

Apart from the inosine binding to adenosine A_1 receptor to reduce pain, a study has shown that spinal administration of inosine increased the CFS levels of ATP, ADP, AMP, adenosine, hypoxanthine, xanthine, and uric acid (de Oliveira et al., 2016). These results suggest that inosine when reach the CNS could induce antinociceptive effect through these other nucleosides, because most of them have presented antinociceptive effects too (Sawynok and Liu, 2003).

Regarding the effects of inosine in humans, the clinical studies evaluating the involvement of inosine on pain are quite scarce. There are no clinical trial studies evaluating the analgesic effect of inosine in any kind of pain. However, there is some evidence that inosine could participate or be involved in clinical pain mechanisms. A study by Schmidt and coworkers found changes in extracellular inosine levels in patients experiencing pain. The authors suggest that shifts in neuronal and glial energy metabolism might be correlated with pain transmission mechanisms. CSF levels of inosine and other purines associated with ATP metabolism are significantly increased in chronic pain patients compared to control patients and are significantly correlated with pain intensity measured using a visual analog scale (Schmidt et al., 2010). Conversely, serum purine metabolites are increased in fibromyalgia patients (i.e., the dysregulation of pain pathways leading to central sensitization) compared to controls. In these patients, the magnitude of inosine detection was substantially higher than that of other metabolites (Fais et al., 2013).

Inosine is also a potential biomarker in plasma from patients experiencing non-traumatic chest pain or unstable angina due to potential acute cardiac ischemia/myocardial infarction (Farthing et al., 2007; Ameta et al., 2016). High levels of this nucleoside have already been reported in mouse hearts (Farthing et al., 2006) and in observational studies in humans (Al-Shamiri et al., 2009; Farthing et al., 2011). However, early evidence has shown that adenosine, but not inosine, may cause chest pain in healthy individuals. Then, the high levels of adenosine would be responsible for these inosine increments (Lagerqvist et al., 1990).

While the mechanisms mediated by inosine in nociceptive and neuropathic pain in animals have been studied in the last years and the results are encouraging, we need further studies to test and to better understand these actions. On the other hand, regarding clinical research on pain, there is a complete avenue to be explored and filled before we can say that this nucleoside could be useful in pain treatments.

INOSINE AND COGNITION

Very few studies have demonstrated that inosine can increase learning or memory, however, two recent non-clinical studies have shown promising results. The study by Ruhal and Dhingra (2018) used behavioral, biochemical, and histological techniques to demonstrate that inosine can induce learning and memory improvement in aged female rats. Inosine given by intraperitoneal route improved the learning and memory of aged rats in the Morris water maze and elevated plus-maze test. The reduced levels of TNF-α explain these results in the hippocampus and cerebral cortex. In addition, inosine increased the levels of GSH in the hippocampus and the activity of SOD in the cortex and hippocampus, and reduced the level of malondialdehyde, a marker of oxidative stress, in the hippocampus and cerebral cortex (Ruhal and Dhingra, 2018). Furthermore, inosine reduced the degeneration of cells in the CA1 region of the hippocampus, the most critical hippocampal region for memory acquisition, consolidation, and evocation (Ruhal and Dhingra, 2018).

Animal models of Alzheimer's disease (AD) have also been used to study the effects of inosine. In one study, AD was induced by streptozotocin (STZ), and the animals received inosine by intracerebroventricular route for 25 days (Teixeira et al., 2020). It is well demonstrated that administration of STZ in the brain induces changes similar to those found in AD such as learning and memory deficit, mitochondrial abnormalities, oxidative stress, neuronal cell damage and brain glucose metabolism alterations (Teixeira et al., 2020). This study demonstrated that inosine prevented memory deficits in the inhibitory avoidance task and the Y-maze test (Teixeira et al., 2020). In addition, inosine increased the serum levels of uric

acid, a potent natural antioxidant. Teixeira et al. (2020) also demonstrated that inosine prevented an increase in Na^+/K^+-ATPase and Mg-ATPase activities and decreased Ca^{2+}-ATPase activity in the hippocampus and cerebral cortex induced by STZ. These actions on ion pumps can be related to the antioxidant effects of inosine and neurotransmitter release. Finally, inosine reduced acetylcholinesterase (AChE) activity and increased choline acetyltransferase (ChAT) activity. ChAT is the enzyme responsible for acetylcholine synthesis from choline and acetyl-CoA, and AChE is an enzyme that breaks down acetylcholine. Acetylcholine is the main neurotransmitter involved in memory processes, and studies have shown that changes in ChAT and AChE levels are correlated with cognitive decline in patients with AD (Gutierres et al., 2014; Pacheco et al., 2018). Another study demonstrated that inosine given by intraperitoneal route reduced the neurological severity score and improved non-spatial cognition and memory in mice undergoing a traumatic brain injury model. In the same study, inosine increased the expression of GAP-43, a marker of axonal growth, in the cerebral cortex (Dachir et al., 2014).

Furthermore, inosine effectively reduces the cognitive decline observed in an animal model induced by 12 Gy ionizing irradiation (Hou et al., 2007). Although there are very few studies evaluating the potential of inosine in learning and memory, the data from Ruhal and Dhingra (2018) have shown that inosine has potential and depends, at least in part, on the anti-inflammatory and antioxidative action of this nucleoside. Although these studies did not measure the inosine brain levels, it is clear the potential of inosine to change and treat cognition-related diseases. Then, this topic is an avenue to study and explore the potential therapeutic effects of inosine on cognition.

INOSINE AND PARKINSON DISEASE

In the last decade, several *in vitro*, *in vivo,* and clinical studies have demonstrated the potential of inosine to treat Parkinson's disease (PD). The studies by Cipriani et al. (2010) and McFarland et al. (2013) seem to be the fundamental studies that led to a series of other articles and a large clinical trial that evaluated inosine's ability to reduce symptoms and the progression of Parkinson's disease. Cipriani and colleagues showed that urate is an endogenous substance with a high antioxidant capacity. This is the final product of the adenosinergic pathway and is a biomarker of PD progression. McFarland and colleagues demonstrated that urate levels in cortical and striatal tissues tended to be lower in PD and AD than in controls (Cipriani et al., 2010; McFarland et al., 2013). These data strongly suggest that the appearance and development of PD may be related to low urate levels (McFarland et al., 2013; Schwarzschild et al., 2014; Chen et al., 2018). This may be due to the oxidation of dopaminergic cells and other markers in the nigrostriatal region of PD (Church and Ward, 1994; Cipriani et al., 2010). In the SURE-PD (safety urate elevation study - Parkinson disease) clinical trial with a 24-month follow-up, it was demonstrated that the elevation of urate (with serum values between 6 and 8 mg/dL) induced by the administration of inosine was safe. It provides a slower progression on the UPDRS scale, which assesses PD signs and symptoms (Schwarzschild et al., 2014). This effect is attributed to the antioxidant capacity of uric acid, given the relationship between oxidative stress and neuronal death in dopaminergic cells (Cipriani et al., 2014; Schwarzschild et al., 2014; Crotty et al., 2017; Paganoni and Schwarzschild, 2017). This study also demonstrated that these elevated urate levels are safe and do not induce urolithiasis, nephrolithiasis, or cardiovascular changes (Schwarzschild et al., 2014; Chen et al., 2018).

The SURE-PD study also demonstrated that inosine led to a significantly higher increase in urate levels in women than that in men, although women started at a lower baseline level (Schwarzschild et al., 2019). In cerebrospinal fluid (CSF), inosine alone was able to increase urate levels in women. The sex of the patients may have been a factor influencing these distinct changes, as inosine induced a more significant reduction in the UPDRS score in women than that in men, suggesting that high urate levels should be more effective in delaying the progression of PD in women than in men (Schwarzschild et al., 2019). In the SURE-PD study, it was found that oral administration of inosine to patients was able to increase the concentration of ferric reducing antioxidant power (FRAP), a measure of antioxidant capacity, in serum levels but not in CSF. FRAP enhancement is inversely proportional to the rate of clinical decline in patients (Bhattacharyya et al., 2016). In another clinical study in an Asian population, administration of inosine for 1 year did not induce any significant side effects, but there were no clinical improvements on the UPDRS scale (Iwaki et al., 2017).

In a cellular model of PD induced by the addition of H_2O_2 to a dopaminergic cells line, called MES 23.5, it was demonstrated that inosine might be a potential treatment for producing anti-inflammatory, trophic, and antitoxic effects *in vitro*. However, these neuroprotective effects of inosine were found only when the MES 23.5 cells were cultured together with a low density of cortical astrocytes, suggesting that this effect is dependent on the release of protective factors from these astrocytes (Cipriani et al., 2014). Another group of researchers evaluated the effect of inosine in an animal model of PD induced by rotenone. In this case, inosine imparted protective effects on protected behavioral, biochemical, and histological parameters. This study suggests that inosine reduced neuroinflammation and oxidative stress due to the suppression of ERK phosphorylation and the downregulation of adenosine A_{2A} receptor expression (El-Shamarka et al., 2020). In another animal model induced by MPTP, it was demonstrated that the use of an adenosine deaminase inhibitor (ADA), which is the enzyme that converts adenosine to inosine, has a neuroprotective effect. Adenosine A_{2A} receptor antagonists also have similar effects.

Furthermore, MPTP has been shown to increase ADA activity to induce significantly higher inosine formation. On the other hand, inosine formation after MPTP can also be considered an attempt to reverse the neurodegenerative effects caused by this toxin (Huang et al., 2019). Taken together, these findings confirm the involvement of the adenosinergic pathway in an animal model of PD.

Taken together, even though the inosine results on PD are quite encouraging in the SURE-PD clinical trial and its results corroborate with some preclinical studies, we can conclude that this nucleoside should be more studied by more research groups in both animals and humans to test its real potential to treat this neurodegenerative disease.

INOSINE AND SCHIZOPHRENIA

Several studies have shown that the adenosinergic system may be altered in schizophrenia (SZ), primarily through the effects of adenosine and nucleotide receptors on dopaminergic and glutamatergic signaling, and as deficiency of inosine is associated with SZ (Malewska-Kasprzak et al., 2019). Moreover, many SZ features may be attributed to purinergic signaling dysfunction, called "purinergic hypotheses," considering that SZ patients present a persistently decreased adenosinergic activity (Lara et al., 2006). Furthermore, adenosine A_{2A} receptor knockout mice show motor disturbances, social and cognitive alterations, and lateral ventricle enlargement related to psychotic symptoms (Moscoso-Castro et al., 2016). Some studies indicate that inosine is involved in SZ development, but the scarcity of more robust results is still evident.

Using generalized singular value decomposition (GSVD), a new algorithm for metabolomic data analysis, Xiao et al. (2011) assessed metabolomic data from the prefrontal cortex (PFC) of *N*-methyl-D-aspartic acid (NMDA) receptor antagonist phencyclidine (PCP)-treated rats, a translational model of SZ. This approach identified a significant disruption in purinergic reactions, denoted by a substantial increase in adenylosuccinate synthase (ADSS), which is responsible for converting IMP to adenylosuccinate, causing imbalance, an increase in inosine levels and downstream metabolites of other enzymes (e.g., IMP, hypoxanthine, and xanthine).

Stress plays a relevant role in the pathogenesis of SZ. In a refined design, Cai et al. (2017) investigated stress-related metabolic pathways in chronic unpredictable mild stress (CUMS) (stress axis activation), long-term dexamethasone exposure (LTDE) (stress axis activation) rat models, and animals treated with clozapine (CLO), risperidone (RIS), and aripiprazole (ARI). These animals showed an imbalance in the bioenergetic pathways responsible for ATP replenishment in the brain. Compared to controls, inosine levels in the PFC and hippocampus were decreased in the CUMS group and increased in the LTDE and CLO groups. Hypoxanthine and uric acid followed the same pattern in the CUMS and LTDE groups. These findings support the rationale that mitochondrial dysfunction and purine metabolism are closely related to stress-induced pathology. Then, the production of uric acid, a scavenger of reactive oxygen species, is reduced when the adenosine pathway is disturbed because inosine and hypoxanthine are its substrates.

Serum adenosine deaminase (ADA) activity, an enzyme that catalyzes adenosine to inosine, is abnormally high in male SZ patients treated with either typical antipsychotics or CLO, even after adjusting for confounding factors (Brunstein et al., 2007). Conversely, increased gene expression levels of ADA and reduced levels of adenosine transporter, equilibrative nucleoside transporter 1 (ENT1), were observed in enriched populations of pyramidal neurons post-mortem sections from the dorsolateral prefrontal cortex (DLPFC) of patients with SZ. However, no changes in inosine levels were detected in DLPFC tissue homogenates in SZ versus controls (O'Donovan et al., 2018).

Adenosine may play a protective role in SZ, particularly through its antipsychotic effects (Ossowska et al., 2020). Lower ADA activity is linked to a functional variant in the ADA gene (*ADA1*2*), which would raise adenosine levels and, by extension, inosine levels (Battistuzzi et al., 1981). As a result, this polymorphism may protect against SZ development. Indeed, this variant presents a lower frequency in male individuals with SZ in a Brazilian cohort. Patients carrying the G/A genotype demonstrate a decrease in about 20–30% of the ADA's enzymatic activity (Dutra et al., 2010).

Inosine may also act as guanosine in cellular processes. Inosine is a modified adenosine in RNA, generated by hydrolytic deamination of adenosine and catalyzed by adenosine deaminase acting RNA (ADAR), called A-to-I RNA editing. Deregulation in this biological process affects pathological conditions (Okada et al., 2015). Genome-wide association studies have pointed out that the etiopathogenesis of neuropsychiatric disorders, such as SZ, is related to the host genetic background (Barešić et al., 2019). Searching for possible deregulation of A-to-I RNA editing in frozen human post-mortem brain samples by Silberg et al. (2011), revealed that SZ and suicide victims presented an increased *ADAR* (e.g., *ADAR2*, *ADAR3,* and *ADRB1*) gene variants that codify enzyme isoforms transcripts with decreased catalytic activity. Additionally, *the ADARB1* rs9983925 single nucleotide variant (SNV) has been associated with suicide attempts in Serbian psychiatric patients exposed to traumatic childhood experiences (Karanović et al., 2015).

To the best of our knowledge, no clinical or experimental study has been conducted to evaluate the biological role of inosine in SZ. We have demonstrated that inosine may act through adenosine A_1, A_{2A}, and A_{2A} receptors (Lapa et al., 2013; Nascimento et al., 2015). The mitogen-activated protein kinase (MAPK), which regulates inflammation, participates in downstream signaling (Welihinda et al., 2016). Considering that inosine negatively regulates oxidative stress (Ruhal and Dhingra, 2018) and neuroinflammation (Junqueira et al., 2017), all crucial components in the pathophysiological course of SZ, this nucleoside could present relevant positive implications in the treatment of SZ.

INOSINE AND EPILEPSY

The effects of inosine on epilepsy have been studied since the 1980s. Early studies on this topic indicated that inosine could interact with benzodiazepine receptors, explaining its antiepileptic effects in several animal models of epilepsy, especially tonic-clonic seizures. Recently, a group of studies has suggested that inosine has a pro-epileptic effect, especially in a rodent absence model of human absence epilepsy. Moreover, some studies have demonstrated that inosine brain levels are

increased in animal models of epilepsy. This section provides an overview of these findings.

There are many different types of seizures; generalized seizures, that involve large bilateral brain areas, comprehend one of the three main categories of epilepsy (Marshall et al., 2021). Generalized seizures can be presented as tonic-clonic seizures or as absence epilepsy. Tonic-clonic seizures are the most common seizures associated with epilepsy. During the tonic phase, there is loss of consciousness and the body is entirely rigid, and in the clonic phase, there is uncontrolled jerking of the limbs and possible difficulty breathing (Marshall et al., 2021). Absence epilepsy is a brief non-convulsive seizure associated with sudden abruptness in consciousness (Jafarian et al., 2020).

Some studies have indicated that inosine may have antiepileptic effects in different animal models of epilepsy. This evidence emerged in the early 1980s when inosine was identified as one of the main compounds of bovine brain extracts responsible for competitively inhibiting the binding of [^{3}H]-diazepam to rat synaptosomal brain membranes (Asano and Spector, 1979). Subsequently, Skolnick et al. (1979) demonstrated that intracerebroventricular (i.c.v.) administration of inosine induced a dose-dependent and time-dependent increase in the latency period to the onset of clonic-tonic convulsions induced by pentylenetetrazol (PTZ). Recently, Ganzella et al. (2011) also demonstrated that i.c.v. administration of inosine protected against quinolinic acid-induced seizures in a time and dose-dependent manner, protecting around 60% of animals at the highest dose tested (400 nmol). Interestingly, intraperitoneal administration of flumazenil, a $GABA_A$ receptor antagonist, or caffeine, an adenosine receptor antagonist, did not change inosine protective effect against quinolinic acid-induced seizures (Ganzella et al., 2011). Considering these findings, inosine seems to exert a partial antagonism on PTZ seizures, which can be explained by its interaction with $GABA_A$ receptors. Moreover, inosine protects against quinolinic acid-induced seizures in a $GABA_A$ receptor-independent manner.

In another approach, i.c.v. administration of inosine in SHR mice 10 min prior to DL-kynurenine significantly reduced the number of mice with DL-kynurenine-induced clonic seizures (Lapin, 1981). Conversely, neither intraperitoneal nor i.c.v. inosine administration prevented seizures induced by PTZ in C57BL/6 or SHR mice (Lapin, 1981).

Systemic administration of inosine has also been shown to have anticonvulsant effects, as demonstrated by pre-treatment with inosine (500–1000 mg/kg), which promoted an increase in the latency of tonic-clonic seizures induced by caffeine in mice and reduced the percentage of animals experiencing seizures (Marangos et al., 1981). In addition, intraperitoneal inosine (1000 mg/kg) reduced the number of mice displaying clonic convulsions and prolonged the latency of clonic convulsions induced by DL-kynurenine C57BL/6 and BALB/c mice (Lapin, 1981). Lewin and Bleck (1985) used BALB/c mice to demonstrate that inosine increased the epileptic dose threshold for PTZ, bicuculline, or picrotoxin and prolonged the time for the first myoclonic contraction induced by these convulsant agents. Interestingly, after subcutaneous administration of the highest dose (1000 mg/kg), an inosine brain concentration of 14.4 μM was reached, suggesting that micromolar concentrations of inosine in the brain are associated with its antiepileptic effects (Lewin and Bleck, 1985).

Recently, Brillatz et al. (2018) demonstrated that inosine also presents antiepileptic effects in a zebrafish epilepsy model with seizures induced by pentylenetetrazol. Interestingly, the authors demonstrated that inosine is the major component of the marine diatom *Skeletonema marinoi*, which also exhibited anticonvulsant effects in zebrafish with PTZ-induced epilepsy (Brillatz et al., 2018). Another research group has proposed a pro-epileptic activity for inosine. Kovács et al. (2015a,b) used Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, a genetically absent epileptic WAG/Rij rat model that spontaneously generates absence-like seizures. The absence-like seizures in WAG/Rij rats, especially those older than 3 months, can be evidenced in electroencephalographic recordings by bilateral synchronous and spontaneously occurring spike-wave discharges (SWDs) (Kovács et al., 2015a). It has been demonstrated that intraperitoneal inosine injection (500 or 1000 mg/kg) significantly increased SWD in WAG/Rij rats. Furthermore, a combined injection of inosine (500 or 1000 mg/kg) and LPS increased the SWD number and the total time of SWD to a more significant extent than LPS or inosine alone, suggesting that inosine potentiated LPS-induced SWD (Kovács et al., 2015b). Interestingly, muscimol (a GABAa receptor agonist)-induced increase in SWD number was potentiated by inosine (500 mg/kg) (Kovács et al., 2015b). Meanwhile, pre-treatment with bicuculline (a GABAa receptor antagonist) before intraperitoneal inosine administration (500 mg/kg) seems to prevent inosine-induced SWD (Kovács et al., 2015a). Taken together, these findings suggest that inosine induces absence-like seizures in WAG/Rij rats, and GABAa receptors could mediate inosine effects. In a different approach, Lakátos et al. (2018) demonstrated that the combined application of allopurinol (a xanthine oxidase inhibitor) and inosine significantly increased the SWD number when compared to allopurinol treatment or inosine treatment. These findings suggest that an increase in the endogenous levels of inosine due to the allopurinol inhibition of the xanthine oxidase enzyme may contribute to absence-like seizures in WAG/Rij rats, reinforcing the possible pro-epileptic effects of inosine in absence seizures (Lakátos et al., 2018).

Some studies have been designed to study inosine brain levels in animal models of epilepsy. Lewin and Bleck (1981) demonstrated that a sub-convulsive series of electroshocks in mice induces clonic movements and tonic extension of the hindlimbs. In this electroshock-induced seizure model, the authors showed that inosine brain levels increased significantly, reaching their maximal value at 5 min (for a 60-Hz stimulus) and 1 min (for a 3-Hz stimulus). Interestingly, phenytoin or phenobarbital administration markedly reduced the increase in inosine brain levels induced by electroshock (Lewin and Bleck, 1981). These results suggest that an early low increase in inosine brain levels following electroshock play a role in seizure generation and propagation. However, high concentrations of inosine after epileptic seizure recovery may contribute to seizure

termination (Lewin and Bleck, 1981). In addition, Lehmann (1987) demonstrated that when folate was bilaterally injected into the amygdala of Albino rabbits, it induced limbic seizures and produced an increase in the hippocampal levels of inosine 10–130 min after injection. The authors suggested that increased inosine levels could be associated with ATP catabolism and indicate a minor perturbation of the cerebral energy state due to epileptic seizures induced by folate (Lehmann, 1987). In contrast, inosine levels in the cerebrospinal fluid (CSF) of patients with a rare disease characterized by progressive myoclonus epilepsy did not differ significantly from inosine levels in the CSF of control patients (Ohisalo et al., 1983). In an interesting case report, Ito et al. described a 4-year-old girl diagnosed with chronic mumps virus infection that presented with generalized tonic-clonic seizures. It was demonstrated that the attacks subsided gradually after administering inosine pranobex (isoprinosine) 100 mg/kg/day (Ito et al., 1991). A marked decrease was observed in slow-wave activity by EEG after approximately 1 month of treatment with isoprinosine, and the patient became seizure-free 9 months after onset (Ito et al., 1991). However, the authors did not rule out the possibility of spontaneous remission (Ito et al., 1991). In addition, considering that inosine pranobex is a combination of inosine, acetamidobenzoic acid, and dimethylaminoisopropanol, it is impossible to conclude the actual contribution of inosine for the improvement of the epileptic seizures presented by the patient.

Considering these findings, there is an evident correlation between inosine and epilepsy. However, further studies are needed to clarify the nature of the aforementioned correlation, since some studies suggest a possible pro-epileptic effect, especially regarding absence seizures. In contrast, others have suggested an antiepileptic effect, mainly in tonic-clonic episodes. In this sense, it would be interesting to evaluate inosine effects in animal models of chronic epilepsy and models used to identify drugs with efficacy against pharmacoresistant seizures. In parallel, identifying the mechanisms involved in the inosine effects in epilepsy is critical and can be performed using genetic, pharmacological, and *in vitro* approaches. Moreover, considering that some studies have identified increased inosine levels in the brains of animals subjected to epilepsy models, it would be essential to clarify whether these inosine levels contribute to the generation/propagation or termination of epileptic seizures. Together, these studies could extend the knowledge regarding inosine in epilepsy.

INOSINE AND ANXIETY

The interaction between inosine and anxiety is not entirely understood. Most studies dealing with this issue have been published in the late 1970s and early 1980s (Asano and Spector, 1979; Slater and Longman, 1979; Paul et al., 1980; Bold et al., 1985). Most of these studies used *in vitro* experiments and suggest a possible interaction between inosine and benzodiazepine receptors. However, few studies have evaluated the *in vivo* effects of inosine on anxiety. In this section, a review of these studies and suggestions for future directions will be provided to better understand the role of inosine in anxiety.

Identification of specific receptors in the brain that mediate the anxiolytic, muscle relaxation, and hypnotic effects of diazepam led to the hypothesis that an endogenous ligand for benzodiazepine receptors must also exist in the brain. In this context, Asano and Spector (1979) identified inosine present in bovine brain crude extracts as an endogenous competitive inhibitor of [^{3}H] diazepam-binding benzodiazepine receptors. Inosine inhibited the binding of about 50% of [^{3}H] diazepam at a concentration of 1.5 mM to the benzodiazepine receptor in the rat brain (Asano and Spector, 1979). Regarding tissue specificity, the authors demonstrated that inosine had a much lower affinity for benzodiazepine receptors in peripheral tissues, such as the kidney and liver, than for those in the brain (Asano and Spector, 1979). Similar results were found previously (Paul et al., 1980) and later (Skolnick et al., 1978), providing evidence that inosine is a compound from brain extracts that competitively inhibits [^{3}H] diazepam and [^{3}H] flunitrazepam (Yarom et al., 1998) binding to the synaptosomal membrane. In addition, inosine inhibited neuronal excitability by increasing membrane conductance in cultured spinal neurons, an effect that was blocked by flurazepam (MacDonald et al., 1979). Together, these findings suggest that inosine could play a neuromodulatory role, mimicking or antagonizing the pharmacological effects of benzodiazepines. However, functional studies such as patch clamp studies or ion flux studies in cells transfected with the benzodiazepine receptors should have been conducted to clarify if inosine acts as an antagonist, agonist or even an inverse agonist of benzodiazepine receptors.

Interestingly, Slater and Longman (1979) used an *in vivo* method to detect drugs with GABA-mimetic properties. In this study, diazepam was injected striatally into the globus pallidus and a highly significant slowing of the head-turn, a behavior that can be modulated by GABA agonists or antagonists, was observed. This behavior was used to demonstrate the *in vivo* GABA-mimetic properties of benzodiazepines (Crossman et al., 1977, 1979). Intrapallidal injection of inosine (10 μg) had no significant effect on the head turn (Slater and Longman, 1979). However, when injected either before or together with diazepam, inosine completely prevented diazepam-induced head-turn slowing (Slater and Longman, 1979). These results suggest that inosine has no GABA-mimetic or benzodiazepine-like properties when tested with the head-turn model.

In contrast, inosine seems to interact with benzodiazepine receptors and can antagonize diazepam actions in the globus pallidus (Slater and Longman, 1979). These findings suggest that inosine could have an anxiogenic effect by antagonizing the actions of diazepam. In this context, Bold et al. (1985) demonstrated that inosine inhibited [^{3}H]-flunitrazepam binding to the benzodiazepine receptor in rat cerebellar cell membranes, with an IC_{50} value of 8 mM and a low affinity for the receptor. Moreover, the same study used the social interaction test in rats, which has been proposed as a valid test for measuring anxiety. In this test, inosine did not increase social interaction in a manner comparable to that of benzodiazepines (Bold et al., 1985), indicating that inosine did not present an anxiolytic

effect in the social interaction test. The authors did not evaluate whether inosine was able to reduce social interactions. It was also not assessed if inosine was able to inhibit the benzodiazepine-induced increase in social interaction. Thus, no conclusion could be drawn regarding the possible anxiogenic effects of inosine. Crawley et al. (1981) used an apparatus that records the number of transitions made by a mouse between an illuminated open-field compartment and a dark enclosed compartment. Benzodiazepine administration prior to the test significantly increased the number of such transitions and could be inferred to be related to the occurrence of an antianxiety effect.

Interestingly, inosine at doses ranging from 10 to 300 mg/kg (below the sedative range, 500–1000 mg/kg) reversed the diazepam-induced increase in the number of transitions between light and dark compartments, suggesting that inosine can antagonize the anxiety-reducing actions of benzodiazepines (Crawley et al., 1981). In corroborating this finding, Wagner and Katz (1983) demonstrated that synthetic purines such as 2′-deoxyinosine and 7-methylinosine, that bind to benzodiazepine receptor, are structurally similar to inosine and seem to present a dose-related anxiogenic effect in Sprague-Dawley rats (Wagner and Katz, 1983). These results reinforce the findings, suggesting that endogenous inosine shows anxiogenic activity due to its interaction with benzodiazepine receptors.

In contrast to most of the findings that suggest a possible anxiogenic effect of inosine, Okuyama et al. (1998) demonstrated that inosine (10–60 mg/kg) administered subcutaneously to male ddY mice (an outbred mouse strain derived from a mouse colony at the Institute of Infectious Diseases of Tokyo University) Yamazaki et al. (2012) presented an anxiolytic-like effect in the Vogel-type anti-conflict test. Interestingly, in this study, inosine and other purines such as adenosine, AMP, c-AMP, and adenine have been isolated from the fruit of *Euphoria longana* (Longan Arillus); however, only inosine and adenosine seemed to have an anxiolytic-like effect (Okuyama et al., 1998). The Vogel-type anti-conflict test presents an excellent predictive value for classical anxiolytic drugs, such as benzodiazepines (Campos et al., 2013). However, the test also responds to non-anxiolytic drugs (Campos et al., 2013), producing false-positive results. It is important to mention that the potential antinociceptive properties of inosine (Nascimento et al., 2010, 2015; Macedo-Junior et al., 2013) could influence the result found by Okuyama et al. (1998), considering the Vogel test involves the response of mice to a noxious electrical stimulus employed as punishment.

According to the findings described here, inosine seems to interact with benzodiazepine receptors, as suggested by studies that used cell membrane binding assays. However, these findings are relatively old, dating back to the late 1970s and throughout the 1980s. Taking that into account, it is crucial to confirm whether inosine acts as a benzodiazepine receptor antagonist. In these sense, functional studies in cells transfected with benzodiazepine receptors and/or in primary cell cultures could not only ensure the interaction of inosine with benzodiazepine receptors but also in particular demonstrate whether this interaction would be able to modify GABA channels functioning in the cell activation/inhibition dynamics (e.g., using patch-clamp assays). Another critical point is to understand whether inosine can change the activity/expression/levels of enzymes, proteins, or second messengers related to the benzodiazepine receptor signaling pathway and whether these findings could be related to its anxiolytic or anxiogenic effects. In addition, the assessment of the anxiolytic or anxiogenic activity of inosine should be performed on animal models associated with the subjacent neurobiology and which meet the criteria of predictive, face, and construct validation. Together, these findings clarify the role of inosine in anxiety.

INOSINE AND DEPRESSION

Several studies have demonstrated a relationship between inosine and depression. These studies showed the effect of inosine treatment in different animal models of depression and paved the way to identify the mechanisms of inosine action in depression. Interestingly, clinical studies have shown that serum inosine levels are associated with major depressive disorders in humans. A review of these studies will be provided in this section, and future directions will be suggested to incorporate inosine in treatment regimens or as a diagnostic biomarker in depressive disorders.

In our database search, the study by Kaster et al. (2013) seems to be among the first to evaluate the possible antidepressant effect of inosine in animal models of depression. In this study, the authors first performed a dose-response curve for inosine in the force swim test (FST) and the tail suspension test (TST). The results demonstrated that inosine significantly reduced the depression behavior in both tests. Similarly, Gonçalves et al. (2017a) demonstrated that inosine given by intraperitoneal route presented antidepressant activity in the TST. Corroborating these findings, Yuan et al. (2018) demonstrated that inosine presented an antidepressant-like effect in the FST in rats. In an interesting approach, an inosine-supplemented diet and an inosine-supplemented tap water presented an antidepressant-like activity, preventing, respectively, chronic unpredictable stress and chronic social defeat stress-induced in set of depression-like behavior in mice (Muto et al., 2014). In addition, inosine improves neuronal proliferation in the mouse brain and promotes neuronal viability and neurite outgrowth in cultured neocortical neurons (Muto et al., 2014).

Regarding action mechanisms related to inosine antidepressant effects, Kaster et al. (2013) demonstrated that both adenosine A_1 receptor antagonist (DPCPX) and adenosine A_{2A} receptor antagonist (ZM241385) were able to prevent inosine antidepressant-like effects. Moreover, activation of PKA, PI3K/Akt, ERK1/2, CaMKII and mTORC1 and the inhibition of GSK-3β and NMDA receptors seems to be involved in the inosine antidepressant-like effects in the TST (Gonçalves et al., 2017a,b). Furthermore, hippocampal CREB phosphorylation, MAPK phosphorylation and BDNF transcription increased 24 h after a single intraperitoneal administration of inosine (Muto et al., 2014; Gonçalves et al., 2017a). Similarly, Yuan et al. (2018) demonstrated that inosine (10 or 50 mg/kg, i.p.) previously administered at 30 min, 6 h, and 24 h in rats induced

a significant increase in phosphorylated ERK and CREB in the rat hippocampus.

From a clinical point of view, it has been suggested that the plasma levels of inosine are significantly reduced in individuals (aged 20–71 years) with major depressive disorder (MDD) when compared with control non-depressive individuals (Ali-Sisto et al., 2016). However, the authors did not observe any statistically significant difference in inosine plasma levels between the remitted and non-remitted groups (Ali-Sisto et al., 2016). On the other hand, Mocking et al. (2021) demonstrated that inosine plasma levels were decreased in both males and females with recurrent major depressive disorder in remission. Zhou et al. (2019) studied the potential biomarkers of depression in children and adolescents with MDD. The authors found that inosine plasma levels are reduced in children and adolescents diagnosed with MDD when compared to health controls. Interestingly, regression analyses suggest that inosine plasma levels are more pronounced in the plasma of boys and among individuals with more severe symptoms of depression (Zhou et al., 2019). Furthermore, inosine plasma levels were significantly higher in individuals treated with antidepressant drugs than in healthy individuals. These studies suggest that inosine could be used as a diagnostic biomarker in individuals with MDD, especially in children and adolescents.

The evidence presented here suggests that inosine induced an antidepressant-like effect in various animal models of depression. However, it is important to note that most studies that evaluated inosine effects in animal models meet only the predictive validity criterion, such as FST and TST. In this sense, it would be essential to extend the test with inosine to animal models that also meet construct and face validities to evaluate its effect on the molecular and biochemical CNS alterations present in depression and classic depression symptoms like anhedonia. It would also be interesting to assess whether inosine increases serotonin/norepinephrine levels in the CNS in animal models of depression. Together, the investigation of these points could confirm, clarify, and extend the role of inosine in depression.

INOSINE AND NEURAL REGENERATION

The therapeutic effects of inosine, which regulates neuron activity, have been described in literature by several research groups. In the last four decades, studies have demonstrated that inosine can be released by cultured neuronal cells, modulate the neurotransmitter release, and activate intracellular signaling pathways that regulate the expression of multiple genes involved in axon outgrowth *in vitro* and *in vivo*. One of the first studies to show that inosine can modulate neuronal activity *in vitro* in adrenergic neurons has been the study by Zurn and Do (1988). The involvement of inosine in other modulatory events, including the growth and regeneration of neuronal connections, was investigated in cultured neural goldfish retinal ganglion cells and rat retinal ganglion cells (Benowitz et al., 1998; Petrausch et al., 2000). These studies have described inosine-stimulating axonal outgrowth through an intracellular mechanism that activates protein kinase–N, GAP-43, protein T alpha-1 tubulin, and L1, a cell adhesion molecule (Benowitz et al., 1998; Petrausch et al., 2000; Cook et al., 2004), mimicking at least one aspect of the molecular changes that underlie axonal regeneration *in vivo*.

These results have led researchers to perform confirmatory *in vivo* studies using animal models, including sciatic nerve injury (Hadlock et al., 1999; Cardoso et al., 2019), controlled cortical impaction (CCI) injury (Smith et al., 2007), closed head injury (Dachir et al., 2014), spinal cord injury (SCI) (Bohnert et al., 2007; Kim et al., 2013; Chung et al., 2015), and ischemic stroke (Chen et al., 2002; Zai et al., 2009, 2011; Benowitz and Carmichael, 2010). In the first published studies investigating the pharmacological effects of inosine, inosine was administered directly into the CNS using osmotic minipumps. The results obtained from these studies corroborate previous *in vitro* data. It has been demonstrated that inosine can stimulate undamaged neurons to extend collateral branches that grow into areas of the brainstem and spinal cord that lose their normal innervation due to injury when administered alone (Hadlock et al., 1999) or together with oscillating field stimulation (OFS) treatment (Bohnert et al., 2007). In addition, inosine combined with NEP1-40, a potent antagonist of the Nogo receptor (the receptor of Nogo-A, a myelin-associated glycoprotein considered to be an inhibitor of neurite growth), doubled the number of axon branches extending from neurons in the intact hemisphere and induced the growth of bouton-like structures in the gray matter into the denervated side of the spinal cord (Zai et al., 2011).

Kim et al. (2013) also demonstrated promising effects of inosine administration on neuron outgrowth. In 2013, inosine was administered intravenously, showing that systemic administration induces axon sprouting in the CNS and increasing serotonergic input to the lumbar spinal cord, indicating that inosine can cross the blood-brain barrier. Thus, in subsequent studies, inosine effects were investigated systemically by intraperitoneal, intravenous, and oral routes in several animal models (Kim et al., 2013; Dachir et al., 2014; Chung et al., 2015; Moore et al., 2016; Cardoso et al., 2019). In these studies, inosine reduced the loss of urinary tract function (Chung et al., 2015), decreased the neurological severity score, and improved motor function (Dachir et al., 2014; Cardoso et al., 2019). All these effects are related to the induction of axon regeneration. Interestingly, when inosine is administered daily via the oral route (500 mg/kg) mixed in flavored yogurt, it induces a more remarkable recovery in terms of a return to standard grasp patterns (finger-thumb pinch) in a cortical injury model (Moore et al., 2016).

Evidence has indicated that the motor improvement observed after inosine treatment *in vivo* was directly related to an increase in the expression of growth-associated membrane phosphoprotein GAP- 43 in neural cells, suggesting that inosine may act similarly *in vivo* and *in vitro*, inducing a program of gene expression that enables axon regeneration. Another protein, Mst3b, is related to the inosine mechanism *in vitro*, and is classified as a purine-sensitive protein kinase that plays a crucial role in axon outgrowth (Irwin et al., 2006).

In addition to a study that revealed a purine-sensitive protein, two exciting studies using an animal model of ischemic stroke and gene microarray analysis have demonstrated that inosine can

affect gene expression in the CNS. The genes that are selectively upregulated by inosine include those encoding tissue inhibitors of metalloproteinase (*timp1*), metallothionein, galectin 3, and complement cascade proteins (Zai et al., 2009). Moreover, genes involved in cell signaling, cell morphology, cell maintenance, assembly and organization, DNA replication, recombination and repair, and nervous system development and function could be upregulated. The authors suggest that chromatin remodeling and genome-wide transcriptional changes may depend on Mst3b activation, a hypothesis that requires further testing (Zai et al., 2011). The data demonstrating inosine effects activating specific proteins and modulating gene transcription are quite promising and may be linked to the enhanced ability of neurons to modify synaptic relationships or promote axon branching. This induction of neural plasticity and axonal sprouting is the basis for behavioral improvement.

Besides the mechanisms involved in inosine-induced plasticity that have been investigated at the molecular level, the contribution of adenosine P1 receptor-signaling pathways to neuronal outgrowth needs to be addressed, since its role in inflammation, immunity, pain, and CNS pathologies has been reported in the literature. The inosine-induced neuronal outgrowth observed in multiple animal models of neurological injury, its clinical safety in humans, and the feasibility of administration, including the oral route, indicates inosine as an excellent candidate for clinical trials of treatment regimens of patients with CNS injuries.

FUTURE ADVANCES AND PERSPECTIVES

A few decades ago, inosine was considered an inert molecule resulting from the degradation of adenosine. In this review, we discuss the current knowledge and describe the myriad scientific evidence that highlight the critical effects of inosine on the central nervous system. Evidence of inosine-binding sites, mainly adenosine receptors, supports an increment of inosine research in the future investigating its physiological role in the CNS disorders and its potential for the development of new therapeutic approaches to treat neurological and psychiatric disorders (see **Figure 1**). We believe that inosine is a great molecule that has been neglected until now and that it is able to treat several physiological disturbs safely. Therefore, we hope that this review can support and promote a path for further investigation of the benefits of inosine through preclinical (*in silico*, *in vitro*, and *in vivo*) and clinical studies.

AUTHOR CONTRIBUTIONS

FN planned and was responsible for the coordination and overall supervision of the study. All the authors contributed equally to writing and editing the manuscript and approved the final manuscript.

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Antitumoral Action of Resveratrol Through Adenosinergic Signaling in C6 Glioma Cells

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Gliomas are the most common and aggressive primary tumors in the central nervous system. The nucleoside adenosine is considered to be one major constituent within the tumor microenvironment. The adenosine level mainly depends on two enzymatic activities: 5′-nucleotidase (5′NT or CD73) that synthesizes adenosine from AMP, and adenosine deaminase (ADA) that converts adenosine into inosine. Adenosine activates specific G-protein coupled receptors named A_1, A_{2A}, A_{2B}, and A_3 receptors. Resveratrol, a natural polyphenol present in grapes, peanuts, and berries, shows several healthy effects, including protection against cardiovascular, endocrine, and neurodegenerative diseases and cancer. However, the molecular mechanisms of resveratrol actions are not well known. Recently, we demonstrated that resveratrol acts as an agonist for adenosine receptors in rat C6 glioma cells. The present work aimed to investigate the involvement of adenosine metabolism and adenosine receptors in the molecular mechanisms underlying the antitumoral action of resveratrol. Results presented herein show that resveratrol was able to decrease cell numbers and viability and to reduce CD73 and ADA activities, leading to the increase of extracellular adenosine levels. Some resveratrol effects were reduced by the blockade of A_1 or A_3 receptors by DPCPX or MRS1220, respectively. These results suggest that reduced CD73 activity located in the plasma membrane in addition to a fine-tuned modulatory role of adenosine receptors could be involved, at least in part, in the antiproliferative action of resveratrol in C6 glioma cells.

Keywords: resveratrol, glioma, CD73, adenosine receptor, brain

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INTRODUCTION

Gliomas are the most common primary tumors of the central nervous system (Wesseling and Capper, 2018). These types of brain tumors have particularly aggressive behavior with a high recurrence rate. Although the current therapeutic approach combines surgical intervention, irradiation, and adjuvant chemotherapy, the prognosis is still very poor for these tumors. Thus, there is a need to find new strategies to improve glioma treatment and reduce its recurrence rate. Adenosine is a key mediator of several biological functions involving multiple signaling pathways (Borea et al., 2018) and mainly operates through four G-protein coupled receptors named A_1, A_{2A}, A_{2B}, and A_3. Adenosine A_1 and A_3 receptors are coupled to Gi/o-proteins and inhibit

adenylyl cyclase activity. In turn, adenosine A_{2A} and A_{2B} receptors are coupled to Gs-proteins and stimulate adenylyl cyclase activity. Adenosine can be formed intracellularly and exported *via* transporters or extracellularly with the participation of CD73 activity as an adenosine-generating enzyme from adenine nucleotides, as ATP, released from cells. Adenosine can be transformed into inosine by adenosine deaminase activity (Fredholm et al., 2011). Because adenosine is considered one of the major constituents within the tumor microenvironment (Di Virgilio and Adinolfi, 2017), adenosinergic signaling has emerged as a potential therapeutic strategy in cancer (Allard et al., 2012). However, the precise procedure to target adenosine-mediated signaling remains under discussion as two different tissues may be affected, the tumoral cells and the immune system. Adenosine seems to be involved in tumor generation, growth, invasion, angiogenesis, and metastasis through activation of all four adenosine receptor subtypes (Gessi et al., 2011; Allard et al., 2012). Nevertheless, adenosine appears to have an opposite biological action as tumor-derived adenosine induces A_{2A} receptor activation from immune cells, leading to an immunosuppressive state of the immune system and, thus, facilitating tumor growth (Ohta et al., 2006). In addition, CD73 is suggested as a key enzyme in tumor growth (Zhang, 2012; Yan et al., 2019). In agreement, its inhibition or depletion causes cell growth inhibition in different *in vitro* (Bavaresco et al., 2008; Zhu et al., 2017) and *in vivo* models (Stagg et al., 2011, 2012). In fact, several drugs and antibodies targeting CD73 are under study in clinical trials due to their potential role in cancer (Buisseret et al., 2018).

Resveratrol (RSV) is a polyphenolic compound present in plants, such as peanuts and grapes, and it shows multiple healthy properties in several diseases, including cancer (Carter et al., 2014; Jiang et al., 2017; Ko et al., 2017). This phytochemical emerged as a promising molecule since the first time its effectiveness was reported against cancer in both *in vitro* and *in vivo* models (Jang et al., 1997; Kiskova et al., 2020). Unfortunately, the action mechanism by which this polyphenol exerts its antitumoral activity remains not well understood. Recently, we reported that RSV acts as a non-selective agonist for adenosine receptors in rat C6 glioma cells (Sanchez-Melgar et al., 2019). Moreover, RSV induces *in vivo* changes in adenosinergic signaling by modulating the functionality of A_1 and A_{2A} receptors in the brain from SAMP8 mice after long-term RSV supplementation in their diet (Sanchez-Melgar et al., 2018). Therefore, the aim of the present work was to investigate whether RSV treatment is able to modulate adenosine-converting enzymes and whether adenosinergic signaling is somehow involved in the antitumoral action of this polyphenol in C6 glioma cells.

MATERIALS AND METHODS

Chemicals

Trans-RSV (ref. R5010), caffeine (CAF) (ref. C-0750), N^6-cyclopentyladenosine (CPA) (ref. C-8031), 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-20-1724) (ref. 557502), and adenosine 5′-triphosphate disodium salt hydrate (ATP) (ref. A7699) were purchased from Sigma Aldrich; 2-[p-(2-carboxyethyl) phenylamino]-5′-*N*-ethylcarboxamido adenosine (CGS21680) (ref. 1063), *N*-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide (MRS1220) (ref. 1217), 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (2-Cl-IB-MECA) (ref. 1104), 4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid (PSB1115) (ref. 2009), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (ref. 0439), 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (BAY606583) (ref. 4472), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) (ref. 1036), and 2′(3′)-O-(4-Benzoylbenzoyl)adenosine-5′-triphosphate tri(triethylammonium) salt (BzATP) (ref. 3312) were purchased from Tocris. Calf intestine adenosine deaminase (ADA) (ref. 10102121001) was purchased from Roche. Other used reagents are indicated in their corresponding section.

Cell Culture

Rat C6 glioma cells were obtained from the American Type Culture Collection (ref-CCL-107) and grown (passages 40–60) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% non-essential amino acids and antibiotics in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. As the potency of adenosinergic ligands depends on the model, species (rat, human, or mouse), tissue, and overall experimental conditions of the assay (Fredholm et al., 2011), C6 cells were subjected to different adenosinergic ligands at concentrations that were selected considering the pharmacological characterization of adenosine receptors (Castillo et al., 2007) and the characterization of RSV as an adenosine receptor agonist in these cells (Sanchez-Melgar et al., 2019). For instance, PSB1115 and CGS21680 used in the range 0.1 nM–1 mM were unable to displace binding of 20 nM [^{3}H]DPCPX to A_1 receptors in intact C6 cells. Similarly, PSB1115 and CPA used in the range 0.1 nM–1 mM were unable to displace binding of 15 nM [^{3}H]ZM241385 to A_{2A} receptors in intact cells (Castillo et al., 2007).

Cell Viability Assays and Cell Counting

Cells were plated in 96-well dishes (10^4 cells/well) and grown overnight before starting treatment. After treatment, cell viability was assessed by the XTT method following the manufacturer's instructions (Roche). Reagents were incubated for 150 min at 37°C, and absorbance was measured at 475 and 690 nm on a Synergy HT (BIO-TEK) plate reader. The results are expressed as percentages relative to the control condition. Cells grown in six-well dishes ($5{\cdot}10^5$ cells/well) were detached and counted on a TC 10^{TM} Automated Cell Counter (BioRad) after treatment and compared with the corresponding controls.

Caspase-3 Activity

Cells from each condition (10^6 cells) were used as indicated by the manufacturer's protocol (Molecular Probes, Barcelona, Spain). Cells were lysed for 30 min at 4°C and centrifuged at

12,000 rpm for 5 min. Supernatant (50 μL) was collected, and a mix containing Z-DEVD, DTT, EDTA, PIPES, and CHAPS was added into the P96-black well. After 30 min of incubation at room temperature protected from light, fluorescence was read at Ex/Em of 340/440 nm, respectively, in a kinetic mode for 4 h. Slope value was used to represent the enzymatic activity. Samples from each condition were analyzed in duplicate at the same cell passage.

Cell Cycle Assays

After treatment, cells were washed with phosphate buffer (PB) and incubated with trypsin to detach cells. After trypsinization, cells were centrifuged, and the obtained pellet was carefully resuspended in 100 μL PB. Cells were then fixed by adding cold ethanol for 4 h at 4°C. After fixation, cells were washed to remove ethanol and staining solution containing 0.1% Triton X-100, 10 μg/ml of propidium iodide (Molecular Probes, Inc.), and 50 μg/ml RNase A in PB was added; samples were incubated for 30 min at room temperature. Fluorescence was detected at 488 nm on a MACSQuant® 10 flow cytometer.

Nuclei Staining

Cell nuclei were visualized by fluorescence microscopy using Hoechst 33258 (Sigma-Aldrich, Madrid, Spain) as a staining method. Briefly, culture media was removed, and cells were washed with PBS (pH 7.4). Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing three times for 10 min in PBS, nuclei were stained with 1 μg/ml Hoechst for 10 min protected from light and mounted with ProLong Gold antifade reagent (Invitrogen, Madrid, Spain). Nuclei were quantified using a DMI6000B microscope and LAS AF software (Leica Microsystems, Wetzlar, Germany).

Plasma Membrane Isolation

Plasma membrane isolation was performed as previously described (Luis Albasanz et al., 2002). Cells were homogenized in isolation buffer (50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$ and protease inhibitors) with Dounce homogenizer (10XA, 10XB). After homogenization, samples were centrifuged for 5 min at 1,000 × *g* in a Beckman JA 21 centrifuge. Supernatants were centrifuged again for 30 min at 27,000 × *g*, and the resulting supernatant was considered to be the cytoplasmic fraction, and the pellet (plasma membrane) was resuspended in isolation buffer. Protein concentration was measured by the Lowry method.

5′-Nucleotidase Activity

Isolated plasma membrane (20 μg) and cytosolic (20 μg) fractions were preincubated in 180 μL of the reaction medium containing 50 mM Tris, $MgCl_2$ 5 mM, pH 9, at 37°C for 10 min. Then, the reaction was initiated by the addition of 20 μL AMP at a final concentration of 500 μM and stopped 20 min later by adding 200 μL of 10% trichloroacetic acid. The samples were chilled on ice for 10 min and then centrifuged at 12,000 × *g* for 4 min at 4°C. The supernatants were used to measure the inorganic phosphate released following the protocol described earlier (Leon-Navarro et al., 2015) using KH_2PO_4 as Pi standard. Non-enzymatic hydrolysis of AMP was used as a blank. Incubation times and protein concentration were selected to ensure the linearity of the reactions. All samples were run in duplicate. 5′-Nucleotidase (CD73) activity is expressed as nmol Pi released/min/mg of protein.

Adenosine Deaminase Activity

An adenosine deaminase (ADA) activity assay (ref. ab204695) was performed in a 96-well plate according to the manufacturer's protocol (Abcam, Cambridge, United Kingdom). The cytoplasmic fraction was diluted 1:100 in ADA buffer assay and assayed in duplicate. Then, the 96-well plate was read at Ex/Em = 535/587 nm as a kinetic curve for 30 min. ADA activity was obtained by interpolation in an inosine standard curve performed in parallel in the same plate. Enzymatic activity was normalized to the amount of protein.

Adenosine and Related Metabolite Detection by HPLC

Chromatographic analysis was performed with Ultimate 3000U-HPLC, and data peaks were processed with Chromeleon 7 (ThermoFisher, Madrid, Spain) as previously described (Alonso-Andres et al., 2019). An HPLC diode array was used working at 254 nm wavelength. Purine standards and samples (40 μL) were injected into a C18 column of 4.6 × 250 mm, 5 μm particle size. Two solvents were used for gradient elution: solvent A, 20 mM phosphate-buffered solution (pH 5.7), and solvent B, 100% methanol. The gradient was 95% (11 min), 80% (9 min), and 95% (2 min) in solvent A. The total run time was 22 min with a constant flow rate of 0.8 mL/min at 25°C. Retention times for inosine and adenosine were 8.4 and 15.5 min, respectively. Each purine level was obtained by interpolation from the corresponding purine standard curve. The standard curves were obtained by using five concentrations of each purine ranging from 0.1 to 500 μM. Data were then normalized to the protein concentration of each sample.

Cell Microscopy and Population Doubling Time Calculation

C6 glioma cell growth was recorded with a digital camera (Leica DFC350FX) attached to a Leica DMI6000B (Leica Microsystems, Wetzlar, Germany) fluorescent microscope using × 20 HCX PL FLUOTAR objective. Cells were maintained at 5% CO_2 and 37°C in a stage-top incubation system (PeCon GmbH, Erbach, Germany) during video recording (one image every 2 min). Cells were counted every 3 h, and the relative increase respect time 0 was calculated. Population doubling time was obtained from the fold increase data by non-linear regression fitting curve to exponential growth equation.

Statistical and Data Analysis

Statistical analysis was according to Student's *t*-test. Differences between mean values were considered statistically significant at $p < 0.05$. GraphPad Prism 6.0 program was used for statistical and data analysis (GraphPad Software, San Diego, CA, United States). Cell cycle histograms were

analyzed with FlowLogic 7.3 software by Inivai Technologies (Victoria, Australia).

RESULTS

Effect of RSV and the Pharmacological Stimulation/Blockade of Adenosine Receptors in C6 Glioma Cell Growth and Viability

Cells were treated with RSV at different times and concentrations to assess the ability of this polyphenol to cause cell death. Cell viability assays were performed based on the XTT reduction method, and the antitumoral activity of RSV was analyzed. Cell viability was diminished in a time- and concentration-dependent manner (**Figure 1A**), revealing that treatment at 100 μM RSV for 24 h reduced about 50% of the measurement of cell viability. The proliferation of cells was gradually decreased as RSV concentration was increased from 0.1 to 100 μM (**Figure 1B** and **Supplementary Video 1**), and the population doubling time (**Figure 1C**) changed from 16.1 ± 1.7 h in control cells to 85.1 ± 22.9 h in the 100 μM RSV treatment. This concentration and time of treatment were used for the next set of experiments with RSV.

Resveratrol is a non-selective agonist for adenosine receptors, showing a strong influence in A_{2A}-mediated signaling (i.e., G protein coupling switch from Gs to Gi) after acute RSV treatment at high concentrations. Therefore, we target adenosine receptors with selective agonists and antagonists (10 μM CPA and 1 and 10 μM DPCX for A_1 receptors; 10 μM CGS21680 and 100 μM ZM241385 for A_{2A} receptors; 10 μM BAY606583 and 100 μM PSB1115 for A_{2B} receptors; 10 μM IBMECA and 10 μM MRS1220 for A_3 receptors) to investigate the role that these receptors play on C6 glioma cell growth. Caffeine (100 μM), a non-selective antagonist for adenosine receptors, was also employed. As **Figure 2A** shows, the activation of A_1 (CPA), A_{2A} (CGS21680), A_{2B} (BAY606583), or A_3 (IBMECA) receptors did not induce significant changes in cell viability after 24 h of treatment. In turn, the blockade of A_{2A} (ZM241385), A_{2B} (PSB1115), or A_3 (MRS1220) receptors significantly reduced the cell viability. Similar results were obtained when the number of cells after treatment was analyzed (**Figure 2B**). The blockade of the A_{2A} receptor significantly reduced the number of cells. Interestingly, blockade of A_1 receptor with DPCPX during RSV treatment significantly reduced the RSV effect on cell viability (**Figures 2A,C**) and the number of cells (**Figure 2B**). In turn, the blockade of the A_3 receptor with MRS1220 significantly reduced the decrease in the number of cells elicited by RSV treatment (**Figure 2B**) but was unable to modify the reduction in cell viability elicited by RSV (**Figures 2A,C**).

To assess whether the reduction in cell viability elicited by RSV or other treatments was associated with the induction of apoptosis, the caspase-3 activity was measured. As **Figure 3A** shows, a strong and significant increase in caspase-3 activity was detected in RSV-treated cells when compared with controls, suggesting the induction of apoptosis by RSV. Caspase-3 activity was also increased after selective blockade of A_{2A} receptor with ZM241385, but more modestly as compared with RSV. Interestingly, blockade of the A_1 receptor with DPCPX or, to a lesser extent, of the A_3 receptor with MRS1220 during RSV treatment resulted in lower caspase-3 activation than achieved with RSV alone (**Figure 3B**). However, no apoptotic bodies were found in stained nuclei after RSV treatment (**Figure 3C**).

The cell cycle of C6 cells was analyzed after treatment with agonists and antagonists of A_{2A}, A_{2B}, and A_3 receptors. The histograms of DNA content obtained by propidium iodide staining and flow cytometry (**Figure 4A**) were used to calculate the percentage of cells in each cell cycle phase (**Figure 4B**). This analysis reveals an accumulation of C6 glioma cells in the G_1 phase after RSV exposure when compared to control conditions, whereas the percentage of cells in the S and G_2/M

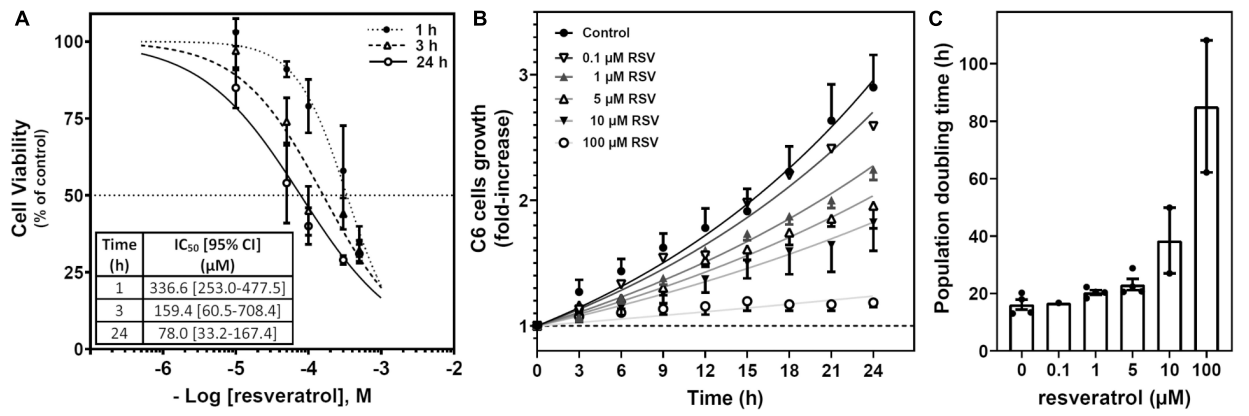

FIGURE 1 | Effect of RSV on C6 glioma cell growth. **(A)** Cell viability based on the XTT method was performed after RSV exposure for 1, 3, and 24 h at different concentrations. Data are means ± SEM of three to six independent assays. **(B)** Cells were treated with different concentrations of RSV as indicated. Cells were counted from phase-contrast images recorded (one frame every 2 min) for 24 h at the indicated interval and relativized to the number of cells at the beginning of treatment. A representative video can be seen in **Supplementary Video 1**. **(C)** Population doubling time derived from three to five video recordings as represented in panel **(B)**.

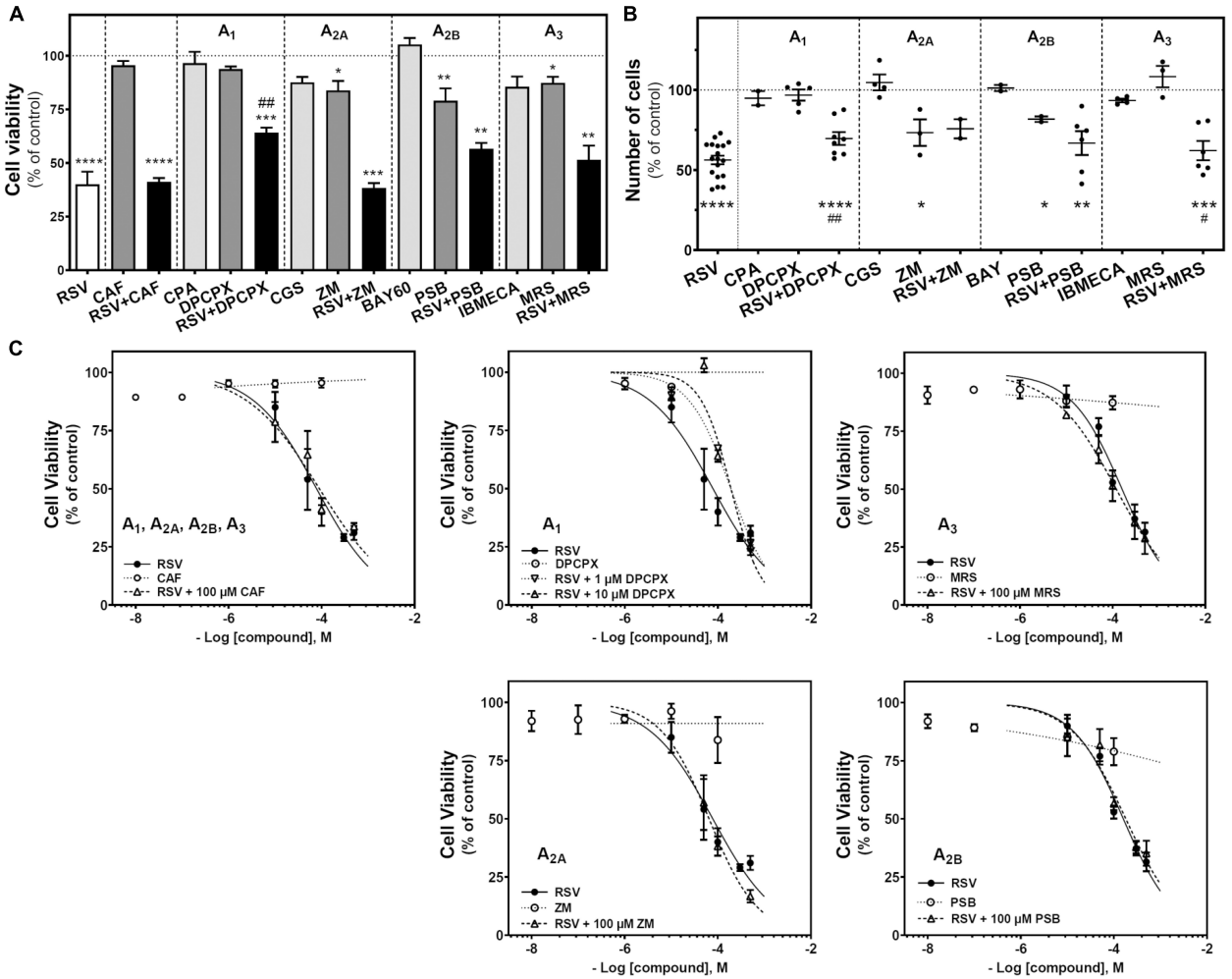

FIGURE 2 | Effect of RSV and adenosinergic ligands on C6 glioma cell growth. **(A)** Cell viability after 24 h of treatment with 100 μM RSV, 100 μM CAF, 10 μM CPA, 10 μM DPCPX, 10 μM CGS, 100 μM ZM, 10 μM BAY60, 100 μM PSB, 10 μM IBMECA, and 10 μM MRS alone or in combination. **(B)** Number of cells after 100 μM RSV, 10 μM CPA, 10 μM DPCPX, 10 μM CGS, 100 μM ZM, 10 μM BAY60, 100 μM PSB, 10 μM IBMECA, and 10 μM MRS alone or in combination after 24 h of treatment. **(C)** Cell viability after 24 h treatment with different concentrations of RSV, CAF, DPCPX, ZM, PSB, and MRS alone or in combination. Data are means ± SEM of 3–10 independent assays. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ significantly different from control condition according to Student's *t*-test. $\#p < 0.05$ and $\#\#p < 0.01$ significantly different from RSV condition. RSV, resveratrol; CAF, caffeine; CPA, N^6-cyclopentyladenosine; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; CGS, CGS21680; ZM, ZM241385; BAY60, BAY606583; PSB, PSB1115; IBMECA, 2-Cl-IB-MECA; MRS, MRS1220.

phases was significantly diminished, suggesting that cellular division was inhibited. Neither pharmacological blockade nor activation of A_{2A}, A_{2B}, and A_3 receptors altered the cell cycle when compared with the control condition, suggesting that RSV action on the C6 cell cycle could not be fully mimicked by selectively targeting these receptors. In addition, no differences were observed when the effect of RSV was analyzed in the presence of MRS1220.

RSV Effect on Adenosine Metabolism

Next, we analyzed the enzymatic machinery involved in adenosine production and degradation. We found a significant reduction in 5′-nucleotidase (5′NT or CD73) activity, which catalyzes adenosine synthesis from ATP, localized in the plasma membrane, whereas no changes were observed in the cytosolic fraction (**Figure 5A**). Likewise, ADA activity, which catalyzes the degradation of adenosine to inosine, was lower in homogenates from RSV-treated cells than in controls (**Figure 5B**). As the decrease of CD73 activity could lead to the accumulation of ATP metabolites that could stimulate P2X receptors, cell viability was assayed after 24 h treatment with ATP as a non-selective P2R agonist and BzATP as P2XR agonist. Results show a slight but significant decrease in cell viability elicited by high concentrations of ATP that were unable to mimic the RSV effect. BzATP did not change cell viability. Moreover, the RSV effect was maintained in the presence of this P2X agonist (**Figure 5C**), In addition, extracellular adenosine levels were significantly increased after RSV treatment (**Figure 5D**).

On the other hand, intracellular levels of adenosine, inosine, xanthine, and hypoxanthine were also quantified (**Figure 6**).

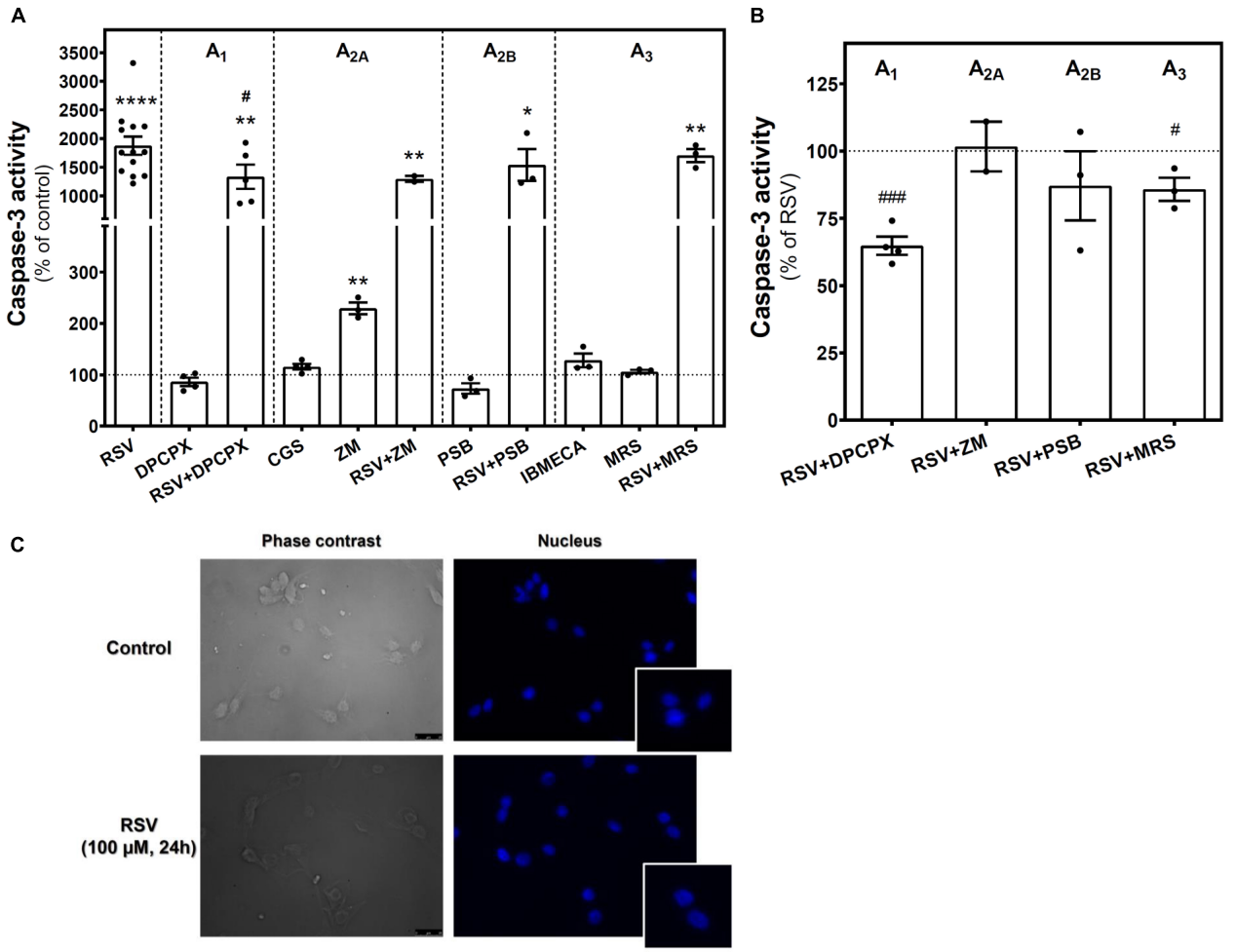

FIGURE 3 | Caspase-3 activity in C6 glioma cells. **(A)** Caspase-3 activity after 24 h of treatment with 100 μM RSV, 10 μM DPCPX, 10 μM CGS, 100 μM ZM, 100 μM PSB, 10 μM IBMECA, and 10 μM MRS alone or in combination with RSV. **(B)** Effect of adenosine receptor antagonists on the activation of caspase-3 elicited by RSV. **(C)** Absence of apoptotic bodies in control and RSV-treated conditions after Hoechst staining of nucleus. Data are means ± SEM of 3–17 independent experiments. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ significantly different from control condition according to Student's *t*-test. #$p < 0.05$ and ###$p < 0.001$ significantly different from RSV condition. RSV, resveratrol; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; CGS, CGS21680; IBMECA, 2-Cl-IB-MECA; MRS, MRS1220; PSB, PSB1115; ZM, ZM241385.

Only inosine levels were strongly and significantly reduced by RSV treatment.

Adenosine Effect on C6 Glioma Cells Growth

After confirming that the extracellular adenosine level was increased by RSV treatment, it was analyzed whether this increase has a role in the reduction of C6 cell growth promoted by RSV. **Figure 7** shows that adenosine is unable to mimic the RSV effect on cell viability. Even at 100 μM adenosine, the inhibition of cell viability is more discrete than that observed after RSV treatment. Moreover, the removal of adenosine from the culture medium with ADA, at two different concentrations (4 and 8 U/mL) that ensure the adenosine breakdown during RSV treatment, did not modify the RSV effect on cell viability. As the effect of RSV on cell viability could not be reversed by ADA, direct activation of adenosine receptors by the increased levels of adenosine could be discarded. Instead, a direct action of RSV in adenosine receptors, mainly the A_1 subtype, could contribute to the antiproliferative effect of RSV.

Associations Between Components of the Adenosinergic System

Considering the link between levels of adenosine and related metabolites and their enzymatic machinery, we compared their mean ± SEM values obtained in control and RSV-treated cells. In this sense, both 5′NT and ADA activities are reduced by RSV treatment, achieving activities of 73% ± 4% and 32% ± 9% of control values, respectively (**Figure 8A**). RSV treatment leads to higher levels of extracellular adenosine that seem not to be attributed to higher 5′NT activity, which is reduced by RSV treatment (**Figure 8B**). Thus, lower inosine levels seem

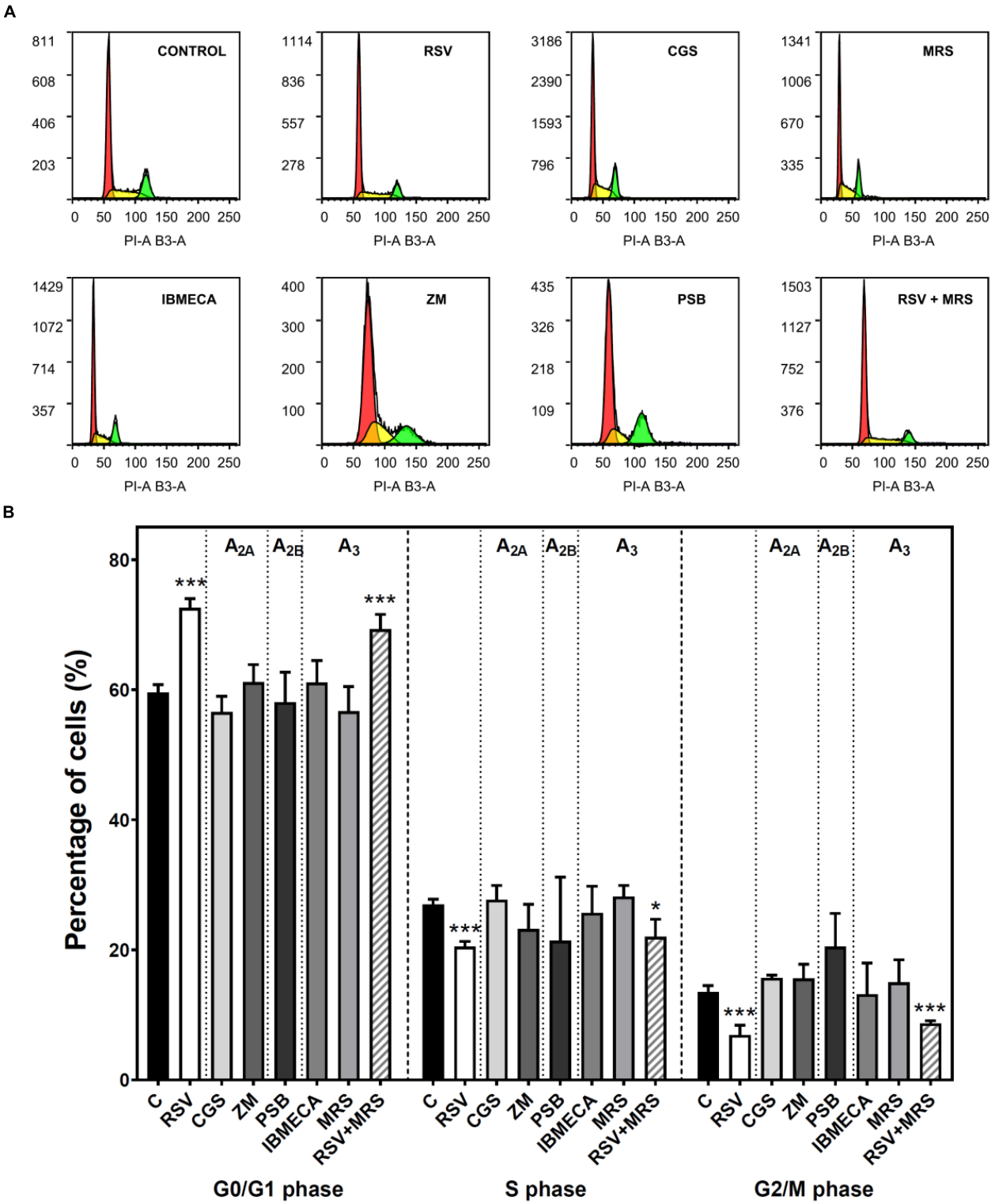

FIGURE 4 | Cell cycle analysis in C6 glioma cells. Cells were analyzed by flow cytometry after 24 h of treatment with 100 μM RSV, 10 μM CGS, 10 μM MRS, 10 μM IBMECA, 100 μM ZM, or 100 μM PSB. **(A)** Representative histogram of cell cycle analysis performed with propidium iodide staining of DNA showing the number of cells versus DNA content (PI-A B3-A). **(B)** Percentage of cells in each cell cycle phase. Data are means ± SEM of 3–15 independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ significantly different from the control condition according to Student's *t*-test. RSV, resveratrol; CGS, CGS21680; IBMECA, 2-Cl-IB-MECA; MRS, MRS1220; PSB, PSB1115; ZM, ZM241385.

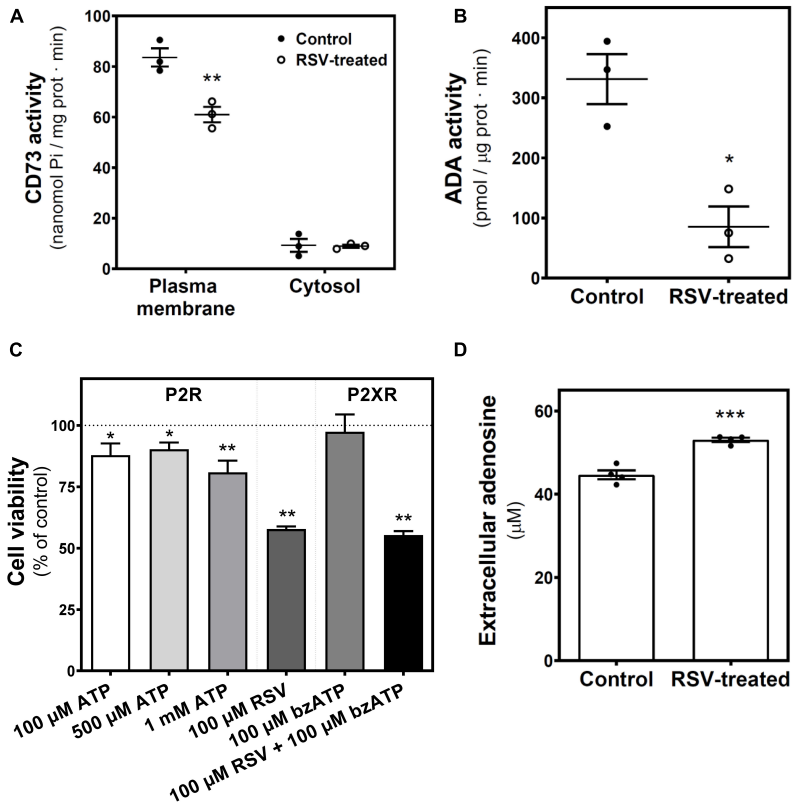

FIGURE 5 | RSV treatment effect on adenosine-related enzymatic activities. 5′-nucleotidase (CD73) and ADA activities were measured in control and 24 h RSV-treated C6 cells. **(A)** 5′-nucleotidase activity localized in the plasma membrane and cytosolic fraction were assayed and represented as nmol Pi/mg prot · min. **(B)** ADA activity was quantified and represented as pmol/µg prot · min. **(C)** Cell viability based on the XTT method was performed after 24 h of treatment with the indicated ligands. **(D)** Quantification of adenosine levels in culture medium by HPLC. Data are means ± SEM of three to five independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ significantly different from the control condition according to Student's *t*-test.

to be associated with lower ADA activity (**Figure 8C**) in RSV-treated cells. Therefore, the decrease in 5′NT and ADA activities after RSV treatment results in lower inosine (9 ± 6% of control cells) and higher adenosine levels (128 ± 1% of control cells) (**Figure 8D**).

Effect of the Inhibition of Phosphodiesterase on C6 Glioma Cells Growth

It is reported that elevation of intracellular cAMP levels through inhibition of phosphodiesterase (PDE) positively correlates with reduced cell proliferation of C6 glioma cells. Therefore, we treated C6 glioma cells with 100 µM RO-20-1724, a selective PDE-IV inhibitor. Our results show that PDE-IV inhibition did not cause a change in the number of cells (**Figure 9A**), the caspase-3 activity (**Figure 9B**), or the cell cycle (**Figure 9C**).

DISCUSSION

Results presented herein indicate that exposure of C6 glioma cells to RSV caused cell growth inhibition in a time- and concentration-dependent manner by accumulating cells in the G_1 phase. Caspase-3 activity was increased after treatment. Furthermore, adenosine-converting enzyme activities (i.e., CD73 and ADA) were significantly reduced in RSV-treated cells. In agreement, increased levels of extracellular adenosine were detected, whereas intracellular adenosine remained unaltered. Interestingly, adenosine A_1 and A_3 receptors seem to contribute in part to the antiproliferative effect of RSV because the blockade of these receptors partially ameliorates the effect of RSV.

Resveratrol is attracting attention in the prevention of several diseases, including cancer (Jang et al., 1997). However, the precise molecular mechanisms behind its antiproliferative action remain to be clarified. Our results show that RSV induced reduction in cell viability in a time- and concentration-dependent manner and a higher activity of caspase-3, an early apoptosis marker. The absence of apoptotic bodies in the nuclei and a cell cycle arrest in the G_1 phase, together with a lower percentage of cells in the S and G_2/M phases, suggests an antiproliferative effect of RSV through cell cycle arrest. A limitation of the cell cycle analysis we performed is that only a single parameter was used for DNA content analysis, and it would be interesting to use an additional specific marker to distinguish between the cell cycle phase. Anyway, we found an IC_{50} value of 78.0 µM (95% CI: 33.2–167.4) for 24 h RSV treatment, very similar to the

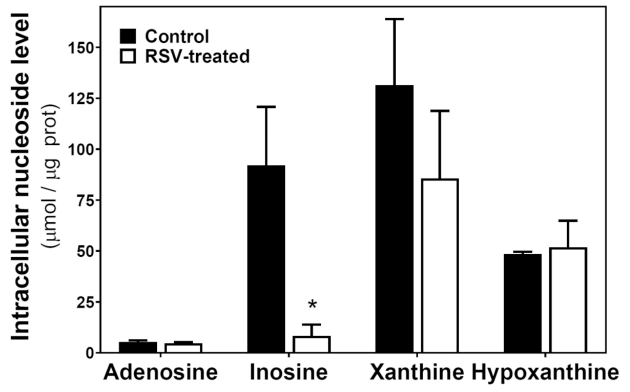

FIGURE 6 | RSV treatment effect on intracellular levels of adenosine and related metabolites in C6 glioma cells. Cells were exposed to RSV at 100 µM for 24 h and adenosine, inosine, xanthine, and hypoxanthine were quantified by HPLC as described in "Materials and Methods." Data are means ± SEM of three to four independent assays. *$p < 0.05$ significantly different from the control condition according to Student's *t*-test.

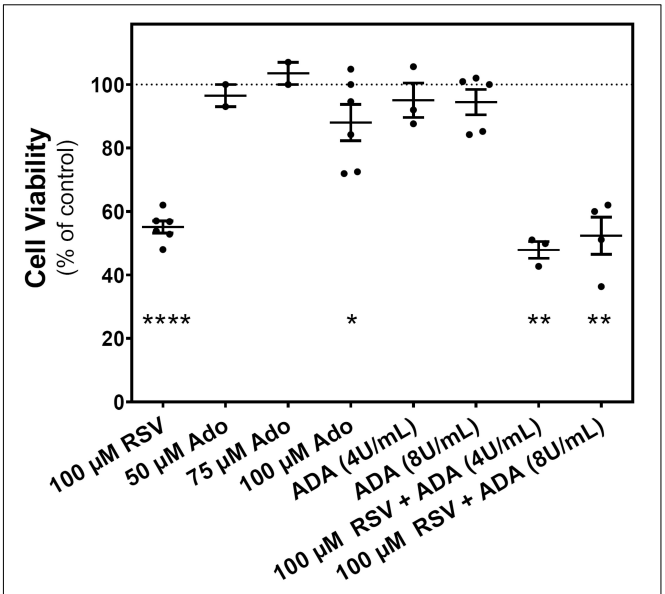

FIGURE 7 | Effect of adenosine on C6 glioma cells growth. Cell viability was measured on C6 glioma cells after 24 h of treatment with 100 µM RSV; 50, 75, and 100 µM adenosine (Ado); 4 or 8 U/mL ADA; or a combination (ADA + RSV). Data are means ± SEM of three to six independent experiments. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ significantly different from the control condition according to Student's *t*-test.

85.26 ± 2.14 µM previously reported in C6 cells by Zielinska-Przyjemska et al. (2017). However, these authors report a cell cycle arrest in the S phase after 100 µM RSV exposure for 24 h. Cycle arrest in the S phase is also reported after 100 µM RSV exposure for 48 h (Wang et al., 2015) or 210 µM RSV for 24 h (Zhang et al., 2007). In agreement with our results, it is reported that 100 µM RSV induced cell cycle arrest in the G_1 phase in breast cancer cells after 24 h but not 48 h treatment (Medina-Aguilar et al., 2016) and in human melanoma cells (Wu et al., 2015) after 48 h. Thus, RSV inhibition of cell cycle progression seems to be cell line specific.

Purinergic signaling is involved in cancer cell proliferation (Di Virgilio and Adinolfi, 2017), and both P2 (Di Virgilio et al., 2018) and P1 (Allard D. et al., 2017) receptors as well as CD39 and CD73 enzymes (Allard B. et al., 2017) could be new targets in cancer. The role of adenosine signaling in cancer is still under debate although it is well accepted that adenosine can promote cancer cell proliferation in several tumors through its receptors (Ohta, 2016; Kazemi et al., 2018). It is reported that adenosine A_{2B} receptors (Sepulveda et al., 2016) and CD73 (Zhang, 2012) are overexpressed in many cancer types. Moreover, adenosine has been found at higher levels in the tumor microenvironment when compared with normal tissue (Ohta et al., 2006) even at a range of 50–100 µM (Vaupel and Mayer, 2016). This tumor-derived adenosine seems to promote cancer cell growth in a receptor-dependent manner as reviewed elsewhere (Ohta, 2016), but it also facilitates immune escape by activating A_{2A} receptors in T cells (Ohta et al., 2006), suggesting a protumor effect of adenosine. However, other authors report a cytotoxic action of adenosine in human cervical cancer cells (Mello Pde et al., 2014), indicating that adenosine might exert a differential action depending on the type of cancer. This cytotoxic effect was also observed in our study when C6 cells were treated with 100 µM adenosine for 24 h. In contrast, it is reported that 100 µM adenosine increased cell proliferation by 36% in U138MG glioma cells (Bavaresco et al., 2008), which might indicate that adenosine action depends not only on the type of cancer (e.g., glioma) but also on the cancer cell line.

Despite the well-known antitumor effect of A_{2A} receptor depletion or pharmacologic inhibition by enhancing the antitumor immune response in mice (Ohta et al., 2006), the specific role of adenosine receptors in the tumor itself remains under debate (Gessi et al., 2011; Di Virgilio and Adinolfi, 2017; Borea et al., 2018; Gorain et al., 2019). Adenosine receptors could display an important action on cancer cell growth, invasion, angiogenesis, and even metastasis (Ohta, 2016; Kazemi et al., 2018). Our work reveals that prolonged pharmacologic blockade of A_{2A} receptors with ZM241385 results in a discrete but significant reduction in the cell viability in conjunction with higher caspase-3 activity, suggesting an antiproliferative effect in cancer cells for A_{2A} receptor antagonists. Other authors report similar data in lung adenocarcinoma tumor cells (Mediavilla-Varela et al., 2013). In a previous study, our group discovered that RSV binds and acts as a non-selective adenosine receptor agonist in C6 glioma cells and that acute RSV treatment altered the A_{2A} receptor/Gs-protein coupling, leading to the inhibition of the cAMP generation upon pharmacologic stimulation of the A_{2A} receptor with CGS21680. Moreover, adenylyl cyclase (AC), PKA protein levels, and basal AC activity were significantly increased after 100 µM RSV treatment for 24 h (Sanchez-Melgar et al., 2019). This dramatic alteration of A_{2A} receptor signaling after RSV treatment makes it difficult to analyze the possible contribution of these receptors to the antiproliferative effect of RSV at least by combining RSV with agonists or antagonists for

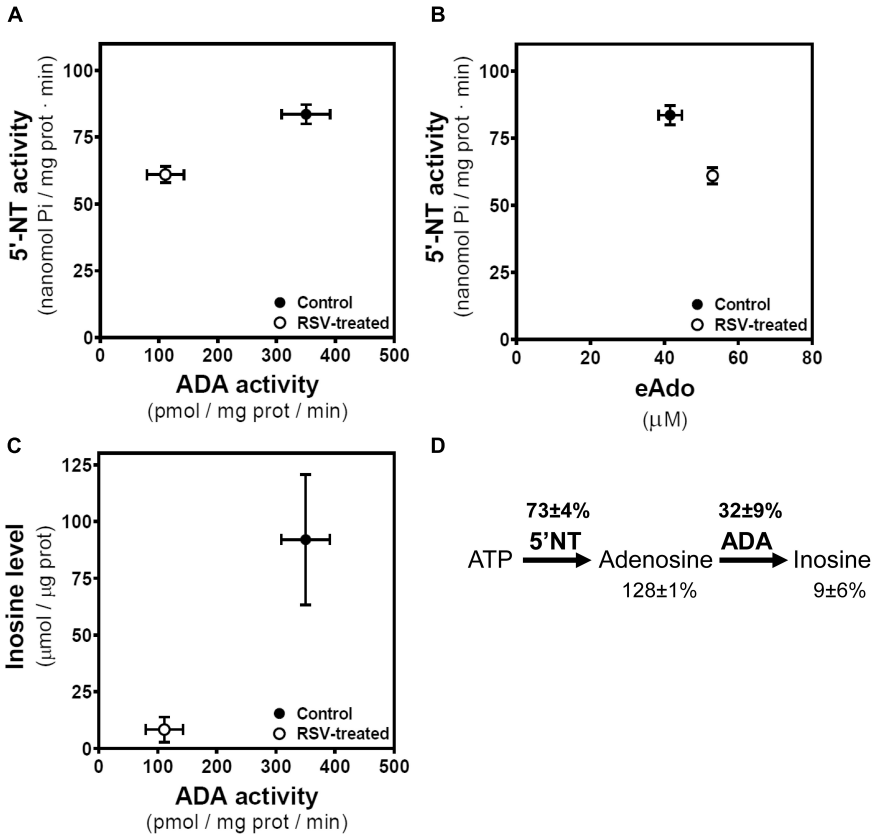

FIGURE 8 | Associations between enzymatic activities and related metabolites. **(A)** Mean values with SEM bars of 5′-nucleotidase (5′-NT) and ADA activities derived from control and RSV treated cells. **(B)** Mean values with SEM bars of 5′-nucleotidase (5′-NT) and extracellular adenosine (eAdo) levels derived from control and RSV-treated cells. **(C)** Mean values with SEM bars of inosine levels and ADA activity derived from control and RSV treated cells. **(D)** Levels of adenosine and inosine and 5′NT and ADA activities in RSV treated cells are expressed as the percentage of the corresponding control value.

A_{2A} receptors as employed here. Diet supplementation with RSV also caused the desensitization of A_{2A} receptors in the brain from SAMP8 mice (Sanchez-Melgar et al., 2018). These results might support that the alteration of A_{2A} receptor signaling could be involved in the antiproliferative action of RSV. It is reported that elevation of intracellular cAMP levels through either activation of AC or inhibition of PDEs leads to PKA activation and positively correlates with reduced cell proliferation of C6 glioma cells. Interestingly, the elevation of cAMP levels with forskolin induces cell cycle arrest of C6 glioma cells in the G_2/M phase. In comparison, inhibition of PDEs not only inhibits cell growth *via* the cAMP/PKA cascade, but also triggers cell death through caspase-3/-7 activation (Safitri et al., 2020). It is described that anticancer agents, such as RSV, may act by modulating cell cycle–associated proteins, such as cyclins, cyclin-dependent kinase (CDK), and CDK inhibitors (Wolter et al., 2001). CDK inhibitors are shown to be the downstream targets of caspase-3 activation, and loss of these inhibitors can result in the aberrant upregulation of CDKs that have been associated with apoptotic cell death (Jin et al., 2000). Therefore, RSV-induced G_1-phase cell cycle arrest could be mediated through the caspase/cyclin-CDK pathways. In agreement, the protein content of the cycle arrest proteins CDK2, CDK4, cyclin D1, PCNA, and P21 is reported to be decreased in a concentration-dependent manner in RSV-treated (100 μM, 24 h) HCT116 and Caco-2 cells compared with control cells (Liu et al., 2014). Moreover, RSV inhibits human U251 glioma cell proliferation and induces G_0/G_1 growth arrest, and these effects are reduced by the CDK inhibitor olomoucine (Jiang et al., 2005). These RSV effects on cell cycle and viability *via* caspase-3 activation could be modulated by different adenosine receptors after their activation by binding of adenosine or even RSV. In fact, treatment of C6 glioma cells with 25 μM Cl-IB-MECA reduced Bcl-2 expression and increased caspase-3 activity after 24 h of treatment. This apoptotic effect was observed only with activation of the A_3 receptor, whereas activation of the A_1 or A_{2A} receptors did not induce significant apoptotic effects (Appel et al., 2001). However, activation of A_1 receptors with CPA increased the cell viability and reduced apoptosis, and the antagonist DPCPX significantly induced apoptosis and caspase-3 expression in MCF-7 cells (Dastjerdi et al., 2016).

Among the molecular targets of RSV reported to date, the AMP-activated protein kinase (AMPK) can be found (Kulkarni and Canto, 2015). The activation of AMPK is reported to suppress the proliferation of various cancers *via* the regulation

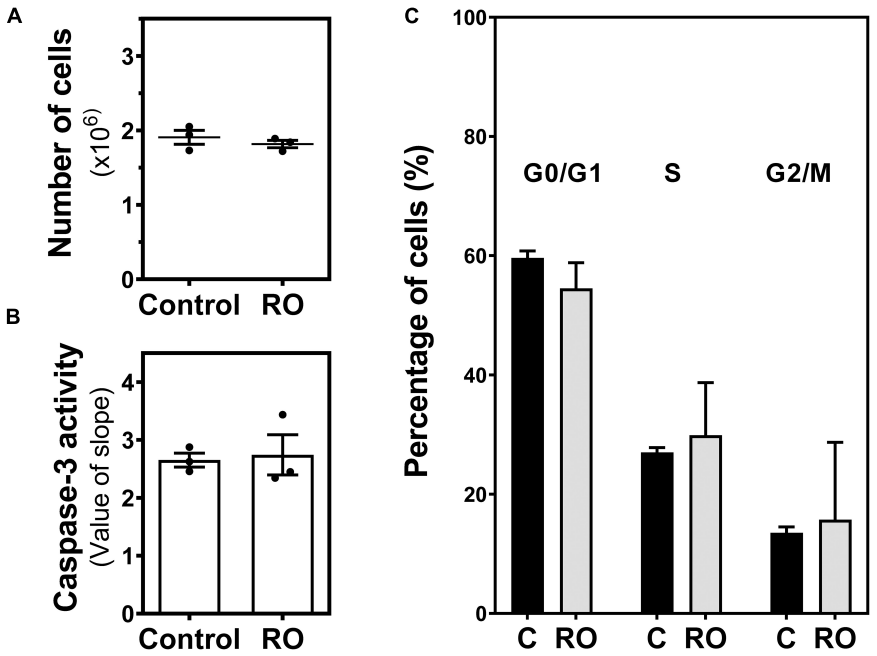

FIGURE 9 | Effect of PDE inhibition on C6 glioma cell growth. Cells were exposed to 100 μM RO-20-1724 (RO) for 24 h and **(A)** the number of cells, **(B)** caspase-3 activity, and **(C)** cell cycle phases were analyzed and measured as described in "Materials and Methods." Data are means ± SEM of three independent assays.

of cell cycle progression, apoptosis, autophagy, inhibition of protein synthesis, and *de novo* fatty acid synthesis. AMPK causes G_1 cell cycle arrest *via* upregulation of the tumor suppressor protein p53, which upregulates p21, a CDK inhibitor (Motoshima et al., 2006). AMPK is a trimer with α-, β-, and γ-subunits. The α-subunit contains the kinase domain, and its Thr^{172} residue is phosphorylated (p-AMPK) by an upstream kinase and determines its regulation. The binding of AMP and, to a lesser extent, ADP to the γ-subunit stimulates AMPK activity. Thus, changes in the ATP/ADP or ATP/AMP ratio lead to the allosteric activation of AMPK (Herzig and Shaw, 2018). Therefore, enzymes involved in the modulation of AMP levels due to the conversion of AMP to adenosine (i.e., 5′NT) or adenosine to AMP (i.e., adenosine kinase) could control AMPK activation. Extracellular adenosine activates AMPK (Aymerich et al., 2006); however, PKA-mediated inhibition of AMPK *via* increased inhibitory phosphorylation of $AMPK^{Ser173}$ and reduced activating phosphorylation of $AMPK^{Thr172}$ is reported (Djouder et al., 2010; Aw et al., 2014). This PKA-mediated inhibition of AMPK could take place in C6 glioma cells as cAMP/PKA signaling is significantly increased, and the AMPK activation (measured as the p-AMPK/AMPK ratio) is not modified in these cells after 100 μM RSV treatment for 24 h (Sanchez-Melgar et al., 2019). Therefore, the cell cycle arrest elicited by RSV in C6 glioma cells seems to be independent of the AMPK/p53/p21 mediated inhibition of CDKs.

The enzyme CD73 has gained attention since it was discovered that adenosine is one of the major constituents in the tumor microenvironment (Di Virgilio and Adinolfi, 2017) and that this tumor-derived adenosine facilitates the immune escape by activating the A_{2A} receptor in T and NK cells (Ohta and Sitkovsky, 2014). CD73 overexpression in tumor cells is associated with the pathogenesis (Yan et al., 2019), progression (Yu et al., 2018), and poor prognosis in several types of cancers, including HNSCC (Ren et al., 2016a), triple-negative breast cancer (Loi et al., 2013), oral squamous cell carcinoma (Ren et al., 2016b), and high-grade serous ovarian cancer (Turcotte et al., 2015), among others. Therefore, targeting CD73 with selective inhibitors or antibodies is being considered as a promising therapeutic strategy against cancer (Stagg et al., 2010). Our data indicate a significant reduction in the CD73 activity located in the plasma membrane fraction after RSV exposure, suggesting that RSV might affect adenosine production in the pericellular space. Nevertheless, a lower enzymatic activity of ADA was also found after RSV treatment, leading to lower deamination of adenosine into inosine and significantly decreased inosine levels. A limitation of the technique employed here to measure CD73 activity could be that we cannot rule out the contribution of alkaline phosphatase in the conversion of AMP to adenosine (Zimmermann, 2021). However, the presence of 100 μM levamisole, a selective alkaline phosphatase inhibitor, during the assay determining CD73 activity in the human cerebral cortex modified the activity of CD73 in neither membranes nor cytosolic fraction by using the same assay conditions as here (Alonso-Andres et al., 2018). This reduced CD73 activity could promote an increased level of ATP. It is well known that extracellular ATP may exhibit a cytotoxic effect in cancer cells depending on the concentration (Vultaggio-Poma et al., 2020). Among P2 receptors, the P2 × 7 receptor subtype seems to be the main player in ATP-dependent biological actions. Prolonged activation

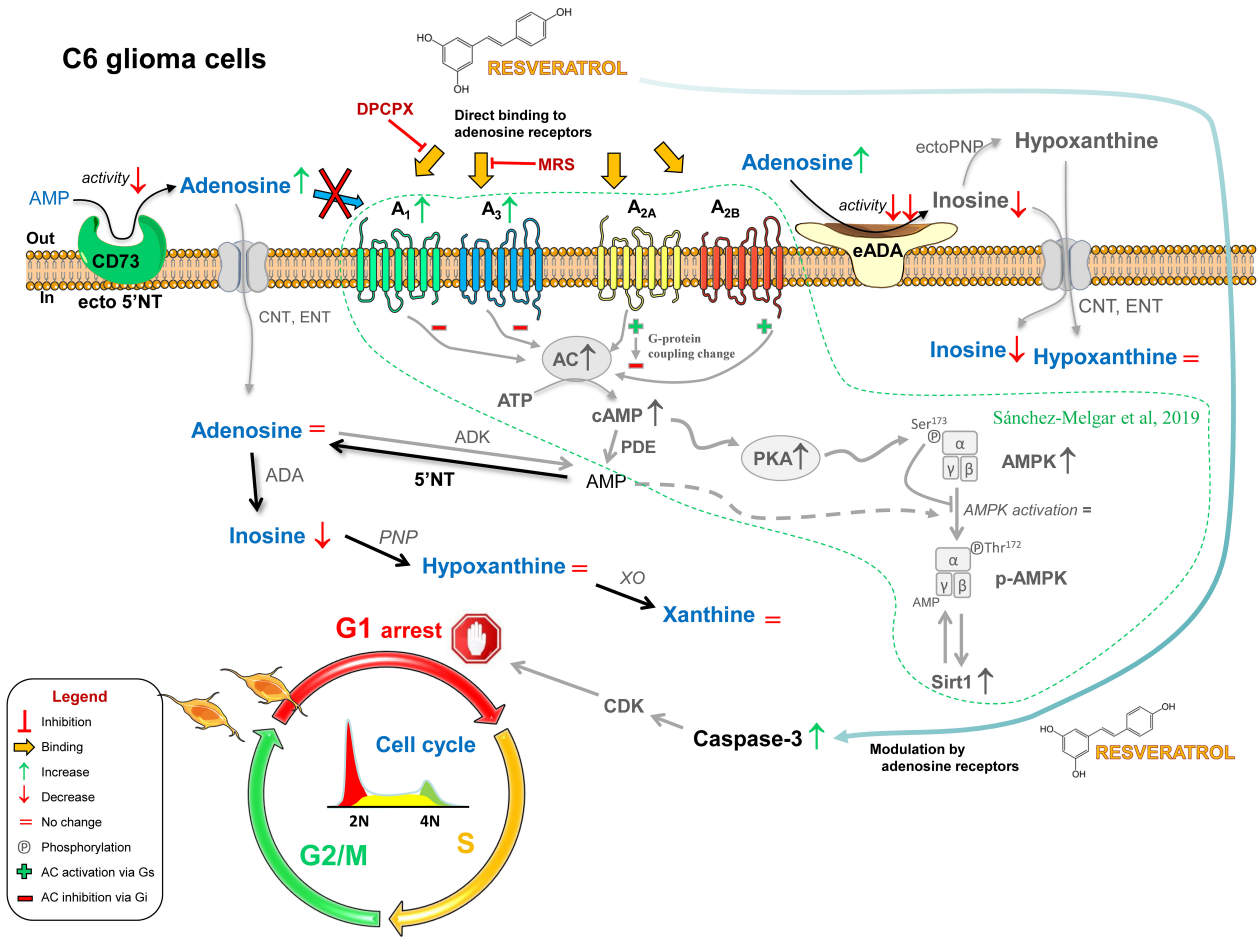

FIGURE 10 | Possible role of adenosinergic signaling in the antitumoral effect of RSV. The exposure of C6 glioma cells to RSV causes the decrease (↓) or the increase (↑) of several compounds and proteins that ultimately lead to cell cycle arrest in the G_1 phase. RSV upregulates A_1 and A_3 receptors; changes G-protein coupling of A_{2A} receptor from activation (Gs, +) to inhibition (Gi, –) of adenylate cyclase (AC) activity; upregulates AC, increases basal AC activity, and upregulates protein kinase A (PKA); higher levels of cAMP inhibit AMPK activation *via* PKA-mediated phosphorylation (Ser^{172}); reduces adenosine-converting enzymes (i.e., CD73 and ADA), leading to increased extracellular levels of adenosine; caspase-3 is activated as a result of the direct binding of RSV to adenosine receptors and the modulatory action of these receptors on other RSV-elicited pathways, leading to CDK-mediated cell cycle arrest in the G_1 phase. Previous results reported by our group in these cells are shown in gray and surrounded by a dotted line.

of P2 × 7, *via* high levels of extracellular ATP over an extended time period, can lead to the formation of a macropore, leading to depolarization of the plasma membrane and, ultimately, to cell death (Lara et al., 2020). However, our results show that, after 24 h of P2X stimulation with BzATP, C6 cell viability did not change, and this prolonged stimulation was unable to modify the RSV effect.

A highly active CD73 enzyme has been detected in glioblastoma (Ludwig et al., 1999) and glioma (Bavaresco et al., 2008). Independently of its enzymatic role, CD73 can mediate cell–cell adhesion being a coreceptor in T cell activation or regulate cell interaction with ECM components and migration on them. Acting as a docking molecule, CD73 mediates migration and invasion of A375 melanoma cells (Sadej and Skladanowski, 2012) and glioblastoma invasiveness (Fenoglio et al., 1997) through focal adhesion kinase activation. Interestingly, RSV was able to regulate the invasion of cancer cells by modulating such focal adhesion kinase (Buhrmann et al., 2017), which could be mediated by the RSV effect on the CD73 enzyme.

The precise molecular mechanism by which RSV modulates CD73 activity is not clarified yet. It is described a possible link between hypoxia-inducible factor-1 alpha (HIF-1α) and CD73 (Sotnikov and Louis, 2010). Hypoxic conditions in the tumor trigger HIF-1α activation and, in turn, an upregulation of the CD73-adenosine pathway (Li et al., 2017), which is able to promote tumor growth and metastasis (Zhang, 2012). RSV treatment reduced HIF-1α in cancer cells *in vitro* (Zhang et al., 2005), which might explain the reduction in the CD73 activity observed in RSV-treated cells in our study. Of interest, moderate hypoxia (24 h at 5% O_2) produces increased endogenous adenosine levels in C6 glioma cells and the downregulation and upregulation of A_1 and A_{2A} receptors, respectively. However,

HIF-1α was not modulated by moderate hypoxia, and C6 cells were resistant to cell death elicited by hypoxic insult (Castillo et al., 2008).

A growing body of evidence indicates that dual blockade of CD73 and A_{2A} receptors could enhance the antitumor response (Beavis et al., 2015; Young et al., 2016). In this sense, RSV induces the reduction in CD73 activity as observed in our study and changes the A_{2A} receptor downstream signaling from activation to inhibition of adenylyl cyclase as we reported previously (Sanchez-Melgar et al., 2019).

Reduced CD73 and ADA activities as reported herein result in increased extracellular levels of adenosine after RSV exposure. However, RSV-induced cell growth inhibition seems to be independent of the activation of adenosine receptors by extracellular adenosine because of (1) an adenosine concentration of 50 μM, similar to that achieved after RSV treatment, and 75 μM were unable to reduce cell viability, (2) 100 μM adenosine treatment slightly decreased cell viability but to a lesser extent than observed with RSV treatment, and (3) ADA presence during RSV treatment did not impede the RSV effect on these cells. Instead, direct activation of adenosine receptors by RSV itself acting as a non-selective agonist seems to be involved as we recently suggested (Sanchez-Melgar et al., 2019). Pharmacological inhibition of the A_1 receptor with DPCPX or the A_3 receptor with MRS1220 during RSV treatment resulted in a significantly reduced RSV effect, suggesting possible participation of reduced levels of cAMP after RSV binding to these Gi-protein coupled receptors (i.e., A_1 and A_3). Moreover, prolonged pharmacologic inhibition of the A_{2A} receptor with ZM241385 or the A_{2B} receptor with PSB1115 partially mimicked the RSV-induced effect on C6 cell viability. As mentioned, RSV treatment altered the A_{2A} receptor/Gs-protein coupling, leading to the inhibition of the cAMP generation upon pharmacologic stimulation of the A_{2A} receptor with CGS21680 after RSV treatment (Sanchez-Melgar et al., 2019). All these data might indicate that the reduction of C6 cell growth upon RSV treatment could be related to the inhibition of cAMP levels through adenosine receptor modulation, mainly A_1 and A_3 receptors.

In summary, our study suggests that a reduced CD73 activity located in the plasma membrane in addition to a fine-tuned modulatory role of adenosine receptors could be involved, at least in part, in the antiproliferative action of RSV in C6 glioma cells (**Figure 10**).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MM and JA: conceptualization, writing—review and editing. AS-M, MM, and JA: formal analysis. MM: funding acquisition. AS-M and SM-L: investigation. AS-M and MM: writing—original draft. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.702817/full#supplementary-material

Supplementary Video 1 | Growth of C6 glioma cells in control conditions or in the presence of 0.1–100 μM RSV. Time-lapse movie (one frame/2 min) of cells growing for 24 h since the start of treatment. Bar size: 100 μm.

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REVIEW
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Adenosinergic Signaling as a Key Modulator of the Glioma Microenvironment and Reactive Astrocytes

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Astrocytes are numerous glial cells of the central nervous system (CNS) and play important roles in brain homeostasis. These cells can directly communicate with neurons by releasing gliotransmitters, such as adenosine triphosphate (ATP) and glutamate, into the multipartite synapse. Moreover, astrocytes respond to tissue injury in the CNS environment. Recently, astrocytic heterogeneity and plasticity have been discussed by several authors, with studies proposing a spectrum of astrocytic activation characterized by **A1**/neurotoxic and **A2**/neuroprotective polarization extremes. The fundamental roles of astrocytes in communicating with other cells and sustaining homeostasis are regulated by purinergic signaling. In the CNS environment, the gliotransmitter ATP acts cooperatively with other glial signaling molecules, such as cytokines, which may impact CNS functions by facilitating/inhibiting neurotransmitter release. Adenosine (ADO), the main product of extracellular ATP metabolism, is an important homeostatic modulator and acts as a neuromodulator in synaptic transmission via P1 receptor sensitization. Furthermore, purinergic signaling is a key factor in the tumor microenvironment (TME), as damaged cells release ATP, leading to ADO accumulation in the TME through the ectonucleotidase cascade. Indeed, the enzyme CD73, which converts AMP to ADO, is overexpressed in glioblastoma cells; this upregulation is associated with tumor aggressiveness. Because of the crucial activity of CD73 in these cells, extracellular ADO accumulation in the TME contributes to sustaining glioblastoma immune escape while promoting **A2**-like activation. The present review describes the importance of ADO in modulating astrocyte polarization and simultaneously promoting tumor growth. We also discuss whether targeting of CD73 to block ADO production can be used as an alternative cancer therapy.

Keywords: tumor microenvironment, glioblastoma, tumor-associated astrocyte, A2-like astrocyte, adenosine, CD73

INTRODUCTION

In the last few years, astrocytes have received increased attention, with many studies aimed at in-depth understanding of their functions in the healthy brain and in central nervous system (CNS) pathologies (Khakh and Sofroniew, 2015; Escartin et al., 2019). Reactive astrocyte responses have been described as detrimental in different pathologies, including neuroinflammatory and neurodegenerative diseases and brain tumors (Liddelow and Barres, 2017).

In this regard, glioblastoma (GB) is the most common and aggressive primary tumor in the CNS (Huse and Holland, 2010). Although many researchers have attempted to develop new therapeutic strategies for GB, patients continue to show a short median survival time (Huse and Holland, 2010; Nørøxe et al., 2016; Di Carlo et al., 2019). The tumor microenvironment (TME) contains not only GB cells, but other normal cells such as immune cells and astrocytes that contribute to cancer progression and may shape the tumor cell response to radio-chemotherapy (Wang et al., 2017; Najafi et al., 2019; Wei et al., 2020). Therefore, GB-astrocyte crosstalk may explain the poor prognosis of patients by improving the understanding of how astrocytes contribute to GB progression, which can provide new biological treatment targets (Brandao et al., 2019).

The presence of extracellular adenosine triphosphate (ATP) and its hydrolysis products, namely ADP, AMP, and adenosine (ADO), triggers the purinergic signaling cascade (Burnstock, 2007; Di Virgilio et al., 2009). The biological effects of extracellular purines and pyrimidines are mediated by P1 and P2 purinoceptors (Zarrinmayeh and Territo, 2020). ADO mediates the sensitization of the P1 receptors named A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R (Fredholm et al., 2011). The P2 family is subdivided into ionotropic P2X (1–7) and metabotropic P2Y ($P2Y_{1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13,\ 14}$) receptors (Burnstock, 2007; Di Virgilio et al., 2018). ATP plays a dual role in the TME by exhibiting anti- or pro-tumor effects depending on its extracellular concentration, the presence of purinergic receptors, and the combined activity of ectonucleotidases, such as ecto-nucleoside-triphosphate-diphosphohydrolases (E-NTPDases) and CD73, which metabolize ATP to ADO in the extracellular space (Allard et al., 2016, 2017). The amplitude of the agonist effects of ATP and its metabolite ADO is critical for maintaining the TME, as these signaling molecules have tumor-promoting activities in immune escape, angiogenesis, cell proliferation, and migration (Allard et al., 2016, 2020; Di Virgilio et al., 2016, 2018). In addition, extracellular ATP and ADO have important roles in neurodegenerative, cognitive, and psychiatric disorders. ADO modulates synapse function by altering neuron firing in different brain regions, contracting the local vasculature, and exerting immune/neuromodulatory effects (Dunwiddie, 1985; Redzic et al., 2010; Hinton et al., 2014; Allard et al., 2016; Di Virgilio et al., 2016). In this regard, selective participation of purinergic signaling in astrocytes has been well-established and recently reviewed (Illes et al., 2019; Agostinho et al., 2020; Lopes et al., 2021). However, astrocytic polarization has recently gained attention, although little is known about the contribution of extracellular purines to **A1**/neurotoxic or **A2**/neuroprotective astrocyte polarization. The present review summarizes the available evidence on the participation of purinergic signaling, mostly involving ADO, in modulating the astrocyte phenotype in the TME and its further impact on GB progression.

ROLE OF REACTIVE ASTROCYTES IN THE BRAIN

Astrocytes are abundant and complex glial cells in the CNS and are key elements involved in brain homeostasis (Sofroniew and Vinters, 2010). Astrocytes establish and maintain the blood–brain barrier with their cell end-feet (Ballabh et al., 2004; Verkhratsky and Nedergaard, 2018). Moreover, these glial cells are tightly integrated into neural networks, participating in synaptic transmission regulation via uptake of the neurotransmitter glutamate, and communicate with neighboring cells through Ca^{2+} signals (Clarke and Barres, 2013). Astrocytes form a fundamental part of synaptology together with the pre- and postsynaptic neuronal compartments as part of the multipartite synapse (Verkhratsky and Nedergaard, 2018).

Astrocytes undergo a set of morphological changes in their normal state in response to CNS insults (Wilhelmsson et al., 2006; Sofroniew, 2009; Burda et al., 2016; Liddelow et al., 2017). The reactivity of astrocytes is a universal reaction that occurs in response to cerebral injury. Under stress condition, these cells assume a wide range of new characteristics in brain, becoming hypertrophic, upregulating intermediate filaments such as nestin, vimentin, and glial fibrillary acidic protein, and in some cases, activating proliferation process (Pekny and Pekna, 2014; Boccazzi and Ceruti, 2016; Guan et al., 2018). Reactive astrocytes are observed in the brains of patients suffering from various pathological conditions, including trauma, infection, neurodegeneration, and ischemia (Zamanian et al., 2012).

Because of their variable roles in different pathological conditions, reactive astrocytes remain controversial. Although they are recognized as the pathological hallmark of CNS structural lesions, previous studies reported that, in addition to supporting CNS recovery, astrocytes could inhibit axon regeneration after CNS injury and produce proinflammatory cytokines that exacerbate neuroinflammatory damage (Sofroniew and Vinters, 2010; Zamanian et al., 2012; Liddelow et al., 2017). Recently, astrocytes were shown to react to insults in an activation spectrum, similar to that observed in macrophages and microglia in the CNS, assuming two opposite phenotypes in a spectrum of polarization: **A1**-astrocyte and **A2**-astrocyte (Liddelow and Barres, 2017; **Figure 1A**). **A1**-astrocytes are neurotoxic and associated with injuries and neurodegenerative pathologies such as Alzheimer's disease (Goetzl et al., 2018; Carter et al., 2019; Grimaldi et al., 2019), Parkinson's disease (Hinkle et al., 2019), and Huntington's disease (Diaz-Castro et al., 2019). Moreover, **A1**-astrocyte polarization has been associated with normal brain aging, which supports the involvement of **A1**-astrocytes in neuroinflammation (Clarke et al., 2018). In contrast, **A2**-astrocytes tend to be neuroprotective and promote neuronal survival (Singh et al., 2019). Therefore, once induced, these two extremes of cell phenotypes diverge in gene expression, cell

structure, signaling, and overall function (Escartin et al., 2019). The plasticity of astrocytes that enables them to exert diverse responses to injuries has become a topic of research interest, representing an opportunity to explore specific therapeutic strategies for brain pathologies including GB (Pavlou et al., 2019).

TUMOR MICROENVIRONMENT OF THE BRAIN: THE IMPORTANCE OF MICROGLIA-ASTROCYTE CROSSTALK

Glioblastoma is considered as the most common and aggressive primary brain tumor, accounting for more than 40% of neoplasms of the CNS (Huse and Holland, 2010; Di Carlo et al., 2019). Clinically, gliomas are divided into four degrees, grade I as benign, which show slow proliferation rate and good prognosis after surgical removal; grade II gliomas, which are slightly more severe and capable of progressing to higher grades; and grade III and IV gliomas, which are the most aggressive and characterized by a high rate of cell proliferation, spreading rapidly through the normal parenchyma of the CNS (Nørøxe et al., 2016; Verano-Braga et al., 2018). Moreover, GB (grade IV glioma) exhibits higher angiogenesis and necrosis when compared to grade I–III gliomas, resulting in a short survival time of approximately 15 months in patients (Huse and Holland, 2010; Di Carlo et al., 2019). The first-choice treatment for GB involves surgical removal combined with radio-chemotherapy with temozolomide (TMZ), a DNA-alkylating agent (Stupp et al., 2005).

The TME directly influences tumor growth and proliferation. The GB microenvironment comprises infiltrating and resident immune cells, vascular cells, and glial cells such as astrocytes and microglia (Chen and Hambardzumyan, 2018; **Figure 1B**). The interactions of tumor cells with non-malignant cells in the TME occur via direct cell-cell communication, shape tissue reorganization, and tumor ecosystem modulation (Tirosh and Suvà, 2018), and impact the biology and aggressiveness of GB (Azambuja et al., 2020a).

Tumor-associated macrophages are the dominant infiltrating immune cell population in the tumor mass, constituting ∼30–40% of total cells of the GB bulk (Hambardzumyan et al., 2016; Chen and Hambardzumyan, 2018). Microglia, as the resident innate immune cell of the CNS, is also abundant in the GB TME (Charles et al., 2011). Both macrophages and microglia display an alternative phenotype of activation or M2-*like* polarization when subverted by the tumor signals associated with growth, invasion, and angiogenesis, in addition to contributing to the establishment of an immunosuppressive environment (Da Fonseca et al., 2016; Placone et al., 2016; Matias et al., 2017; Roesch et al., 2018). In addition to macrophages and microglia, lymphocytes are recruited to the tumor site, a relevant part of the TME. A higher proportion of $CD4^+$ than $CD8^+$cells in the TME is associated with a high tumor grade and worst prognosis (Gieryng et al., 2017; Strepkos et al., 2020).

Other non-neoplastic cells are found in smaller numbers, such as oligodendrocyte precursor cells and neurons. Although there is limited evidence explaining the contribution of glial cells to immunosuppression, these cells have the potential to alter the TME (Henrik Heiland et al., 2019). Communication among these different cell types is an important process carried out by the exchange of information via extracellular vesicles, including exosomes, containing signaling proteins or regulatory RNAs (Rajendran et al., 2014).

The interaction of GB cells with astrocytes around the peritumoral area has become a topic of great interest. Reactive astrocytes exhibit intimate crosstalk with activated microglia and similar secreted factors such as interleukin-1β, interleukin-6, and nitric oxide, which are related to GB progression

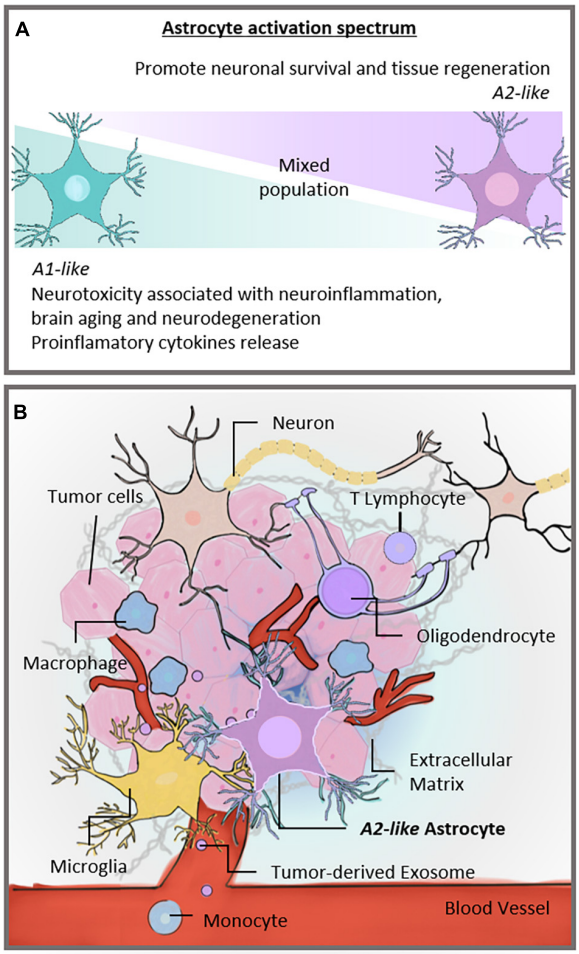

FIGURE 1 | Activation spectrum of astrocytes and the brain TME. **(A)** Astrocytic spectrum of activation. Recent studies proposed that astrocytes became reactive and assumed an activation spectrum characterized by two extremes, the **A1** and **A2** phenotypes. The **A1**-astrocyte phenotype is associated with neurotoxic and neuroinflammatory effects, whereas the **A2**-astrocyte phenotype is related to neuroprotection. **(B)** Different cells constitute the TME of GBs. Neurons, microglia, and glial cells, such as astrocytes and oligodendrocytes, share the brain milieu with tumor cells, establishing a complex ecosystem. These cells are surrounded by extracellular matrix and susceptible to signals carried by tumor-derived exosomes. In addition, monocytes can be recruited to the brain site and contribute to forming the TME. Our hypothesis is that astrocytes in the TME assume an **A2**-like astrocyte phenotype, partly explaining GB malignancy. TME, tumor microenvironment; CNS, central nervous system; GB, glioblastoma.

(Liddelow and Barres, 2017; Jha et al., 2019) and a poor prognosis for patients (Mega et al., 2020). This relationship may also be essential for the immune aspects of the brain tumor TME. Placone et al. (2016) suggested that astrocytes promote the proliferation and invasion of tumor cells and decrease chemotherapeutic efficiency by protecting malignant cells from apoptosis (Chen et al., 2015). Moreover, astrocytes cleave the inactive form of matrix metalloproteinase-2 (pro-MMP2) to matrix metalloproteinase 2, which is involved in tumor invasiveness (Le et al., 2003). Brandao et al. (2019) demonstrated that astrocytes are involved in GB progression, identifying reactive astrocytes around the tumor as tumor-associated astrocytes characterized by high proliferation, migration, and invasion to support tumor cell survival. Studies have demonstrated that astrocytes release exosome vesicles (EVs) containing promoters of angiogenesis and immune modulation, further supporting the novel capabilities of astrocytes in communicating with the TME (Proia et al., 2008; Hajrasouliha et al., 2013). Additionally, Leiss et al. (2020) highlighted astrocyte-glioma crosstalk, indicating that platelet-derived growth factor is a potential astrocytic biomarker associated with a poor prognosis in patients with GB.

Astrocytes in the peritumoral areas can be affected by GB, causing leakage through the blood–brain barrier and contributing to the entrance of a set of immune cells at the tumor site (Watkins et al., 2014; Nørøxe et al., 2016). The recruitment of new tumor-associated macrophages and regulatory T-cells to the TME and overall immunosuppressive microenvironment are crucial for GB development and progression and are factors that affect the poor response of this tumor to conventional immunotherapy (Joyce and Fearon, 2015; Broekman et al., 2018; Pasqualini et al., 2020).

In summary, tumors control their microenvironment to establish an immunosuppressed niche. The tumor capacity to "educate" non-transformed cells in the TME contributes to escape from surveillance by avoiding an effective immune response against tumor and preventing the recruitment of other adaptive and innate immune cells that may inhibit tumor growth (Da Fonseca et al., 2016). Therefore, tumor cells can effectively alter the immune system from a protective to a detrimental state and favor tumor progression (Chen and Hambardzumyan, 2018). In accordance with these observations, increasing evidence has revealed that astrocytes contribute to the immunosuppressive characteristics of the TME, and that microglia-astrocyte crosstalk is an important subject that requires further study (Placone et al., 2016). Besides that, the discussion about astrocytic activation states into the GB microenvironment needs to be amplified in order to comprehend the complexity of GB phenotypes and the diversity of the tumor ontogeny (Nørøxe et al., 2016).

PURINERGIC SIGNALING IN ASTROCYTES: FROM ADENOSINE TRIPHOSPHATE TO ADENOSINE

ATP is known for its classic function in energy metabolism. However, in the extracellular environment, this nucleotide is crucial for the maintenance of physiological functions including neuro/gliotransmission and immune/inflammatory responses (Khakh and Burnstock, 2009). ATP can be metabolized to ADO by ectonucleotidases, such as CD39 (NTPDase1) and CD73 (ecto-5′-nucleotidase), which play key roles by producing active hydrolyzed products and recycling nucleotides (Burnstock, 2007; Allard et al., 2017). Adenosine deaminase (ADA) is responsible for converting ADO to inosine, and both ADO and inosine can be transported across cellular membranes through nucleoside transporters (ENTs) (Boswell-Casteel and Hays, 2017; Pastor-Anglada and Pérez-Torras, 2018; **Figure 2A**).

ATP is considered as a danger-associated molecule that is released by injured cells into the extracellular medium during inflammation (Minkiewicz et al., 2013). In addition, ATP can be released by astrocytes through specific and regulated pathways such as exocytosis (Pangršič et al., 2007), vesicular release (Coco et al., 2003; Zorec et al., 2016), or diffusion through ion channels, specifically pannexin/connexin channels (Stout et al., 2002; Suadicani et al., 2012). Increasing evidence has demonstrated that both astrocytes and tumor cells express purinergic receptors and purine-metabolizing enzymes, which can trigger a set of biological effects in the CNS (Wink et al., 2003, 2006; Rodrigues et al., 2015; Azambuja et al., 2019a; Illes et al., 2019; Campos-Contreras et al., 2020). In line with this, ATP and ADO signaling may have significant impacts on therapies for brain tumors such as GB (Azambuja et al., 2019a,b, 2020c).

Astrocytes release various gliotransmitters, such as glutamate, D-serine, and ATP (**Figure 2B**). Notably, after activation, a single astrocyte releases both glutamate and ATP in the multipartite synapse, leading to biphasic modulation of synaptic transmission (Ma et al., 2016). Extracellular ATP is well-known for its role in mediating astrocytic Ca^{2+} waves (Guthrie et al., 1999) as well as in astrocyte-mediated wound healing in glial scars (Singh et al., 2015). Moreover, the catabolism of ATP likely represents the source of ADO inactivating postsynaptic $A_{2A}R$ (Agostinho et al., 2020), as well as the mechanism by which the release of gliotransmitters by astrocyte exerts neuronal feedback actions and eventually modulates synaptic transmission and plasticity (Illes et al., 2019; Scemes et al., 2019).

As described above, microglia-astrocyte crosstalk is instrumental to CNS functions and determines the fate of astrocytes and microglia activation (Jha et al., 2019). Microglial activation by lipopolysaccharide, a TLR4 agonist, induces the release of ATP, which in turn stimulates $P2Y_1$ in astrocytes, leading to the release of glutamate and further modulation of neuronal activity (Pascual et al., 2012). Cortical astrocytes respond to extracellular ATP under *in vitro* experimental conditions, and astrocytic activation into a reactive phenotype, including cell proliferation and glial fibrillary acidic protein remodeling, depends on ATP levels (Suadicani et al., 2012). Similarly, Ficker et al. (2014) found a correlation between P2X7 receptor sensitization and the development of inflammatory and neuropathic pain, possibly involving oxidative stress.

ATP can be converted into its breakdown product ADO in the extracellular environment by ectonucleotidases. A previous study reported a purinergic enzyme profile for E-NTPDases

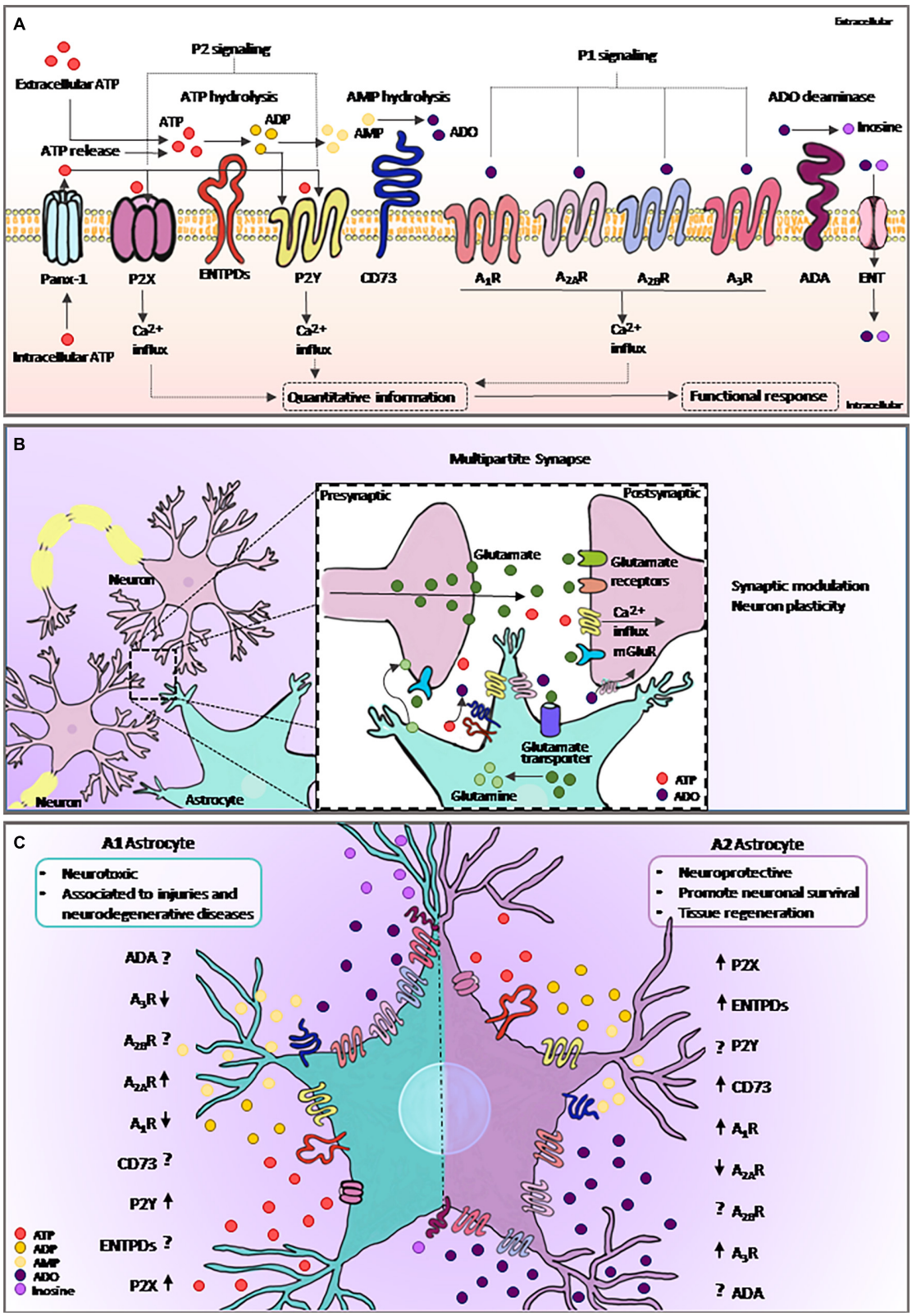

FIGURE 2 | Purinergic signaling and astrocytes. **(A)** Intracellular ATP is released from astrocytes via specific channels such as Panx-1. Extracellular ATP and its breakdown products, ADP and ADO, are agonists of purinergic receptors, and the extracellular levels of nucleotides and nucleosides are regulated by

(Continued)

FIGURE 2 | ectonucleotidases such as ENTPDs and CD73. ADA catalyzes irreversible deamination of ADO into inosine. ADO can return to the intracellular space trough specific transporting channels, named as ENTs. Purinergic signaling plays an important role in many biological processes, including astrocytic functions. Both ATP released from astrocyte and from neighboring cells can selectively bind to P2 receptors, whereas ADO binds to P1 receptors. Activation of purinergic receptors in astrocytes can induce several functional responses, including the regulation of neural communication and immune/inflammatory responses. **(B)** ATP is an important gliotransmitter released by astrocytes in combination with glutamate. The multipartite synapse is a key mechanism of neuron communication and plasticity. **(C)** Astrocytes became reactive in response to injury. Here, we show the mechanism by which the elements of purinergic signaling are expressed or face this response. The graphical representation of purinergic signaling shows **A1**-astrocyte in the left panel and **A2**-astrocyte in the right panel. Arrows represent the described modulation of the elements found in reviewing the literature on the topic, and the interrogation points are represented as components without data already described in the literature. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ADO, adenosine; Panx-1, pannexin channel 1; ADA, adenosine deaminase; ENTPDs, ecto-nucleoside-triphosphate-diphosphohydrolases; ENT, equilibrative nucleoside transporter.

and CD73 in rat astrocytes, demonstrating that extracellular ATP is rapidly converted to ADP, and ultimately, to AMP by NTPDase2, an ectoenzyme predominantly expressed in these cells (Wink et al., 2003, 2006). As high levels of extracellular ATP are neurotoxic, NTPDase2 activity in astrocytes may protect neurons from damage, in addition to promoting ADO production (Wink et al., 2006). In addition, CD73 overexpression was observed in reactive astrocytes as a possible protective astrocyte repertoire during the symptomatic phase of an experimental autoimmune encephalomyelitis model, demonstrating the relationship between the ATP/ADO ratio and acute inflammatory situations (Lavrnja et al., 2015). CD73 activity is also involved in astrocyte adhesion and migration, possibly via interactions with the extracellular matrix (Adzic et al., 2017; Adzic and Nedeljkovic, 2018).

In general, ADO is directly related to cellular processes such as viability and adaptability (Cunha, 2016). However, under pathological conditions, the ADO concentration acts as a risk factor for CNS pathologies, including the development/progression of neurodegenerative diseases and brain tumors (Cunha, 2016; Azambuja et al., 2020e). Thus, studies have focused on investigating astrocyte plasticity to understand the role of ADO in pathological situations.

The ability of astrocytes to detect patterns of neural activity is attributed to the fine adjustment of astrocyte signaling, which in turn modulates synaptic transmission and contributes to the most diverse forms of synaptic plasticity (Covelo and Araque, 2018; Mederos et al., 2019). Brain injuries caused by trauma or stroke increase extracellular ADO levels (Haskó et al., 2005; Fusco et al., 2018). In this regard, the P1 receptors A_1R and $A_{2A}R$ are located at synapses, particularly at excitatory ones, and cross-communication between astrocytes and neurons regulates the neural networks (Rebola et al., 2008; Illes et al., 2019). Indeed, astrocytes depress excitatory synapses and potentiate inhibitory synapses by activating A_1R and $A_{2A}R$, respectively (Martin-Fernandez et al., 2017). Interestingly, astrocytic $A_{2A}R$ modulates glutamate-mediated signaling through a variety of mechanisms (Rebola et al., 2008). For example, $A_{2A}R$ influences glutamate and GABA uptake by neurons, and selective $A_{2A}R$ activation in astrocytes inhibits glutamate uptake by decreasing Na^+/K^+-ATPases (NKAs) and the α2 subunit of NKA (Matos et al., 2013). Based on these results, $A_{2A}R$ overexpression observed in astrocyte reactivity contributes to the progression of brain diseases, making this receptor a potential therapeutical target for Alzheimer's disease, Parkinson's disease, and Sandhoff disease, as previously reported and extensively revised (Ohta et al., 2006; Matos et al., 2015; Scharbarg et al., 2016; Basanta and Anderson, 2017; Liddelow and Barres, 2017; Quail and Joyce, 2017; Wesseling and Capper, 2018; Allen et al., 2019; Okada et al., 2019; Schalla and Stengel, 2019; Azambuja et al., 2020d; data summarized in **Table 1**).

Although $A_{2A}R$ has shown a protagonist effect in studies of neurodegenerative disease, some neuroprotective actions have been reported for $A_{2B}R$ sensitization. Moidunny et al. (2012) observed a positive correlation among astrocytic leukemia inhibitory factor protein expression, $A_{2B}R$ sensitization, and further Gq/11-PLC-PKC-MAPK-NFκB cascade activation, supporting the neuroprotective effect of $A_{2B}R$ under excitotoxic conditions. In contrast, in an ischemia model, the use of $A_{2B}R$ antagonists (MRS1754 and PSB603) partially limited astrocyte proliferation, thus preventing neurodegenerative effects on neurons (Fusco et al., 2018).

In addition to the relation with astrocytes, purinergic signaling is also an important player in the microglial reactivity. In the CNS, the P2X7 receptor is preferentially located on microglia (Illes, 2020). Besides that, several authors have also been reported the expression of P1 receptors in microglia, describing ADO as a crucial modulator of microglia phenotype of CNS pathologies, including cancer (Hammarberg et al., 2003; Synowitz et al., 2006; Ferreira-Silva et al., 2020).

Extracellular ADO is ultimately converted to inosine by ADA, which exerts many biological regulatory functions such as protecting the brain against neuronal diseases. A very informative study applied an experimental strategy for profiling fibroblasts and inducing neuronal progenitor-derived human-induced astrocytes from patients with amyotrophic lateral sclerosis, to evaluate dysfunctional astrocytic energy metabolism. The investigation revealed that induced human astrocytes had reduced ADA activity, making the astrocytes more susceptible to ADO-induced toxicity. In contrast, restoration of ADA activity and/or supplementation with inosine stimulated the aerobic state of astrocytes, increased the bioenergetic capacity, and decreased neurotoxicity, thereby demonstrating a beneficial therapeutic approach (Allen et al., 2019).

Considering the collective results of previous studies, A_1R, $A_{2B}R$, and ADA activity in astrocytes is crucial for neuronal maintenance and may contribute to the **A2**/neuroprotective profile of astrocytes. In contrast, overexpression of $A_{2A}R$ disrupts adenosinergic homeostasis and is involved in neuronal degeneration, representing a classic condition of **A1**/neurotoxic activation in astrocytes. These data are summarized in **Table 1**. An imbalance of the adenosinergic pathway is common

TABLE 1 | Purinergic signaling in astrocytes.

Nucleotide or nucleoside/receptor/channel/enzyme	Biological process	Experimental system	Effect	References
ATP	Neuroinflammation	*In vitro*	Induction of astrocytic reactivity by ATP	Adzic et al., 2017
ATP	Neuron and glia plasticity	*In vitro*	ATP released as an activation process of astrocytes	Singh et al., 2015
ATP/ADO	Neuron and glia plasticity	*In vivo*	ATP/ADO are released downstream of the GABA-mediated astrocyte Ca^{2+} signal	Covelo and Araque, 2018
Panx1	Neuron and glia plasticity	*In vivo*	Related to ATP release in astrocytes and better outcome of seizures	Scemes et al., 2019
P2X7	Neuroinflammation and pain	*In vivo*	P2X7 receptors as a key mechanism in inducing pain during inflammation	Ficker et al., 2014
CD73	Cell adhesion/migration	*In vitro*	Upregulation of CD73	Adzic and Nedeljkovic, 2018
CD73	Neuroinflammation	*In vivo*	Neuroprotection of CD73 overexpression during acute inflammation	Lavrnja et al., 2015
ADO	Neuron and glia plasticity	Cerebral cortex human astrocytes	Inhibition of astrocyte proliferation independent of P1 receptor sensitization	Marcelino et al., 2020
$P2Y_1 + A_{2A}R$	Neuron and glia plasticity	*In vivo*	ATP/ADO release impact synaptic plasticity and enhance cognitive functions	Mederos et al., 2019
rENT-1	Neurodegeneration	*In vitro*	Neuroprotection role of ADO after hypoxia and glucose deprivation	Redzic et al., 2010
ENT1	Neurodegeneration	*In vitro* + *In vivo*	Reduced GFAP expression in astrocyte cultures ENT1-knockdown, and in $ENT1^{-/-}$mice	Hinton et al., 2014
AR	Neuron and glia plasticity	*In vivo (Drosophila)*	Neuromodulation by ATP release from astrocytes and subsequent activation of AR on dopaminergic neurons	Ma et al., 2016
A_1R	Neuron and glia plasticity	*In vitro* + *In vivo*	Activation of A_1R receptors is increased by wakefulness	Schmitt et al., 2012
A_1R	Neuron and glia plasticity	*In vivo*	Astrocytic ATP and ATP-derived adenosine involved in the cognitive deficits following sleep deprivation	Florian et al., 2011
A_1R	Neuroinflammation	*In vivo*	Induction of inflammation via LPS and control of sleep	Nadjar et al., 2013
A_1R	Neuroinflammation	*In vivo*	Neuroprotective effects	Kong et al., 2020
$A_1R - A_{2A}R$	Neuron and glia plasticity	*In vivo*	Neuromodulation by regulating specific synapses via ATP/ADO	Martin-Fernandez et al., 2017
$A_{2B}R$	Neuron and glia plasticity	*In vitro*	Role of ADO in neuronal protection	Moidunny et al., 2012
$A_{2A}R$	Neuron and glia plasticity	*In vitro*	Increase in extracellular glucose concentration induces astrocytic ADO release, regulating the need for sleep	Scharbarg et al., 2016
A_1R-$A_{2A}R$	Neuroinflammation	Surgical specimens from glioma patients	Neuroprotective and anti-convulsive effect	Huang et al., 2016
$A_{2A}R$	Neurodegeneration	*In vivo*	Neuroprotection after $A_{2A}R$ antagonism using MSX-3	Cerri et al., 2014
$A_{2A}R$	Neuron and glia plasticity	*In vivo*	Interaction between $A_{2A}R$ and D2-dopamine receptors	Cervetto et al., 2018
$A_{2A}R$	Neuron and glia plasticity	*In vivo*	Dysfunction of astrocytic $A_{2A}R$ triggers the crosstalk between astrocyte and neuron	Matos et al., 2015
$A_{2A}R$	Neuroinflammation	*In vitro* + *In vivo*	Astrocytic activation via $A_{2A}R$ as an important mediator of inflammation mediated by microglial activation	Ogawa et al., 2018
$A_{2A}R$	Neuron and glia plasticity	*In vitro*	Astroglial glutamatergic transmission	Okada et al., 2019
$A_{2A}R$	Neurodegeneration	*In vitro*	Overexpression upregulates astrocytic genes related to aging and astrocytic reactivity	Paiva et al., 2019
$A_{2A}R - A_{2B}R$	Neuron and glia plasticity	*In vitro*	Activation of receptors in affecting synaptic networks and neuronal activity	Eusemann et al., 2015
$A_{2B}R$	Neurodegeneration	*In vivo*	ADO as a neuroprotector	Fusco et al., 2018
ADA	Neurodegeneration	*In vitro*	Decrease of ADA activity	Allen et al., 2019

ADA, adenosine deaminase; ADO, adenosine; AR, adenosine receptor; ATP, adenosine triphosphate; ENT1, equilibrative nucleoside transporter 1; LPS, lipopolysaccharide; Panx1, Pannexin 1; rENT1, rat equilibrative nucleoside transporter 1.

in neuronal disorders. ADO exerts a strong inhibitory or excitatory influence on neuronal synapses. Notably, neuronal-stem cell-derived astrocytes can develop into GB (Yao et al., 2018). Therefore, it is essential to understand the activation mechanisms of astrocytes and influence of ADO on their proliferation and function.

ADENOSINERGIC SIGNALING IN GLIOBLASTOMA MICROENVIRONMENT

Considering the lack of effective therapies for treating patients with GBs, new biological targets must be identified, and adenosinergic signaling has emerged as a candidate target (Azambuja et al., 2019a,b, 2020c,d,e; Yan et al., 2019). Studies by our group reported that CD73 overexpression in GB favors tumor progression (Azambuja et al., 2019b, 2020c,d). Moreover, A_1R sensitization potentiates *in vitro* GB cell proliferation, migration, and invasion and contributes to TMZ resistance (Azambuja et al., 2019a). The importance of this protein in cell adhesion and invasion has been verified, as it was observed that CD73 in GB interacts with the extracellular matrix of the TME (Cappellari et al., 2012b). Using an astrocytoma cell line (U373), $A_{2B}R$ was described as a low-affinity receptor activated only by high concentrations of ADO, which occurs in pathological conditions such as hypoxia, and in contrast, $A_{2A}R$ was expressed under physiological ADO levels in the brain (Eusemann et al., 2015). Studies of CD73-FLK mice detected positive regulation of $A_{2B}R$ in GB, and its blockage potentiated TMZ-induced tumor cell death (Yan et al., 2019). A_1R and $A_{2A}R$ are highly expressed in high-grade gliomas, specifically in grade III astrocytoma. In low-grade gliomas, A_1R and $A_{2A}R$ exhibit low expression. ADO acts as a neuroprotective agent and prevents hypoxic toxicity, that is, deregulation of A_1R and $A_{2A}R$ axis influences the metabolism of ADO in the invasive process of GB (Huang et al., 2016).

The tumor niche contains sites of hypoxia formed by the high rate of cell proliferation, without a corresponding increase in the rate of new blood vessel formation. This condition contributes to increased extracellular ATP levels, and consequently, selects the population of immunosuppressive cells present in the TME (Trabanelli et al., 2012). Interestingly, hypoxia conditions and the subsequent release of hypoxia-inducible factor-1α have been associated with the accumulation of extracellular ADO in the TME via the CD39-CD73 axis and further signaling through $A_{2A}R$ (Hatfield et al., 2014). Torres et al. (2019) reported that extracellular ADO production was higher in hypoxia conditions to promote cell migration and invasion in a hypoxia-inducible factor-2 dependent process, whereas A_3R blockade reversed this effect. This corroborated the findings of Niechi et al. (2019), who showed that ADO depletion decreases tumor aggressiveness. In addition, clinical trials of cancer immunotherapy targeting the "hypoxia-adenosinergic pathway" through hyperoxic breathing stimulation and $A_{2A}R$ blockage in combination with conventional anti-PD1/PDL1 immunotherapy are underway (Ohta et al., 2006; Hatfield et al., 2015; Hatfield and Sitkovsky, 2020). These trials are reporting promising results in patients refractory to current therapies (Fong et al., 2020; Willingham et al., 2020). However, it is important to highlight the lack of studies on the therapeutic effect in human CNS tumors.

The release of EVs from tumor cells supports the ability of GBs to communicate with distant cells. Interestingly, the content of EVs reflects the activation state of the mother cell; hence, tumor cells can proliferate and modulate acceptor cells (Matarredona and Pastor, 2019; Zhang et al., 2019; Schuurmans et al., 2020). Studies of EVs are emerging and have shown promising results regarding their ability to maintain a propitious niche for tumor development, as they can modulate immune, epithelial, and glial cell signaling (Benito-Martin et al., 2015; Whiteside, 2017; Almeida et al., 2019; Boomgarden et al., 2020). Particularly, glioma cell lines as well as primary cultured cells released EVs capable of promoting M2-like activation of tumor-associated macrophages (Azambuja et al., 2020b). Moreover, exosomes, a subclassification of EVs, carry ADO and its products, such as inosine, hypoxanthine, and xanthine, which further contribute to independent adenosinergic signaling (Azambuja et al., 2020a; Ludwig et al., 2020).

Among the non-neoplastic cells comprising the TME, astrocytes stand out because of their high phenotypic similarity to GB cells (Galland et al., 2019). As mentioned earlier in this review, the astrocyte spectrum of activation of the **A2**-like phenotype is associated with the success of GB progression. Astrocyte phenotypic modulation can be attributed to GB, further supporting the invasion and resistance to therapy (Oushy et al., 2018; Hallal et al., 2019). One of the main functions of ADO in the extracellular environment is to act as a potent immunosuppressive mediator, which benefits tumor progression (Linden, 2011; Tozaki-Saitoh et al., 2011; Allard et al., 2016; Arab and Hadjati, 2019).

Therefore, in agreement with the results of studies using other non-transformed cells comprising the TME, such as macrophages and microglia, the purinergic receptors and ectonucleotidases may be integral components affecting the phenotypic differentiation of tumor-associated astrocytes (**Figure 2C**). A better understanding of the crosstalk among microglia, astrocytes, and tumor cells may reveal innovative therapeutic options based mainly on adenosinergic signaling to overcome GB progression.

ADENOSINERGIC SIGNALING AS A THERAPEUTICAL STRATEGY FOR GLIOBLASTOMA

Considering the present review, it is possible to understand the importance of astrocytes in maintaining homeostasis in the brain and the effective participation of ATP and ADO signaling in several processes related to health and disease. As the main source of ADO in the CNS is extracellular ATP metabolism, regulation of the hydrolysis of AMP to ADO may play a crucial role in maintaining GB-associated immunosuppression and may be an additional alternative for GB treatment in combination with standard radio- and chemotherapy with TMZ. Indeed, studies have shown the potential of P1 receptor antagonism for anti-glioma therapy. For example, the $A_{2B}R$ blockage would

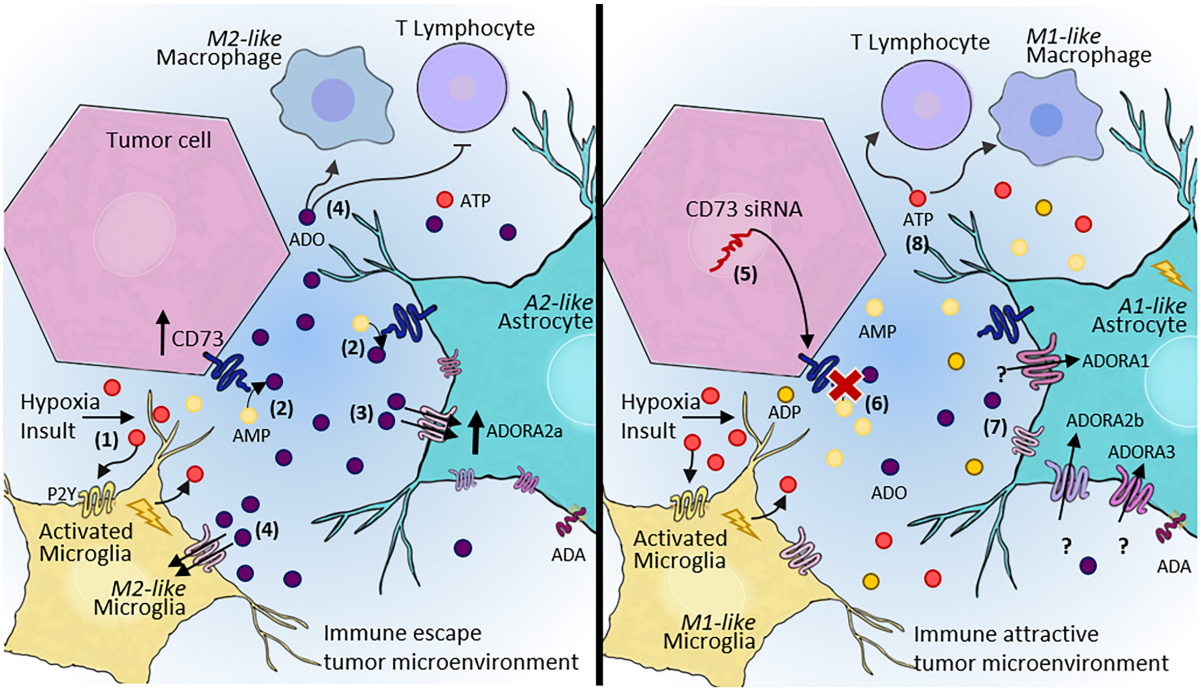

FIGURE 3 | Adenosinergic signaling as a therapeutic target for GB treatment. **(1)** After hypoxia or other insults, microglia and astrocytes become reactive, contributing to the release of ATP into the extracellular space. **(2)** Our hypothesis is that rapid conversion of ATP to ADO via CD73 activity expressed by both tumor cells and **A2**-astrocytes in the TME promote immune escape. **(3)** ADO can sensitize P1 receptors, mainly $A_{2A}R$, contributing to the maintenance of escape from immune surveillance, and consequently, increasing tumor malignancy and progression. **(4)** ADO promotes an immunosuppressive TME, which inhibits T lymphocyte recruitment and induces M2-like macrophage/microglia polarization. **(5)** In contrast, a therapeutic approach blocking CD73 may alter this entire pathway, **(6)** leading to decreased extracellular ADO. **(7)** A decreased ADO concentration in the TME may contribute to A1-astrocyte polarization and consequent impairment of tumor growth. **A1**-astrocytes overexpress A_1R, $A_{2B}R$, and A_3R, but this effect has not been elucidated in tumors. **(8)** In summary, a therapeutic approach aimed at blocking adenosinergic signaling would establish an immune-attractive tumor microenvironment, resulting in the recruitment of effective immune cells to combat tumor cells. ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADO, adenosine; siRNA, small interfering RNA; TME, tumor microenvironment.

contribute to recovering GB chemosensitivity to TMZ (Yan et al., 2019). In addition, the pharmacological blockade of CD73 using a selective inhibitor, the α,β-methylene adenosine 5′-diphosphate (APCP) have been described as a potential therapy for several tumors, such as melanoma, breast cancer, and gliomas (Koszałka et al., 2014; Tomczyk et al., 2018).

By using CD73 as a molecular target and nanotechnology as a strategy for siRNA delivery to the CNS, our group demonstrated the potential of CD73 inhibition and knockdown for controlling *in vitro* and *in vivo* tumor progression (Azambuja et al., 2019a). According to our studies, the pharmacological inhibition as well as the silencing of CD73 impaired the protumor activities of CD73, decreasing the tumor volume and providing an opportunity to overcome chemoresistance and/or improve the TMZ effect (Azambuja et al., 2019a, 2020c,d). We also recently reported the impact of blocking CD73 and consequent decrease in ADO availability on the levels of M2-like macrophages in the TME in a preclinical rat GB model (Azambuja et al., 2020e; **Figure 3**). These data strongly indicate the potential of using therapeutics to modulate the TME via purinergic constituents to re-establish the antitumor activity of immune cells. Nonetheless, the CD73 inhibition may not be always therapeutically helpful for tumors as seen for the GB and the specific biological characteristics of each tumor should be considered. For example, in medulloblastoma, the CD73 expression is a marker associated with better prognosis of patients (Cappellari et al., 2012a, 2015).

Considering the similar features and responses of microglia and astrocytes in the TME, the ADO pathways may also be altered in astrocytic polarization, providing a therapeutic approach for treating patients with GB. Moreover, the participation of ADO and P1 receptors in the blood-brain barrier-associated properties is another interesting point for discussion about the therapeutical potential of ADO signaling blockage. As extensively described by Bynoe et al. (2015), both astrocyte's end-feet and endothelial cells of the blood-brain barrier express those receptors, especially A_1R and $A_{2A}R$. Therefore, P1R mediated-signaling is decisive to regulate the permeability of macromolecules, inflammatory cells, and even therapeutic drugs/cells into the CNS (Bynoe et al., 2015).

CONCLUDING REMARKS

The present review raises the hypothesis that reactive astrocytes are involved in the progression of brain tumors, such as GB. As astrocytes are instrumental to the microenvironment of the CNS,

they can be corrupted by tumor cells and directly and indirectly participate in the TME, thus regulating crosstalk among tumor, glial, and endothelial cells.

Notably, CD73 knockdown or inhibition decreases *in vitro* and *in vivo* GB growth, and CD73 is an interesting target for brain tumor therapy. Additionally, adenosinergic therapy can be applied in most TME cells to inactivate not only transformed cells but also tumor-associated cells, including A2-astrocytes that support tumor progression.

OPEN QUESTIONS

Considering the recent advances described in this review, although astrocyte participation in healthy and diseased brain biology has been well-established, there is no general agreement on astrocytic polarization in different phenotypes according to the nature of the injury, nor specifically in brain tumors. Recently, astrocytes were proposed to be involved in neuroinflammation. Further studies are needed to better describe their full characteristics and key features in different disorders involving immune components. Moreover, studies of astrocytic polarization are necessary to understand if different injury conditions lead to the production of different subpopulations of **A1**/neurotoxic and **A2**/neuroprotective astrocytes, or to transition stages until the fully reactivated cell state is reached.

Few studies have focused on the link between purinergic signaling and astrocyte participation in the tumor niche constitution. Considering the key role of the gliotransmitter ATP and its metabolite ADO on astrocyte functions, the specific factors involved in purinergic signaling, particularly adenosinergic signaling, as potential makers of astrocyte polarization should be further examined. Additionally, the contribution of ADO as a potent modulator of immune responses may directly influence astrocytic polarization, which requires further analysis. Moreover, the contribution of astrocyte-microglia crosstalk may be another key element in the outcome of GB, as well as for other CNS pathologies.

Finally, considering the genetic and phenotypic similarities of astrocytes and GB cells, tracing possible molecular markers or determining the signature of different astrocytic phenotypes is a crucial strategy for determining the contribution of non-malignant astrocytes in brain tumor physiology and establishing more effective therapeutic approaches.

AUTHOR CONTRIBUTIONS

GD and DR selected articles in PubMed and analyzed articles. GD created **Table 1**. DR assembled the figures. EB acquired the funding, supervised, and guided the entire work. All authors conceived the idea of studying the role of purinergic signaling in astrocytes and tumor progression, wrote and edited the article, and reviewed and edited the final text.

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