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The Role of NO/cGMP Signaling on Neuroinflammation: A New Therapeutic Opportunity

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Abstract

The nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling appears to play a key role in inhibiting neuroinflammation and preventing the activation of a proapoptotic pathway, thereby promoting neural cell survival. In addition, evidence indicates that cGMP/protein kinase G (PKG) pathway is involved in the modulation of glial cell activity. Phosphodiesterase 5 (PDE5), which hydrolyzes cGMP in the inactive form, 5'GMP, is present throughout the body and brain and has emerged as a potential therapeutic target for diseases related to neuroinflammatory and neurodegenerative processes, since their inhibition leads to accumulation of cGMP. The objective of this chapter is to review current knowledge of NO/cGMP signaling pathways on neuroinflammation and the potential therapeutic use of PDE5 inhibitors (PDE5-Is) in neurological diseases. The extensive, while recent, literature on the effects of PDE-Is on Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), Huntington's disease (HD), and stroke has been reviewed.

Keywords: PDE5 inhibitors, neurological diseases, glial cells, cGMP signaling, neuroinflammation, neurodegeneration

1. Introduction

A growing number of studies have explored the interaction between the nervous and immune systems during the development of neurological disorders. The central nervous system (CNS) is an environment considered "immunologically privileged," as many antibodies and peripheral immune cells are blocked by the blood-brain barrier (BBB), a highly specialized brain endothelial structure composed of pericytes, astrocytes, and microglia, which does not allow

the passage of peripheral immune cells and whose resident cells express little major histocompatibility complexes I and II (MHC-I and MHC-II) receptors, as well as low levels of pro-inflammatory cytokines. However, in damage situation, glial cells show increased expression of MHC, Toll-like receptors (TLRs), and proinflammatory cytokines (such as TNF- α , IFN- γ , IL-1 β , and IL-6). Innate immune response mediated by glial cells seems to be crucial for the progression of many neurodegenerative diseases including Alzheimer's disease (AD), multiple sclerosis (MS), and Parkinson's disease (PD). Thus, neuroimmunology emerged as an intersection between the nervous system disease mechanism and therapeutic targets.

The nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling appears to play an essential role in inhibiting neuroinflammation and in preventing the activation of a proapoptotic pathway, thus promoting neural cell survival. Phosphodiesterase type 5 inhibitors (PDE5-Is) have recently emerged as a potential therapeutic strategy to modulate neuroinflammation. Mechanistically, PDE5-Is exert anti-inflammatory and neuroprotection effects by inhibiting PDE5 with subsequent accumulation of cGMP and activation of protein kinase G (PKG). The objective of this chapter is to review present knowledge of the NO/cGMP signaling pathways on neuroinflammation and the potential therapeutic use of PDE5-Is on neurodiseases.

2. The role of NO/cGMP signaling on inflammation

Cyclic nucleotides, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) exert many physiological roles such as the regulation of ion channels, relaxation of smooth muscle, immunomodulation, inflammation, cell proliferation and apoptosis, insulin secretion and glycogen synthesis/glycogenolysis, lipogenesis and lipolysis steroidogenesis, phototransduction as well as neuronal survival, and consolidation of memory. Both cAMP and cGMP can alter cell function by activating or inactivating proteins by phosphorylation. The most important regulation of cyclic nucleotides is achieved in negative feedback by activating phosphodiesterases (PDEs), which hydrolyses the cAMP and cGMP in their inactive forms, 5'AMP and 5'GMP, respectively [1–3].

Synthesis of intracellular cAMP from adenosine 5'-triphosphate (ATP) by membrane-bound adenylyl cyclase (AC) is mainly regulated by G proteins. The response to activation of G-protein-coupled receptors (GPCRs) transduces a variety of extracellular signals and then to intracellular signals, regulating cellular responses [4]. The key transducer of cAMP signals is the cAMP-dependent protein kinase A (PKA). Upon binding of cAMP to the regulatory PKA subunits, it dissociates into two free regulatory and two catalytic subunits. The liberated active catalytic PKA subunits can phosphorylate serine and threonine residues on substrate proteins, including the transcription factor cAMP-response element-binding protein (CREB). There are some alternative PKA-independent cAMP actions, such as the immunomodulatory effects in monocytes and macrophages of guanine exchange proteins directly activated by cAMP (EPAC-1 and EPAC-2) [5, 6].

Synthesis of cGMP is mediated by membrane-bound/particulate (pGC) and cytosolic/soluble (sGC) guanylate cyclases, which convert guanosine 5'-triphosphate (GTP) into cGMP. sGC is

activated by NO released by the endothelium and neurons, whereas pGCs (GC-A, GC-B, and GC-C) are activated by binding of specific peptides. GC-A present in the kidney is responsible for controlling natriuresis and blood pressure through stimulation by atrial natriuretic peptide (ANP) and brain-type natriuretic peptide (BNP), which are released from the heart. In the small intestine, GC-C stimulates secretion of fluids through activation by intestinal peptide, guanylin [7]. The physiological effects of cGMP activities are determined by three types of intracellular targets: cGMP-dependent kinases (PKG), cyclic nucleotide-gated channels, and cGMP-binding PDEs [8]. In some cell types, it modulates the concentration of cAMP by activating PDE2 or inhibiting PDE3 activity [9, 10].

cGMP plays an important role as a mediator of the action of NO. NO is highly reactive and unstable free radical, which regulates a variety of cellular functions by diffusion from its originating cell to surrounding cells [11]. The NO can be synthesized by three NO synthase (NOS) isoforms, namely, neuronal synthase (nNOS or NOS-I), inducible form (iNOS or NOS-II), and the endothelial form (eNOS or NOS-III). The constitutive isoforms, eNOS and nNOS, are anchored on the internal surface of the cell membranes, and their activities by the endothelial cells and neurons are responsible for maintenance of physiological homeostasis such as blood pressure and blood flow, platelet aggregation, leukocyte adhesion to the endothelium, and neuronal signaling. eNOS and nNOS produce NO under physiological conditions and are primarily regulated by intracellular Ca^{2+} /calmodulin levels. The inducible isoform iNOS is Ca^{2+} independent and represents a newly synthesized enzyme, which is expressed in response to specific stimuli, such as endotoxin and cytokines. iNOS is present in macrophages, hepatocytes, smooth muscle, endothelium, and glial cells and produces NO after immunological stimulation [i.e., $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, lipopolysaccharide (LPS)]. Whereas eNOS and nNOS produce NO for a short period of time (seconds or minutes), iNOS produces NO for long period of time (hours to days) and typically synthesizes 100–1000 times more than constitutive NOS [12, 13]. At high levels, NO produced by iNOS exerts cytotoxic and pro-inflammatory effects; however, the low nanomolar concentrations of NO produced by the eNOS isoform exhibit anti-inflammatory effects via the cGMP signaling and perhaps other mechanisms [14, 15]. The NO pathway can inhibit vascular nuclear factor-kappaB (NF- κ B), a key transcriptional mediator of inflammation, by increasing the expression of cytoplasmic and nuclear levels of its inhibitor, the I κ B- α [16], or by directly inhibiting the NF- κ B binding [17]. Moreover, eNOS regulates NF- κ B expression in a negative feedback mechanism, limiting local inflammation [18].

Studies developed on knockout mice for NOS isoforms indicate that NO derived from eNOS and nNOS is critical in the regulation of leukocyte-endothelial cell interactions in postcapillary venules [19, 20]. NO produced by the vascular endothelium exerts a cytoprotective and antithrombotic role by preventing the activation and adherence of leukocytes and platelets. The anti-inflammatory effects of NO are mediated predominantly via the activation of sGC and subsequent formation of cGMP. The production of cGMP causes specific downregulation of the expression of P-selectin on endothelial cells and platelets to prevent leukocyte rolling, adhesion, and migration [21].

The NF- κ B is the generic name of a family of transcription factors that functions as dimers and regulates gene expression of a plethora of inflammatory and immune mediators, including

cyclooxygenase-2 (COX-2) and iNOS, both considered important mediators in the recruitment of inflammatory cells [22–24]. The NF- κ B proteins are sequestered in the cytoplasm through physical interaction with I κ B family proteins. Proinflammatory cytokines (IL-1, TNF- α), B- and T-cell activators, pathogen-associated molecular patterns (PAMPs), and oxidative stress activate I κ B kinase (IKK), a cytoplasmic kinase complex, that phosphorylates the I κ B molecules, leading to their subsequent degradation through the ubiquitin–proteasome pathway. NF- κ B dimers then translocate to the nucleus where they can bind to κ B consensus sequences and activates the transcription of various genes [4]

Cyclic AMP/PKA modulates the NF- κ B function through several events; some of them include CREB-mediated transcription of the I κ B gene, thus elevating the expression of resynthesized I κ B, inhibiting I κ B degradation via blocking of IKK activity, and enhancing I κ B levels by interfering with I κ ubiquitination and/or subsequent proteasomal degradation [25–28].

Intracellular levels of cGMP also exert a role in modulating inflammatory response. Initially, some studies demonstrated that inhibition of endogenous NO production markedly increased monocyte chemoattractant protein-1 (MCP-1) mRNA levels in endothelial cells, whereas exogenous addition of NO dose dependently decreased MCP-1 mRNA expression and secretion [29]. This NO modulating effect of MCP-1 expression occurs via suppression of NF- κ B by reducing the degradation of I κ B [30, 31]. In sequence, a detailed study described the NO/cGMP role in regulating the inflammatory response. According to Aiwaza and cols [9], NO and C-type natriuretic peptide (CNP) inhibit NF- κ B activity via cGMP-dependent activation of PKA, but not of PKG. In summary, the cGMP elevated levels by NO donor or natriuretic peptide inhibited PDE3 activity, which lead to the increase of cAMP and activation of PKA. PKA inhibited NF- κ B transcription activity and, subsequently, the downstream MCP-1 and vascular cell adhesion molecule-1 (VCAM-1) gene expressions.

Moreover, there are other mechanisms by which NO/cGMP regulates the NF- κ B activation and MCP-1 expression, such as activation of mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) and, ultimately, inhibition of p38 MAPK, suggesting a counter-regulatory action of p38 MAPK and NF- κ B [32].

In addition to anti-inflammatory effects, NO can have both pro- and anti-tumorigenic activities depending on NOS uncoupling that can occur under some conditions, such as low [Arg] or elevated levels of endogenous NOS inhibitors. Uncoupled NOS produces oxidants like peroxynitrite and O₂, which initiates different downstream signaling that for tumor cells are pro-proliferative and antiapoptotic, e.g., NF- κ B. However, when the primary product of NOS is NO, downstream signaling is dominated by anti-tumorigenic NO-dependent pathways (sGC/cGMP/PKG) [33]. The NO/cGMP/PKG pathway appears to play an essential role in promoting apoptosis, thus inhibiting tumor growth. Activating cGMP/PKG pathway by PDE5 inhibitors selectively inhibits colon tumor growth, as well as the knockdown of PDE5 in colon cancer cell (HT-29) by siRNA efficiently promotes apoptosis and delayed proliferation [34, 35]. Recently, it was also demonstrated that increased intracellular levels of cGMP induced by the inhibition of PDE5 significantly inhibit colonic tumorigenesis dependent on inflammation [36].

However, NO/cGMP/PKG actions appear to be highly cell type and context dependent. In some neural cells, the NO/cGMP/PKG pathway has an essential role as an antiapoptotic/prosurvival factor [37]. This neuroprotective mechanism may be especially important during brain ischemia, inflammation, or trauma. In retinal neuroglial progenitor cells, NO/cGMP/PKG antiapoptotic cascade is activated through Akt-induced CREB1 activation [38, 39]. CREB is a transcription factor involved with neurotransmitters, growth factors, and other signaling molecules with essential functions for memory and neuronal survival [40, 41]. In cerebellar granule neurons, there is evidence that NO plays an active role in sustaining the neuronal survival through NO/cGMP/PKG [42].

cGMP/PKG1 is also considered as a key effector in cardioprotection induced by PDE5 inhibitors against ischemic injury in the infarcted heart and cardiomyocytes. The potential mechanisms include its antiapoptotic effect as is evident by increased phosphorylation of Akt (pAkt) and glycogen synthase kinase 3 β (pGSK3 β), Bcl-2 expression, and prevention of caspase-3/caspase-7 activation [43–45]. Other studies provided the evidence that PDE5 inhibition prolonged survival of transplanted of bone marrow-derived mesenchymal stem cells in ischemic heart via cGMP/PKG signaling, contributing to regeneration of the ischemic heart [46].

3. The role of glial cells on neuroinflammation: the modulation by NO/cGMP

Several lines of evidence strongly suggest that neuroinflammation is a crucial process involved in the progression of neuronal degeneration, a common feature observed in several neurodegenerative disorders. Therefore, the involvement of the local innate immune response can be a very complex process, contributing to perpetuate the damage to the CNS [47].

In the inflammatory process, there is an increase in blood flow and vascular permeability, venular dilatation, and recruitment of cells to the inflammatory site. Reactive oxygen species (ROS) play an important role in the inflammatory process, including endothelial cell damage and increased microvascular permeability, chemotactic factor production, neutrophil recruitment, oxidation, and lipid peroxidation [48]. These inflammatory mediators play a regulatory role in the growth, differentiation, and activation of immune cells [49]. Glial cells (microglia, astrocytes, and oligodendrocytes) define brain homeostasis and are responsible for defense against neural tissue injury [50, 51].

3.1. Astrocytes

Astrocytes constitute a very heterogeneous population of cells, which regulate pH, extracellular levels of ions, neurotransmitters, and energy metabolism. They are involved in the formation and functioning of BBB [52] and also actively participate in neurotransmission [53]. In situations of CNS damage, the typical response is some degree of reactive gliosis [54], an astrocytic response involving positive gene regulation of cytoskeletal proteins (e.g., glial fibrillary acid protein, GFAP), hypertrophy, hyperplasia, and rearrangement of astrocytes, which may form glial scars [50].

In addition, astrocytes play an important role in central immunity. The innate immune response is precisely adjusted by identifying the type of threat that is present. Molecular structures associated with the threat are recognized by pattern recognition receptors (PRRs). PRRs recognize PAMPs, expressed by bacteria, fungi, and viruses, or damage-associated molecular patterns (DAMPs), expressed by cells and tissues under stress or injury. One of the major classes of PRRs in mammals is TLRs. The response is rapid both to the presence of pathogens and to other types of damage to the tissue, activating the immune system, which releases cytokines and chemokines, and modulating the BBB [50].

Astrocytes express TLRs [55]. The brain and spinal cord of multiple sclerosis patients showed increased TLR3 and TLR4 in astrocytes in regions of inflammation [56]. Most TLRs, after detecting their respective ligands, initiate a signal that is mediated by the myeloid differentiation gene 88 (Myd88) and result in the activation of nuclear transcription factor NF- κ B. Translocation of NF- κ B to the nucleus culminates in the secretion of proinflammatory molecules (IL-1 β , IL-6, TNF- α , and IL-12). Activated astrocytes may also produce chemokines that recruit microglial cells, lymphocytes, and dendritic cells to the site of injury [57].

In the astrocytic response, in addition to increased TLR4 levels, leading to the expression of a variety of chemokines and cytokines [55, 58], other important processes occur, such as alteration of intracellular calcium signaling. Under conditions that lead to neuroinflammation in the CNS, as in exposure to LPS, Ca²⁺ signaling in the astrocyte network is over activated, triggering astrocyte activation. The inhibition of the communicating junctions (gap junctions), with changes in intercellular Ca²⁺ waves and Na⁺/K⁺-ATPase activity, results in disorganization of the actin filaments (stress fibers) [58, 59], and these effects are hallmarks of astrocyte reaction on neuroinflammation.

Evidence indicates that the cGMP/PKG pathway is involved in the regulation of astrocytic activity [60]. The NO, through the cGMP/PKG, decreased intracellular Ca²⁺ in astrocytes, reducing intercellular Ca²⁺ waves [61]. In addition, cGMP inhibited IFN- γ -induced MHC-II expression, as well as the expression of LPS-induced matrix metalloproteinase-9 (MMP-9) and TNF- α in cultured astrocytes [13, 60, 62]. According to these studies, MMP-9 expression is dependent on extracellular signal-regulated kinase (ERK) activation via NF- κ B. This data supports the hypothesis that the NO/cGMP/PKG pathway plays a role in astrocytic cells that contributes to the resolution of neuroinflammation.

3.2. Microglia

Microglia constitute the cells that are part of the innate immune system and are therefore considered as the pathological sensors of the CNS damage. The phenotypic changes of the microglia after activation are functionally identical to those observed in macrophages [63, 64]. The physiological functions of microglia are important for the maintenance of homeostasis. In addition, they have been shown to be responsible for the secretion of neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF) [65] and for removing aggregates of proteins [66]. However, when exposed to infections, lesions, or dysfunction of the nervous system, microglial cells become activated. In the absence of pathology, the microglia “at rest”

are small cells with long and thin processes (“branched phenotype”). When activated, the microglia loses the long extensions typical of the inactive microglia and exhibits ameboid extensions (“ameboid phenotype”) [67]. Protein Iba-1, expressed on the microglia surface, is used as a marker of its activated state [68]. This physiological transformation is associated with changes in the expression of surface receptors and the release of cytokines, which may contribute to the damage of synaptic plasticity and the neurodegenerative disease aggravation [69].

Activated microglial cells become a source of TNF- α , IL-1 β , IL-1 α , superoxide, NO, chemokines, and glutamate, which may promote neuronal death. TNF- α , secreted both by microglia and astrocytes, can directly promote neuronal death by binding to its corresponding receptors (TNFRs). Evidence indicates that TNF- α induces apoptosis of mature oligodendrocytes in inflammatory demyelinating diseases such as multiple sclerosis [61, 70] and plays a key role in neurodegeneration process observed in Parkinson’s and Alzheimer’s diseases [57].

An *in vitro* study using N9 microglial cells demonstrated that the treatment with the PDE5 inhibitor, sildenafil, suppressed NO, IL-1 β , and TNF- α production induced by LPS, due to suppression of the MAPKs/NF- κ B pathways through the inhibition of NADPH oxidase, mediated ROS generation [71]. These results indicate that cGMP accumulation as a result of PDE5 inhibition might participate in the inhibition of microglial activation.

3.3. Oligodendrocytes

Oligodendrocytes are myelinating CNS cells that arise from oligodendrocyte progenitor cells (OPCs). OPCs differentiate in mature or myelinating oligodendrocyte, fixing extensions in axons to generate the concentric membrane layers to produce myelin. The presence of oligodendrocytes is more common in the white matter of neuronal tissue, such as the corpus callosum and cerebellum, and less frequent in gray [72]. In both compartments, myelin is necessary for the saltatory conduction of action potentials along axons [73].

Oligodendrocyte dysfunction and myelin abnormalities are found in a wide variety of neurological diseases and may be involved in the pathophysiology of various diseases, including genetic leukodystrophies [74], schizophrenia and bipolar disorder [75, 76], brain injury [77], and endocrine and metabolic abnormalities [78, 79] and neurodegenerative conditions such as strokes [80, 81], Parkinson’s disease [82], Alzheimer’s disease [83–85], multiple sclerosis [86], and diabetic encephalopathy [87].

In an attempt to repair myelin damage, increased differentiation of OPCs into mature oligodendrocytes promotes remyelination [88]. In later stages of injury, however, OPCs also enter into apoptosis. Recent studies have shown that treatment with sildenafil increases the levels of the protein expressed by oligodendrocytes, myelin basic protein (MBP), and also restores myelin sheath morphology, indicating remyelination. In addition, sildenafil induces the differentiation of OPCs into mature oligodendrocytes, as demonstrated by the increase of glutathione S-transferase pi (GST-pi, a marker of mature oligodendrocytes), indicating that cGMP signaling can modulate OPC survival and myelin production [89].

Oligodendrocytes are not inert immune cells, but secrete a wide variety of inflammatory mediators, such as the proinflammatory cytokines IL-1- β and IL-6 and CCL-2 and IL-8 chemokines involved in the recruitment of immune cells during inflammation [90]. In experimental multiple sclerosis models, oligodendrocytes in apoptosis also express increased levels of COX-2 at the demyelination beginning, which seem to make these cells more susceptible to death by glutamate-mediated excitotoxicity [91].

4. Therapeutic applications of phosphodiesterase 5 inhibitors in central neurological diseases

Faced with rising costs for the development of new drugs, researchers are looking for ways to repurpose older ones. Taking medications that have been developed for one disorder—and even some that fail in initial trials—and “repositioning” them to tackle another are a growing strategy for researchers in industry and academia [92].

The administration of selective PDE5-Is increases the levels of cGMP [93, 94], with effects on multiple organs and systems. The PDE5-I, sildenafil, is a medication for angina pectoris developed in 1989 [92]. For many years, sildenafil (Viagra[®], Pfizer) has been the most representative molecule of the class of drugs to treat erectile dysfunction (ED) [95]. Under the trade name Revatio[®] (Pfizer), it was also approved for pulmonary artery hypertension therapy in June 2005 [96] and, more recently, for the Raynaud’s phenomenon [97]. Therefore, sildenafil is a classic success story of repositioning.

PDE5 is present throughout the body and brain [95, 98] and has emerged as a potential therapeutic target for diseases related to neuroinflammatory and neurodegenerative processes because of its reported relation with them (for review, see [99]). To date, only four PDE5-Is have been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency: sildenafil, vardenafil, tadalafil, and avanafil. Sildenafil is reported to clearly cross the BBB [100], whereas evidence for vardenafil is indirect [101] and, while it was first considered that tadalafil does not cross it [102], later was demonstrated that this drug is able to cross the barrier [103]. Several studies indicate that sildenafil and other PDE5-Is may offer novel strategies for the treatment of neurological pathologies [12, 102, 104]. The beneficial effects of PDE5-Is were initially attributed to its mechanism in smooth muscle (regulating blood flow) and improving synaptic plasticity and neurogenesis. However, recent studies point to an important effect of these drugs on neuroinflammation, which may be, at least in part, responsible for their protective effects on central neurological diseases.

Thus, five major mechanisms of PDE5-Is have been described in neurological disease models: (1) by modulating the CREB pathway, inducing the formation of new synaptic connections and neurogenesis, improving cognition and memory; (2) through the modulation of Akt/GSK3 β and calpain/p25/CDK5 pathways, decreasing aggregate formation of proteins; (3) through apoptosis inhibition; (4) by inducing angiogenesis and improving blood flow; and (5) through the modulation of neuroinflammation. Targeting multiple elements in the network underlying complex diseases, such as neurological diseases, may produce benefits

beyond those of representative monotherapies [105, 106]. Repositioning PDE5-Is as therapeutic approaches that can be used in combination with other drugs can therefore be useful. This section aims to name and classify representative preclinical and clinical studies of PDE5-Is in central neurological diseases (Alzheimer's disease, multiple sclerosis, Parkinson's disease, Huntington's disease (HD), and stroke) and to describe the main known mechanisms, with emphasis on neuroinflammation (following a search of the Medline®/PubMed® database, during the period between 2000 and 2016).

4.1. Alzheimer's disease

Alzheimer's disease (AD) has become the fourth leading lethal disease among the elderly after cancer, heart disease, and stroke. It is an age-related neurodegenerative disease characterized by the presence of senile plaques (consisting of β -amyloid filaments, A β), neurofibrillary tangles (composed of hyperphosphorylated tau deposits), and neuronal degeneration accompanied by significant loss of synapses [107, 108] (**Figure 1A**). While early studies focused on assessing the beneficial effects of PDE5-Is on AD through the formation of synapses, neurogenesis, and protein aggregation pathways, more recent studies have shown that the role of these drugs in neuroinflammation may be an important mechanism in AD.

4.1.1. PDE5-Is' beneficial effects in AD through CREB/BDNF/Arc pathway, Akt/GSK3b pathway, and calpain/p25/CDK5 pathway modulation

cGMP/PKG pathway contributes to phosphorylation of the transcription factor CREB; Prickaerts et al. [109] suggested that the cGMP/PKG/CREB pathway induces the synthesis of proteins essential for memory consolidation, probably through the formation of new synaptic connections [110]. Therefore, the chronic administration of PDE5-Is may lead to gene transcription through CREB activation, by raising cGMP levels (**Figure 2A**).

Puzzo et al., in 2009 [102], and Cuadrado-Tejedor et al., in 2011 [111], showed that sildenafil has beneficial effects on AD models, modulating the CREB pathway. Puzzo et al. [102] demonstrated that sildenafil (3 mg/kg, i.p., for 3 weeks) may be beneficial against cognitive loss in the APP/PS1 mouse model of amyloid deposition, producing an immediate and lasting improvement of synaptic function, CREB phosphorylation, and memory. This effect was associated with a reduction in A β levels. Cuadrado-Tejedor et al. [111] showed that sildenafil (15 mg/kg, i.p., for 5 days) restored cognitive deficits in aged rat model of AD (Tg2576-AD transgenic mice); however, whereas pCREB was not significantly induced in mice treated with sildenafil, the BDNF and Arc (CREB downstream target molecules) increased, confirming that the drug acts through this pathway (CREB/BDNF/Arc), inducing synaptic formation and improving memory.

Cuadrado-Tejedor and coworkers [111] showed, however, that sildenafil did not affect A β -burden while decreased tau phosphorylation. The formation and aggregation of A β and tau involve some pathways, which can be plausible therapeutic target for the treatment of AD. GSK3 β , which is inhibited by Akt, and cyclin-dependent kinase 5 (CDK5), which is activated by p25, are the most relevant kinases involved in the pathogenic mechanisms of AD

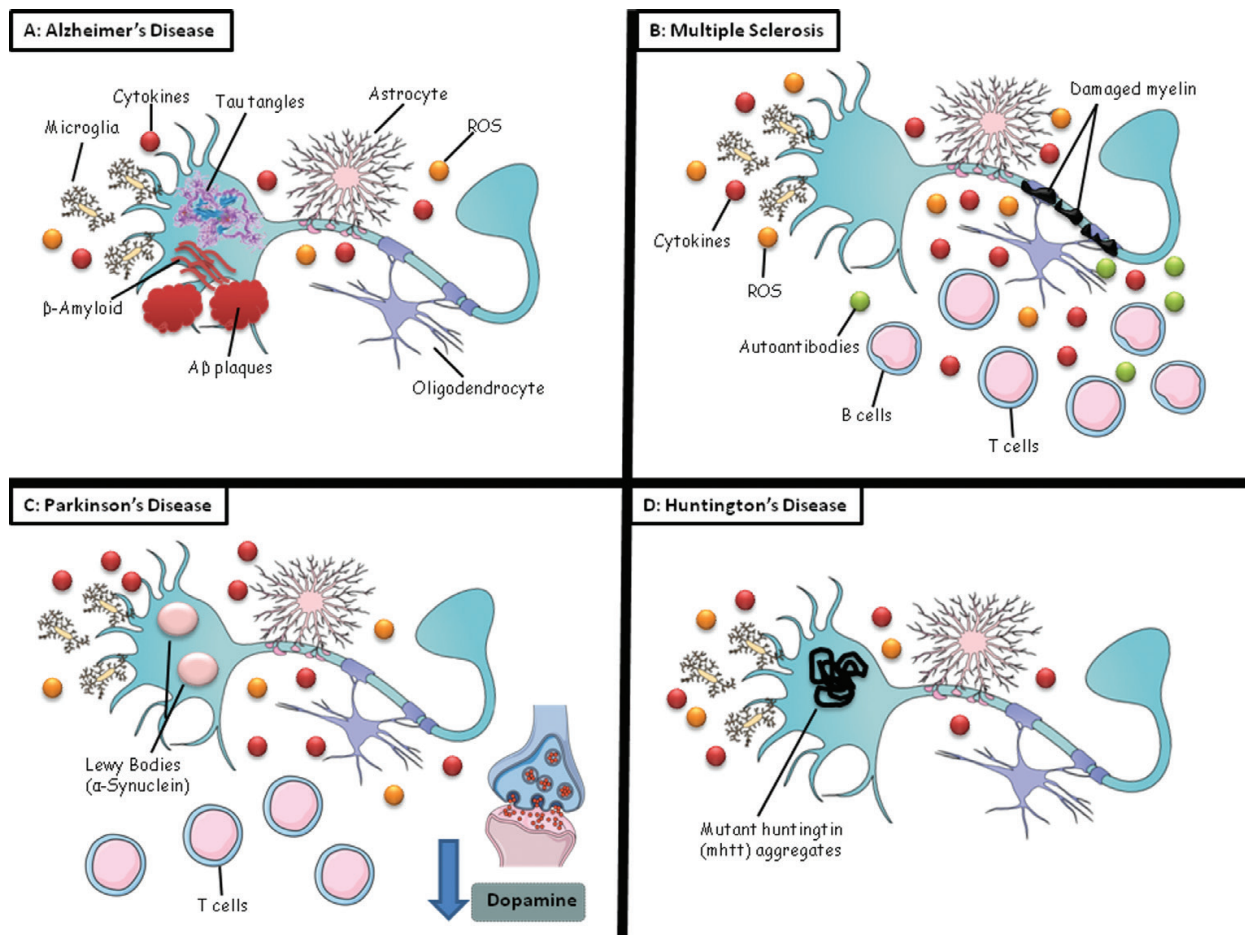


Figure 1. Hallmark pathologies of (A) Alzheimer's disease (AD), (B) multiple sclerosis (MS), (C) Parkinson's disease (PD), and (D) Huntington's disease (HD). (A) In AD, neurons contain intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein and extracellular plaques of amyloid β ($A\beta$). The inflammatory reaction, with activation of microglia and astrocytes and the subsequent release of inflammatory cytokines and reactive oxygen species (ROS), plays a significant role in the pathological processing of AD. (B) MS is a chronic autoimmune/inflammatory disorder characterized by demyelination of axons, with associated acute and chronic inflammatory events involving the recruitment/activation of microglia/macrophages, astrocytes and B and T cells and release of proinflammatory cytokines, ROS, and autoantibodies. (C) In PD, neurons contain α -synuclein aggregates, forming Lewy bodies. Neuronal loss leads to lower production of dopamine. There is a persistent inflammatory response, T-cell infiltration, and glial cell activation in patients with PD and animal models, which play a crucial role in the degeneration of dopaminergic neurons. (D) In HD, mutant huntingtin protein (mhtt) containing an extended polyglutamine repeat, caused by at least 36 CAG repeats in the huntingtin gene, leads to intraneuronal aggregates. In all four diseases, the pathological events ultimately result in neuronal death. Over time, this either causes or contributes to neuroinflammation.

by phosphorylation at multiple sites of the microtubule-binding protein, tau [112, 113]. The activities of GSK3 β and CDK5 were reduced by sildenafil, whereas the drug increased Akt and decreased p25. The decrease in kinase activity of GSK3 β and CDK5 due to sildenafil may lead to a reduction in tau phosphorylation, possibly contributing to the reestablishment of cognitive function (**Figure 2B**). Then, according to Cuadrado-Tejedor et al. [111], sildenafil reversed the marked memory deficits of elderly Tg2576 animals by regulating the Akt/GSK3 β /pTau and p25/CDK5/pTau pathways, not resulting from any decrease in the $A\beta$ -load. However, the contrasts between Cuadrado-Tejedor et al. [111] and Puzzo et al. [102] may be

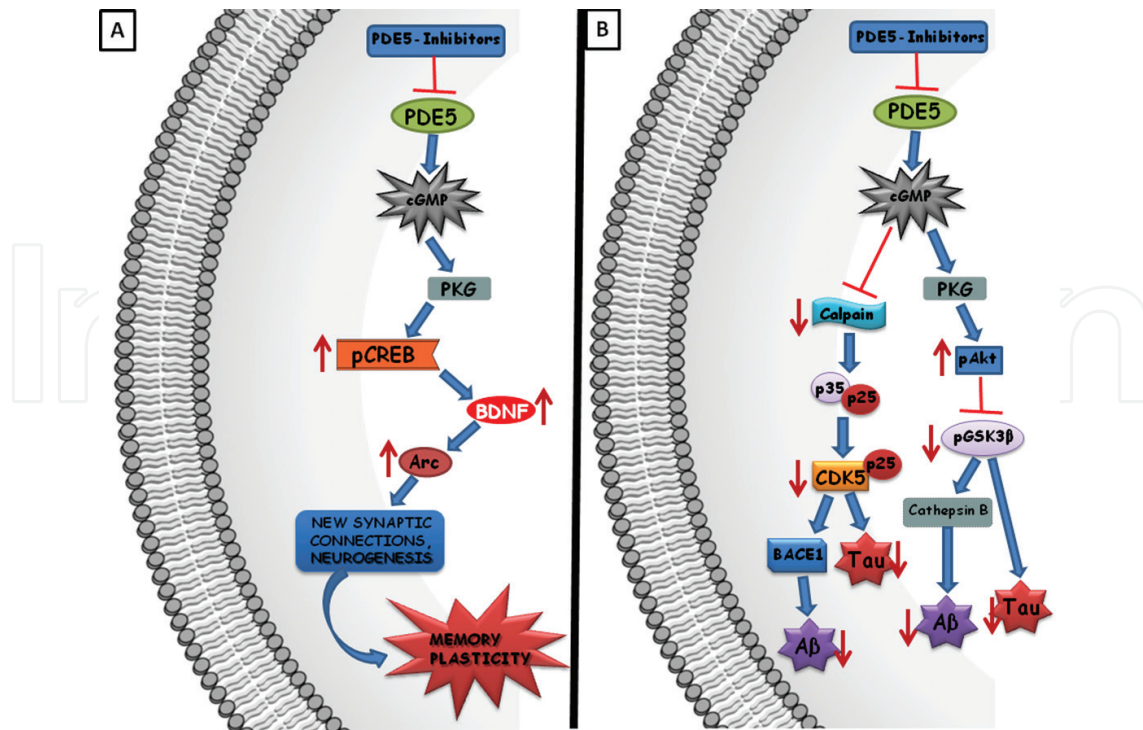


Figure 2. PDE5-I mechanisms in the CREB pathway and protein aggregation. (A) The PDE5 inhibitors (PDE5-Is) modulate the CREB pathway, increasing the expression of CREB and the downstream targets, BDNF, and Arc. The result is the induction of new synaptic connections and neurogenesis, leading to the restoration of pathological cognitive signs of neurological diseases, such as Alzheimer’s disease and Huntington’s disease. (B) PDE5-Is also modulate pathways involved in the protein aggregation. Calpain is an enzyme that cleavage p35 in its more stable isoform, p25. The formation of the p25/CDK5 complex is associated with tau hyperphosphorylation. In addition, p25/CDK5, via the downstream target BACE1, also leads to cleavage of amyloid precursor protein (APP), contributing to the formation of A β plaques. PDE5-Is induce a decrease in the activity of calpain, p25, and CDK5, with consequent decrease in protein aggregation. GSK3 β is a kinase involved in the hyperphosphorylation of Tau. In addition, through the downstream target, cathepsin B, GSK3 β leads to the formation of A β plaques. pAkt is a GSK3 β inhibitor, modulating the pathway. PDE-Is increase Akt phosphorylation and decrease GSK3 β activity and cathepsin B expression, which can contribute to control the protein aggregation. —| Inhibition —| activation; \uparrow increased expression/activity; \downarrow decreased expression/activity.

due to differences between dose, duration of treatment, and animal models. In addition, both Akt/GSK3 β and p25/CDK5 signaling are also involved in the regulation of A β [114], and it is possible that if the treatment was longer, this effect would be detected.

Following the same line of investigation, Orejana et al. [114] treated senescence-accelerated mouse-prone 8 (SAMP8, used as a model of aging, which displays many established pathological features of AD) with sildenafil (7.5 mg/kg, i.p., for 4 weeks) and showed that the mechanism of protection is through A β decrease, by pAkt/GSK3 β /cathepsin B pathway and calpain/p25/CDK5/BACE1 pathway inhibition. pAkt inhibits GSK3 β , which is an important activator of cathepsin B [115]. Calpain is an enzyme that cleaves p35 in p25, and p25/CDK5 regulates BACE1 (protein cleaving enzyme 1) expression levels. BACE1 and cathepsin B (both β -secretases) cleave amyloid precursor protein (APP), contributing to the formation of A β . Sildenafil decreased the activity of calpain, p25, and CDK5 and markedly increased Akt phosphorylation and decreased GSK3 β activity. Consequently, sildenafil decreased the expression

of BACE1 and cathepsin B, leading to a reduction in APP and A β levels (**Figure 2B**). These findings demonstrate that sildenafil modulates calpain/p25/CDK5/BACE1 and pAkt/GSK3 β /cathepsin B pathways, and these mechanisms are probably responsible for beneficial effects of this class of drugs in AD models.

4.1.2. PDE5-Is beneficial effects in AD through the control of neuroinflammation

Although the first PDE5-I studies in AD models have been focused on synapse formation, neurogenesis, and memory improvement, investigating primarily CREB, tau phosphorylation, and A β formation pathways, more recent studies also point to an important anti-inflammatory mechanism of this class of drugs in AD. The work by Orejana et al. [114] was perhaps one of the first studies to suggest and demonstrate that sildenafil modulates inflammatory cells in AD model. They showed that sildenafil decreased the GFAP, a marker of astrogliosis. However, Orejana et al. [114] could not differentiate whether the reduction in GFAP levels resulted from less accumulation of A β or if it was a direct modulation of inflammatory events by sildenafil. A recent study using sildenafil in cultured astrocytes confirmed that sildenafil has a direct mechanism on neuroinflammation [116].

Until 2013, it was unknown whether PDE5-Is reversed A β -induced neuroinflammation in APP/PS1 transgenic mice. Zhang et al. [117] showed that APP/PS1 mice presented impaired cognitive ability, neuroinflammatory response in the hippocampus, and downregulated cGMP; sildenafil reversed memory deficits and cGMP/PKG/pCREB signaling dysfunction and reduced A β levels in this model. In addition, sildenafil decreased the proinflammatory cytokines IL-1 β , IL-6, and TNF- α . The inhibition of hippocampal PKG immediately prior to the injection of sildenafil significantly blocked these effects, further indicating the participation of PKG in the anti-inflammatory effects produced by sildenafil (**Figure 3A, B**).

An ongoing neuroinflammatory process has been considered a marker of AD [117]. The deposition of A β peptides and the activation of glial cells surrounding senile plaques in brain areas involved in cognitive functions are assumed to participate in the onset of a pathological cascade resulting in synaptic dysfunction, synaptic loss, and neuronal death [118, 119]. The inflammatory reaction, with activation of microglia and astroglia, and the subsequent release of inflammatory cytokines (IL-1 β , TNF- α , and COX-2 and so on) play a significant role in the pathological processing of AD [108] (**Figure 1A**). Proinflammatory cytokines, such as TNF- α and IL-1 β , may contribute to brain dysfunction and neurodegeneration, impair synaptic plasticity, and induce memory impairment, while the anti-inflammatory cytokine IL-4 has the opposite effect [120, 121]. NF- κ B is well known as a key regulator that induces the expression of many proinflammatory cytokines and inducible effector enzymes linked to the inflammatory process. The degradation of I κ B- α (NF- κ B inhibitory protein) and NF- κ B phosphorylation were enhanced after the A β injection [108].

Additionally to classical PDE5-Is, other drugs have been demonstrated to act on AD by inhibiting PDE5 and modulating neuroinflammation. It has recently been showed by Li et al. [108] that sodium hydrosulfide (NaHS), a hydrogen sulfide donor, decreased PDE5 levels, attenuated neuronal death, and suppressed apoptosis by inhibiting the activation of pro-caspase-3 in

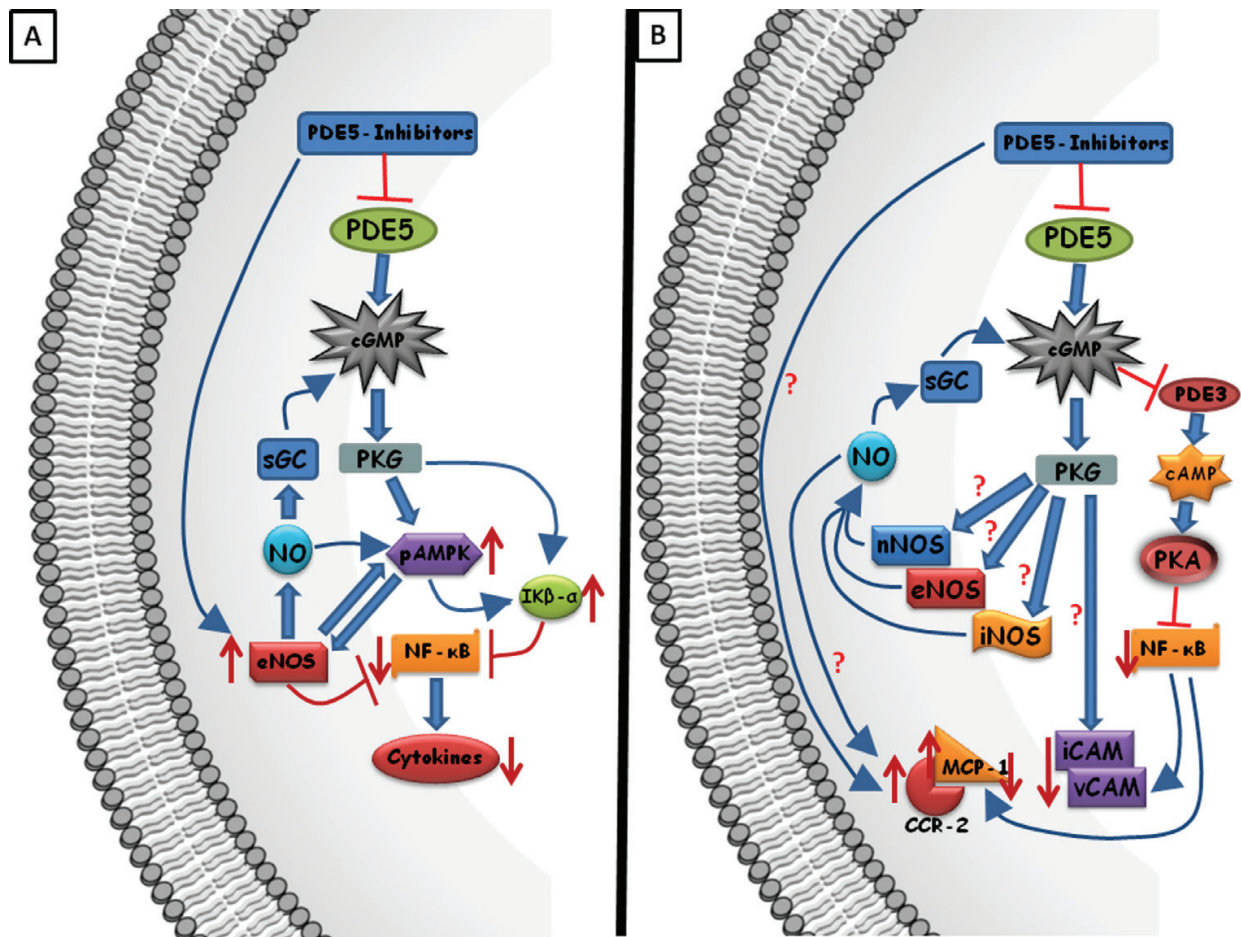


Figure 3. PDE5 inhibitors modulate neuroinflammation. (A) AMPK exerts its anti-inflammatory activity through multiple signaling pathways. The phospho-AMPK (pAMPK) suppresses NF- κ B, by increasing its inhibitory protein, IK β - α . Consequently, the production of cytokines is decreased. In addition, AMPK phosphorylates and activates eNOS, inducing NO production; eNOS increases AMPK, in a positive feedback, and NO can act as an endogenous activator of AMPK, suggesting a reciprocal relationship between AMPK and eNOS. eNOS also inhibits NF- κ B, decreasing the inflammatory response. PDE5-Is increase pAMPK and eNOS expression, increase IK β - α , and decrease NF- κ B. NO activates sGC, inducing cGMP production, which can amplify the effect of PDE5-Is. (B) Some possible mechanisms of PDE5-Is in the control of neuroinflammation have been demonstrated while not fully understood. PDE5-Is increase levels of the chemokine MCP-1 and its receptor, CCR-2, which are typically overregulated in multiple sclerosis models. It is possible that this effect is indirect, through NO, since this gas was shown to increase MCP-1/CCR-2, but it has not been confirmed so far. In addition, PDE5-Is were shown to increase the three isoforms of nitric oxide synthase (nNOS, iNOS, and eNOS), along with increased levels of NO. However, the mechanism of NOS participation on the effects of PDE5-Is is unclear. ICAM-1 and VCAM-1 were decreased by PDE5-Is, which may be important for the control of leukocyte infiltration. It is possible that complex cross signaling is occurring, but although it has been demonstrated in inflammatory models, it has not been confirmed in preclinical studies with PDE5-Is in neurological diseases. For example, elevated levels of cGMP by NO may inhibit PDE3 activity, which leads to increased cAMP levels and PKA activation. PKA inhibits NF- κ B transcription activity and, subsequently, the expression of downstream MCP-1 and VCAM-1. —| Inhibition; —> activation; ↑ increased expression/activity; ↓ decreased expression/activity.

the hippocampus of Sprague-Dawley rats (injected with aggregated A β 25-35). NaHS upregulated the expression of peroxisome proliferator-activated receptors (PPAR- α and PPAR- γ), which antagonize the effects of NF- κ B [122]. Moreover, the A β 25-35-injected rats exhibited a decrease in IK β - α degradation and an increase in NF- κ B p65 phosphorylation levels, whereas

these effects were attenuated by NaHS. NaHS can therefore act as an anti-inflammatory mediator by inhibition of PDE5.

A novel PDE5 inhibitor, Yonkenafil (yonk) (2, 6, or 18 mg/kg i.p.), given daily for 3 months, has been shown to have beneficial effects in APP/PS1 mice through anti-inflammatory mechanisms. Yonk reduced the area of A β plaques, increased neurogenesis, and inhibited over-activation of microglia and astrocytes [119]. A recent study by Yin et al. [123] has shown that Icariside II (ICSII), another new PDE5 inhibitor, derived from the Chinese herb *Epimedium brevicornum*, has protective effects on the AD model induced by intracerebroventricular streptozotocin (ICV-STZ) in Sprague-Dawley rats. ICSII (10 mg/kg for 21 days) improved cognitive deficits, attenuated neuronal death, and decreased A β levels by suppressing BACE1 and APP expression in the rat hippocampus. In addition, ICSII decreased IL-1 β , TNF- α , COX-2, and transforming growth factor- β (TGF- β) levels while increasing I κ B- α and decreasing NF- κ B activation.

It was demonstrated by Jin et al. [124] that Icariin (ICA), a flavonoid extracted from Chinese herb (*Berberidaceae epimedium* L.), an effective oral agent, is also a PDE5-I. Chronic treatment with ICA (30 and 60 mg/kg, twice a day for 4 months) improved the learning and memory of APP/PS1 transgenic mice, and the levels of APP, A β , and PDE5 decreased in the hippocampus and cortex after ICA treatment. Furthermore, ICA-treated mice showed increased expression of three NOS isoforms (nNOS, iNOS, and eNOS), along with increased levels of NO and cGMP. These results suggest that NO itself may be involved in the anti-inflammatory effect of PDE5-Is. NO is an important molecule in supporting neurite outgrowth and synapse remodeling [125, 126]. This study by Jin et al. [124] also showed that the three isoforms of NOS and NO levels decreased in the brain of APP/PS1 mice, reinforcing that NO deficiency may contribute to AD. Thus, ICA has a neuroprotective mechanism, probably due to stimulation of the NO/cGMP signaling pathway through the inhibition of PDE5 activity and coordinated induction of NOS isoform expression. Corroborating this result, Rapôso et al. [89, 127] showed that the absence of iNOS abolished the anti-inflammatory effects of sildenafil in mice brains. Treatment with sildenafil for 4 weeks decreased GFAP, COX-2, and the expression of various pro-inflammatory cytokines in wild-type C57BL/6 mice, although it did not have anti-inflammatory effects in iNOS^{-/-} mice. Also, Nunes et al. [128] reported that eNOS is upregulated following chronic administration of sildenafil. These studies point to the relevance of the physiologic expression of NOS for the anti-inflammatory mechanism of PDE5-Is (**Figure 3B**).

Despite the rich (though recent) literature on the effects of the PDE5 inhibitors on animal models of AD, clinical studies are lacking. However, PCR analysis of postmortem tissue of patients suffering from AD found a considerable increase in PDE5 expression in the temporal cortex of the brain compared to healthy controls of the same age [129]. Also, it was observed that lower levels of cGMP in the cerebrospinal fluid of patients with AD were associated with cognitive decline and amyloid pathology [129]. In addition, a clinical study demonstrated that chronic administration of udenafil (Zydena; available in Korea, Russia, and the Philippines) to 27 patients with ED (100 mg at 3-day intervals for 2 month) has shown to lead to an improvement in cognitive function [130]. This has led to suggest that sildenafil could improve cognitive function in AD patients.

Thus, the efficacy and safety of treatment with repeated doses of PDE5-Is have been demonstrated in several animal models of AD. Since the side effects of PDE5-Is are widely known and do not preclude its administration to a senile population, and considering the lack of effective treatments for AD, PDE5-Is have been proposed as potential alternatives as cognitive enhancers [99, 131].

4.2. Multiple sclerosis

Multiple Sclerosis (MS), the most common neurological disorder in young adults in the Western world, is a chronic autoimmune/inflammatory disorder characterized by demyelination of the nerve cells, which leads to severe psychomotor impairment [132]. CNS demyelination is frequently associated with acute and chronic inflammatory events involving the recruitment-activation of microglia/macrophages, astrocytes, and leukocytes, with the release of pro-inflammatory cytokines, ROS, and NO (**Figure 2B**) [133, 134]. Neuroinflammatory responses appear to begin before any significant loss of neuronal populations in the progression of MS [135].

It has been demonstrated that NO/cGMP signaling is involved in the regulation of neuroinflammation and myelination [89]. The intracellular accumulation of cGMP in different models of inflammation reduces the production of proinflammatory cytokines and oxidative stress, modulating the inflammatory response [136]. In addition, inhibition of PDEs seems to block the inflammatory response of microglia, reducing myelin sheath changes [137, 138]. Therefore, neuroinflammation mediated by glial cells (astrocytes and microglia) appears to be an important phenomenon that perpetuates neural damage in MS, and since cGMP-mediated pathways regulate inflammatory responses in immune and CNS cells, PDE5-Is are potential tools for treating MS.

In fact, it has been reported that patients suffering from ED, and in parallel MS, showed an improvement in clinical status for both pathologies after treatment with sildenafil [139]. The effect of sildenafil on improving the clinical status of patients with MS was initially attributed to the induction of neurogenesis [140]. However, studies have shown that sildenafil is a modulator of inflammation in the central and peripheral nervous systems and protects the myelin sheath both in pathological and healthy conditions [89, 116, 127, 128, 140–144]. This anti-inflammatory mechanism should better explain the protective effect of PDE5-Is in MS, considering the nature of the disease.

In 2011, Pifarré et al. [142] showed that sildenafil (10 mg/kg, s.c., for 18 days) reduced the clinical signs of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, developed in female C57BL/6 mice. Sildenafil prevented axonal loss and promoted remyelination. Furthermore, sildenafil decreased CD3⁺ leukocyte infiltration and microglial/macrophage activation in the spinal cord, while increasing T regulatory cells expressing fork head box transcription factor 3 (Foxp3 Tregs) and decreasing ICAM-1 in the infiltrated cells of the spinal cord. Autoreactive T cells infiltrating the CNS are the initiator and early effector cells in EAE development, but infiltrated macrophages, dendritic cells, and resident microglia constitute the ultimate effector cells that amplify neuroinflammation and tissue injury.

ICAM-1, a type-1 membrane-bound glycoprotein expressed in the majority of leukocyte subtypes, endothelial and CNS glial cells, is involved in leukocyte entry, lymphocyte activation, and other immune responses and plays a central role in the development of MS and EAE [145, 146]. The decrease of ICAM-1 induced by sildenafil was also reported by Rapôso et al. [89]. Pifarré et al. [142] also showed that the presence of astrocytes forming scar-like structures around infiltrates was enhanced by sildenafil, suggesting a possible mechanism for the restriction of the leukocyte dissemination in healthy parenchyma. However, this result does not corroborate other studies showing that PDE5-Is decrease GFAP expression and astrocyte activation [89, 114, 127, 140].

Continuing the investigation, Pifarré et al. [143] demonstrated that sildenafil treatment (10 mg/kg, s.c., for 18 days) preserved axons and myelin and increased the number of remyelinating axons in the EAE model; also, sildenafil protected immature and mature myelinating oligodendrocytes. However, if the protective effect of sildenafil on myelin and axons is secondary to its effect, controlling inflammation remains unknown. In addition, sildenafil upregulated YM-1, a marker of the macrophage/microglial M2 phenotype that has neuroprotective and regenerative properties, while Iba-1, a classical macrophage/microglial activation marker, was downregulated. In vitro analyses of spleen cells from sildenafil-treated animals showed downregulation of Th1/Th2/Th17 responses, while Tregs were upregulated and prevented accumulation of MOG-specific IgG2b in serum. These results suggest that sildenafil has a protective role, modulating central resident and peripheral immune cells.

A sequence of studies has characterized the effects and mechanisms of sildenafil in a cuprizone-induced demyelination and neuroinflammation in rodents, which has been widely used as a model of MS. Nunes et al. [140] and Rapôso et al. [127] demonstrated that sildenafil (25 mg/Kg administrated in the drinking water for 4 weeks) ameliorates cuprizone-induced demyelination in C57BL/6 mice. Sildenafil modulated the neuroinflammatory response (mediated by glial cells), reducing GFAP and Iba-1, IFN- γ , TNF- α , IL-1 β , IL-2, and COX-2 expressions. However, the anti-inflammatory effect of sildenafil was abolished in the cuprizone model induced in iNOS^{-/-} mice [127], showing that iNOS plays an important role in the mechanism of PDE5-Is. Sildenafil preserved the myelin and axons' ultrastructure and elevated GST-pi, indicating that sildenafil protects mature oligodendrocytes. However, it is not clear if sildenafil induces oligodendrogenesis or if it inhibits cell death/apoptosis or both. Myelin protection and oligodendrocyte proliferation have also been demonstrated in ischemic models [147, 148], and several studies showed that PDE5-Is inhibit apoptosis in central neurological disease models [122, 148, 149].

Contributing to the understanding of the mechanism by which sildenafil acts in the control of neuroinflammation in MS model, Nunes et al. [128] investigated the involvement of the AMPK/I κ B- α /NF- κ B signaling pathway and the eNOS. AMPK, the regulatory protein of the lipid and glucose metabolism, is upregulated in activated astrocytes during reactive gliosis [150], whereas AMPK activators downregulate inflammation in vitro and in vivo in various animal models [151–153], and the loss of AMPK exacerbates the effects of EAE model [154]. The anti-inflammatory activity of AMPK is exerted through multiple signaling pathways, including phosphorylation and activation of eNOS and production of NO. NO may act as

an endogenous activator of AMPK, suggesting a reciprocal relationship between AMPK and eNOS [155]. In addition, recent evidence suggests that the activation of AMPK can suppress NF- κ B, thus contributing to the regulation of inflammation [71] (**Figure 3A**). Nunes et al. [128] showed that sildenafil treatment (25 mg/Kg administrated in the drinking water for 4 weeks) improved the clinical status of the cuprizone-MS mice model. The treatment reduced unphosphorylated (inactive) AMPK and increased phospho-AMPK (pAMPK, active). Moreover, sildenafil decreased NF- κ B p65 expression and increased its inhibitory protein, IK β - α . However, if AMPK induces NF- κ B inhibition and which downstream targets may be involved in this inhibition require further investigation. The same study showed that sildenafil reduced the expression of GFAP, IL-1 β , and TNF- α and increased the expression of the anti-inflammatory cytokine IL-10. Besides, the level of eNOS was increased by sildenafil, suggesting reciprocity between AMPK and eNOS. This study then provides evidence that sildenafil has anti-inflammatory effects probably through modulation of AMPK/IK β - α /NF- κ B signaling (**Figure 3A**). However, the involvement of downstream proteins, such as AMPK-SIRT1-NF- κ B, and other pathways, such as MAPK-NF- κ B, should also be further investigated. In addition, Nunes et al. [128] showed that eNOS may play a role in the sildenafil mechanism. The possible role of NOS in the mechanism of sildenafil corroborates with other studies [89, 124, 127].

The ongoing investigation, in 2016, by Nunes et al. [141] demonstrated that sildenafil increased levels of the chemokine MCP-1 and its receptor, CCR-2, in the cuprizone-induced MS model. This may be part of the anti-inflammatory mechanism, since CCR-2 is a chemokine closely related to the pathology of MS and MS-animal models. In general, during the first weeks of cuprizone exposure, it undergoes a typical overregulation of the chemokine, and both microglia and astrocytes produce CCR-2 [156]. Also, an increase in CCR-2 may be associated with a reduction of macrophage infiltrates after stroke, showing the neuroprotective effects of this receptor [157]. Moreover, mediators in the microenvironment define at what time microglia/macrophages can assume an active and phagocytic phenotype [157]. The expression of MCP-1/CCR-2 by glial cells may promote this change in microglia phenotype in an attempt to repair the injured environment [158]. Sildenafil can, therefore, modulate inflammation by playing a role in the regulation of glial cell morphology and activation through MCP-1/CCR-2 signaling (**Figure 3B**).

Borán et al. [60] estimated that stimulation of cGMP/PKG pathway acts beneficially in microglia, inducing the phagocytic phenotype (M2) and decreasing expression of inflammatory genes, in detriment to the proinflammatory phenotype (M1). The cGMP/PKG pathway stimulated the regulation of microglial cell morphology, inducing a dramatic reorganization of the actin cytoskeleton compatible with a protective phenotype, which is more effective in the removal of dead cells. cGMP-mediated pathways have been implicated in the regulation of the actin cytoskeleton and cell morphology in different cell types, including macrophages and astrocytes [159, 160]. Borán and García [160] demonstrated that the stimulation of the PKG pathway by NO regulates cytoskeleton dynamics and motility in cultured rat astrocytes, and evidence indicates that cGMP is involved in the regulation of astrocyte cytoskeleton through Na⁺/K⁺-ATPase activity, IP3 receptor (IP3R), and ankyrin B. Ankyrin B, a protein associated with the cytoskeleton, interacts with Na⁺/K⁺-ATPase and IP3R, connecting the pump to the Ca²⁺ responses from internal cell stores and to the integrity of the cytoskeleton [160] (**Figure 4A**). This suggests

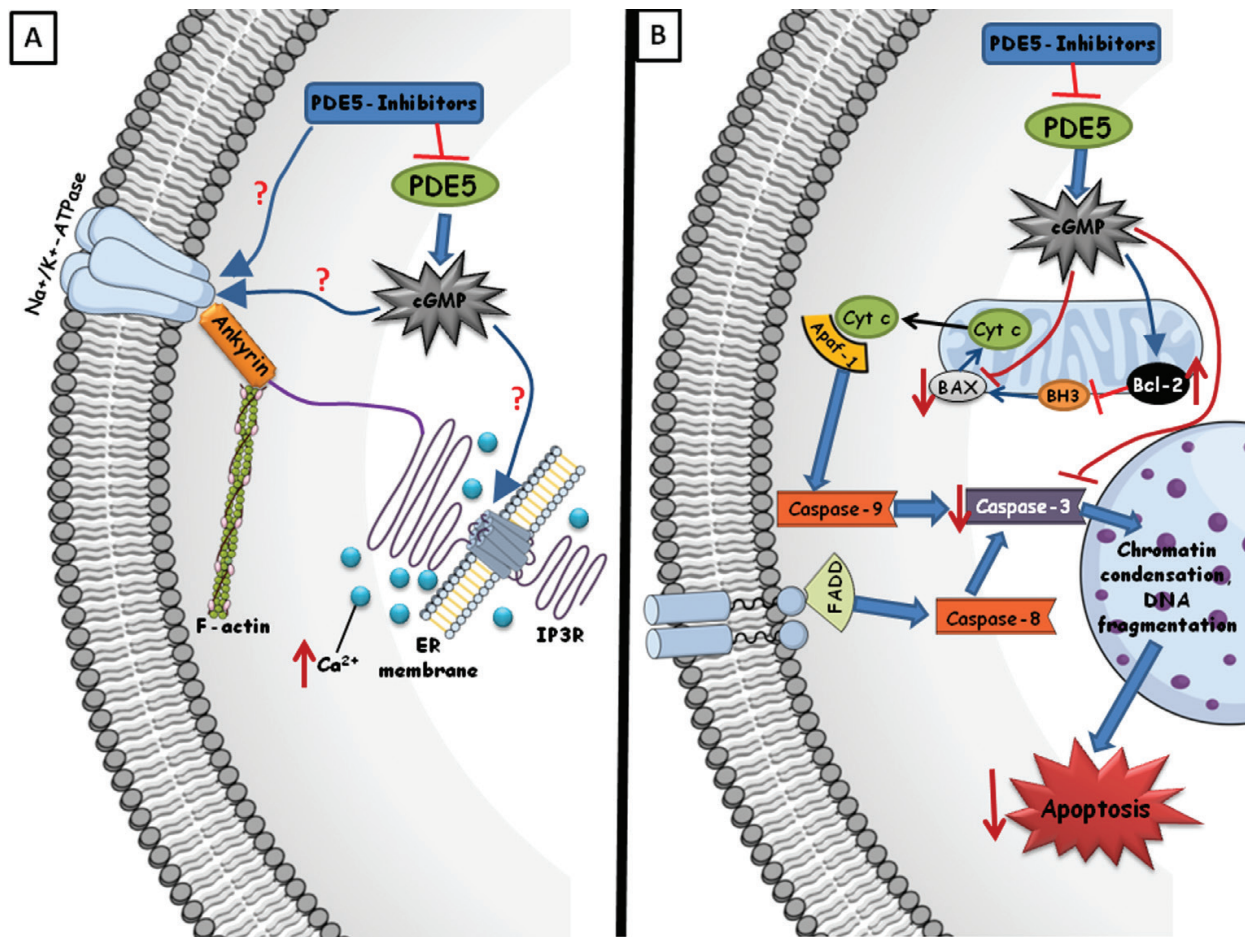


Figure 4. Mechanisms of PDE5-Is in the cytoskeleton and in the apoptosis pathways. (A) PDE5-Is induce Ca²⁺ response and the stabilization of F-actin in astrocytes; however, the mechanism behind this effect is not elucidated. Na⁺/K⁺-ATPase interacts with ankyrin B, a cytoskeleton-associated protein, and with the IP3 receptor (IP3R) [coupled to the endoplasmic reticulum (ER) membrane], connecting the pump to the Ca²⁺ responses from internal cell stores and to the integrity of the cytoskeleton. It is possible that this mechanism contributes to the dramatic reorganization of actin cytoskeleton observed in microglia, macrophages, and astrocytes after stimulation of the cGMP/PKG pathway, leading to a more protective phenotype of these inflammatory cells. (B) PDE5-Is have an antiapoptotic effect by enhancing the expression of the antiapoptotic Bcl-2 protein and reducing the proapoptotic BAX and caspase-3 proteins. Whether the PDE5-Is mechanism involves caspase-mediated apoptosis by extrinsic and/or canonical intrinsic pathway is unclear. In the extrinsic pathway, the death receptor-ligand binds to the associated protein with death domain (FADD), which activates the initiator pro-caspase-8. Caspase-8 activates caspase-3, inducing apoptosis. The intrinsic apoptotic pathway is characterized by mitochondrial changes in response to various stress signals, such as severe genetic damage, hypoxia, and oxidative stress, which activate the initiator pro-caspase-9. Proapoptotic mitochondrial proteins, BH3-only members, activate other proapoptotic proteins, such as BAX, and antagonize antiapoptotic proteins, such as Bcl-2. Subsequently, the mitochondrial outer membrane is disrupted, and its permeability increases, resulting in cytochrome-c (Cyt-c) leakage into the cytosol. Cyt-c in the cytosol forms a complex with Apaf-1, called the apoptosome, which assists in auto-activation of initiator pro-caspase-9. Caspase-9 activates caspase-3, leading to apoptosis. —| Inhibition; —|> activation; ↑ increased expression/activity/level; ↓ decreased expression/activity.

that stress fibers and Ca²⁺ waves could be changed by sildenafil. The involvement of the cytoskeleton in the sildenafil mechanism has been demonstrated by Nunes et al. [116]. Sildenafil induced Ca²⁺ response and a more organized actin fiber pattern in cultured astrocytes, compared to LPS stimulated cells. It is possible that the mechanism behind sildenafil effects in the cytoskeleton involves Na⁺/K⁺-ATPase, IP3R, and ankyrin B (Figure 4A). In addition, this study

showed for the first time that sildenafil has astrocytes as target cells [116], confirming that the control of inflammation is not an indirect effect, secondary to neurogenesis, myelin repair, or improvement of blood flow.

Although there is no clinical report investigating the use of sildenafil chronically in patients with MS, one study has shown the potential of the drug to improve motor impairment. Cocchiarella [161] chronically administered sildenafil (100 mg per day for 7 months) to a 42-year-old man, who developed a generalized motor deficit with spasticity that made him a quadriplegic (but grew normally, including normal intellectual development). The diagnosis was inconclusive. Physical therapy evaluation for muscle strength and manual measures (scale from 0, no muscle activity whatsoever, to 5, muscle activity with full range of motion and against maximal resistance) by a physical therapist indicated a positive change in muscle activity, following sildenafil administration. After stopping the treatment, the patient kept all gains. The patient experienced common drug-induced events associated with sildenafil treatment, such as erection, headache, and nausea. This study indicates that sildenafil has potential to improve other motor deficiencies, such as MS.

Despite the autoimmune/inflammatory nature of MS that has already been described, the control of the disease through the use of immunosuppressants and immunomodulators has proven to be unsatisfactory. PDE5-Is, being sildenafil the most representative, are widely used and well-tolerated drugs, which may be a useful therapeutic intervention to ameliorate the neuropathology of MS. Therefore, well-designed clinical trials may demonstrate that oral administration of PDE5-Is can be appropriate for individuals with MS and other neuroinflammatory/neurodegenerative diseases, providing additional benefits to current treatments.

4.3. Parkinson's disease and Huntington's disease

Parkinson's disease (PD) is a common, slow-progressing neurological disorder that leads to a constant loss of motor function. Its clinical features include resting tremor, slow movements (bradykinesia), rigidity, impaired balance, difficulty initiating movement (akinesia), and loss of postural reflexes [162]. PD is characterized by the death of dopaminergic neurons in the substantia nigra, which results in the absence of dopamine release in striatum and therefore in motor impairment. The remaining neurons contain intracellular inclusions (Lewy bodies), composed of α -synuclein [163] (**Figure 1C**).

Studies by Uthayathas et al. [164] and by Janis et al. [165] evaluated the use of sildenafil as a neuroprotective agent in the murine model of PD induced by chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The hypothesis was that the cGMP accumulation would attenuate the loss of nigrostriatal dopamine neurons induced by the model. The analysis revealed that sildenafil did not prevent neurotoxicity and did not protect against dopamine depletion induced by chronic exposure to MPTP. Also, Uthayathas et al. [164] showed that a single dose of sildenafil (10 mg/kg i.p.) had no effect on fatigue as seen by swimming time. On the other hand, sildenafil did not produce any deleterious effects on nigrostriatal dopamine neuron function, nor did it potentiate the neurotoxic effects of MPTP, suggesting that sildenafil would not accelerate cell loss when used as a treatment of ED in men diagnosed with PD as this drug is used therapeutically to treat sexual dysfunction in PD patients. However,

contradictorily, in 2010, a case report by Perkovic et al. [166] described choreoathetotic movements that were most likely induced by sildenafil in a 56-year-old patient with PD. The man presented strange, involuntary movements and anxiety after taking sildenafil 100 mg, 50 min after the last daily dose of levedopa/carbidopa. These adverse effects were considered to be elicited by the administration of sildenafil (drug abuse) in a previously stabilized responder to levedopa therapy. This effect may be a predisposition for pharmacokinetic interaction in short-time interval between levedopa and sildenafil applied in high dosage.

Despite these negative results using a single dose of sildenafil, therapy with the aim of modulating the immune response and neuroinflammation in PD, targeting microglia, astrocytes, and T cells, has recently been proposed [167]. Considerable evidence shows that persistent inflammatory response, T-cell infiltration, and glial cell activation [168, 169] are common features of human patients and animal models of PD and play a crucial role in the degeneration of dopaminergic neurons [170, 171] (**Figure 1C**). As a result, appropriate treatment appears to involve the ability to modulate peripheral and resident immune cells for the purpose of modifying inflammatory response. It is possible that the chronic modulation of neuroinflammation by PDE5-Is may be beneficial for PD. Clinical studies demonstrated that while a single dose of sildenafil does not cause a clear improvement in cognition in healthy adults [172], chronic administration of udenafil has shown to lead to an improvement in cognitive function [130]. This has led some to suggest that the therapeutic benefits of PDE5-Is may be better observed after chronic inhibition rather than after a single dose [173]. However, studies evaluating the anti-inflammatory effects of chronic PDE5-Is in PD models are lacking.

Investigation of the role of cGMP and PDE-Is in Huntington's disease (HD) is also in the beginning. HD is a dominant hereditary neurodegenerative disorder, characterized by progressive impairment of cognitive and motor functions. This disorder is caused by a mutation that encodes an abnormal expansion of CAG-encoded polyglutamine repeats in a protein called huntingtin (htt) [174]. While healthy individuals contain 16–20 repeats, more than 36 are present within the htt gene in HD patients [175]. Toxic protein aggregates are also seen in HD patients, whose brains contain accumulations of mutated HTT protein (**Figure 1D**) [176]. The pathological hallmark of HD involves the loss of neurons in the cortex and striatum that lead to clinical manifestations including involuntary movements known as chorea, behavioral and psychiatric characteristics, and cognitive dysfunction. Mutant huntingtin (mhtt) has been reported to impair cAMP and cGMP/CREB signaling, a transcriptional pathway that has been hypothesized to play a critical role in HD pathology [177, 178].

It was demonstrated by Saavedra et al. [179] that hippocampal cGMP levels were threefold lower in R6/1 mice (heterozygous transgenic mice in B6CBA background, expressing exon-1 of mhtt with 145 repeats), when they present deficits in object recognition memory and in passive avoidance learning. nNOS levels were also downregulated, while there were no changes in the levels of PDE5 and PDE9. A single i.p. injection of sildenafil (3 mg/kg), immediately after training, increased cGMP levels and improved memory in R6/1 mice. The same study demonstrated that cGMP levels were also reduced in the human HD hippocampus (six HD patients and five control cases). These results showed that the regulation of hippocampal cGMP levels may be a suitable treatment for cognitive impairment in HD [179]. Other studies have reported

decreased levels of nNOS in the caudate of HD patients [180] and in the striatum and cortex of HD mouse models [181, 182]. It has to be investigated whether the mechanism of sildenafil protection in HD neural tissue is via NOS, as demonstrated in other neurological disease models [89, 124, 127, 128].

Puerta et al. [183] demonstrated that the PDE5-Is, sildenafil, and vardenafil (both 1.5 mg/kg p.o., given twice a day for 5 days) protected against 3-nitropropionic acid (3NP), which produces striatal lesions that closely mimic some of the neuropathological features of HD (model induced in male Lewis rats). Rats treated with both sildenafil and vardenafil showed improved neurologic scores and reduced lesion volume. In addition, striatal pCREB levels along with the expression of the downstream target, BDNF, were significantly increased in sildenafil-treated rats, and sildenafil reduced death of GABAergic neurons in the brain tissue. In addition, the activation of calpain (involved in aggregates formation through calpain/p25/CDK5 pathway) was reduced, showing that this drug also can avoid huntingtin N-terminal fragment aggregates. The mechanism demonstrated by Puerta et al. [183] in the HD model is similar to that observed in several studies with AD models.

Also in 2013, Thakur et al. [184] showed that sildenafil was beneficial in the 3NP-HD model induced in Wistar rats, improving cognitive and motor functions. Sildenafil (2 and 4 mg/kg i.p., for 14 days) dose dependently restored body weight and improved memory performance and locomotor activity. The PDE5-I attenuated succinate dehydrogenase activity, balancing the cellular energy deficits induced by 3NP. In addition, as far as we know, this study showed for the first time (and was the only one to show) that sildenafil improves oxidative and nitrosative stress in HD model, indicating that inflammatory parameters may also be the target of this drug in HD.

Despite the lack of studies showing the role of PDE-Is in HD neuroinflammation, several studies carried out on postmortem HD brain tissue and mouse models of HD have found altered expression of immunologically active molecules in the CNS [185–187], and imaging studies indicated increased microglial activity in manifest and premanifest HD gene-expansion carriers [188, 189]. The mhtt leads to activation of microglia and complement, resulting in subsequent production and release of ROS, NO, and cytokines [190]. A study of 20 HD patients, of whom 5 were presymptomatic and 15 were symptomatic, as well as 16 age-matched healthy controls, showed that there were increased levels of IL-6, MMP-9, vascular endothelial growth factor (VEGF), and TGF- β 1 in HD patients. These trends were further observed in a murine HD model [191]. Politis and coworkers [192] found an increase in the peripheral plasma levels of the pro-inflammatory cytokine IL-1 β in HD gene carriers compared to normal controls; and increased microglial activation in the somatosensory cortex was associated with augmented plasma levels of IL-1 β , IL-6, IL8, and TNF- α [193]. In addition, the biomarkers of inflammation were shown to be increased in the plasma of HD gene-expansion carriers, and upregulation was observed up to 16 years prior to expected onset [186, 187, 194], although, in a recent study, these findings were not confirmed [195]. On the other hand, Vinther-Jensen et al. [196] showed that biomarkers of neurodegeneration increased in manifest HD disease, but did not provide evidence of neuroinflammation in early pathogenesis of HD. Therefore, the involvement of neuroinflammation in the HD pathology is not confirmed. However, it is possible that

inflammatory events begin years before the onset of the illness. This makes PDE5-Is potential tools to prevent HD development through modulating neuroinflammation, while it is only a speculation and studies need to be developed.

Therefore, despite the important role of neuroinflammation in PD and HD, there is a lack of studies using PDE5-Is to evaluate inflammatory parameters, making this an interesting field for exploration.

4.4. Stroke

Although stroke is the third most common cause of death [197] and the leading cause of permanent disability in adults worldwide [198], the available therapeutic options remain very limited. As vasodilators with good hemodynamic effects, PDE5-Is have been considered potential tools to treat hypoxia and stroke. Due to this obvious effect, these drugs were initially investigated in stroke models considering their mechanisms in cerebral neovascularization and blood flow.

Several studies have shown that administration of sildenafil to animal models of stroke has beneficial effects [147, 199–201]. It was demonstrated that chronic sildenafil elevated cGMP levels in the brain [147], increased angiogenesis in the ischemic border regions, induced capillary-like tube formation, and increased VEGF [199]. Correspondingly, the relative cerebral blood flow in the lesion boundary area has also been improved [147, 200]. Sildenafil also evoked neurogenesis, increased neuronal and oligodendrocyte progenity, and reduced neurological deficits [147, 201]. However, the drug did not alter the size of the lesion [200, 201]. In contrast to these early works, Novitzky et al. [202] reported that sildenafil did not improve the conditions of C57BL/6 mice induced model of occlusion of the middle cerebral artery. However, in this study, sildenafil was given in a single peritoneal dose (Revatio®, Pfizer; 0.8 mg/ml), while in other ones, the drug was administered chronically.

To clarify the mechanism of PDE5-I protection in stroke model, a work by Barros-Miñones et al. [203] showed that sildenafil reduced the activation of calpain and CDK5 and increased the p25/p35 ratio, showing that the protective effects of sildenafil in the ischemia model are, at least in part, by similar mechanism observed in other neurological diseases. As described above, calpain cleaves p35 in its more stable isoform, p25. Cleavage of p35 to p25 and formation of the p25/CDK5 complexes are associated with aggregate formation (**Figure 2B**). As expected, sildenafil prevented tau hyperphosphorylation. This study also showed that sildenafil increased the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL and reduced cell death. The effect of sildenafil on the decrease of apoptosis (through reduction of proapoptotic proteins Bax and caspase-3 expression and increasing the antiapoptotic protein Bcl-2) has also been demonstrated in physiological aging mouse model [149] (**Figure 4B**).

Following the same sequence of investigation of other neurological conditions, more recent studies have shown that the role of sildenafil in promoting stroke recovery is, at least in part, related to the anti-inflammatory mechanism. In 2014, Charriaut-Marlang et al. [148] surgically induced ischemia model in P7 Sprague-Dawley rat pups by occlusion in the right common carotid artery and tested sildenafil. The animals were treated with a single dose of Viagra®

(Pfizer, 10 or 5 mg/kg i.p.). They found that sildenafil increased mean blood flow, reduced brain tissue loss, and decreased apoptosis (demonstrated by TUNEL). In addition, sildenafil increased the index of myelinated fiber density and improved motor capacity. Associated with these beneficial effects, sildenafil had anti-inflammatory effects, reducing astrogliosis and GFAP-positive cell density and decreasing microglial density. A very recent study by Moretti et al. [204] also demonstrated that sildenafil modulates neuroinflammation in the ischemia model induced in C57BL/6 mice P9 pups by permanent middle cerebral artery occlusion. Animals were treated with a single dose of sildenafil (Viagra®, Pfizer, 10 mg/kg i.p., given 5 min after artery occlusion), which provided a reduction of the mean lesion 8 days after ischemia; also, it reduced the number of GFAP-positive cells, decreased microglial density, and modulated the M1 and M2 profiles of microglia/macrophages in the late phase after ischemia. The number of activated microglia/macrophages (M2) increased 72 h after artery occlusion, while it decreased 8 days after ischemia in sildenafil-treated animals. However, despite the clear anti-inflammatory action of sildenafil in ischemic model, the mechanism behind this effect is still unexplained.

A reported clinical study by Silver and coworkers [205] tested the chronic administration of sildenafil (25 mg per day, for 90 days) in ten ischemic stroke patients aged 18 to 80 years, with a score of 2 to 21 (mild to moderately severe stroke; National Institutes of Health Stroke Scale, NIHSS). Sildenafil appeared to be safe in this group of patients, and all of them presented an improvement from baseline NIHSS score. However, despite the success in preclinical and some clinical studies, PDE5-Is have not been more fully investigated in studies with humans and have not moved into clinical practice until now.

5. Conclusion

In conclusion, the relevant role of NO/cGMP signaling in the control of neuroinflammation and in the modulation of glial cell activity has lead researchers to investigate the effects of PDE5 inhibitors on central neurological diseases. These drugs (sildenafil being the most representative and studied among them) have been shown to be safe and effective in the treatment of central neurological disorders, and its mechanisms have been clarified. Modulation of neuroinflammation appears to be a relevant mechanism of PDE-Is, mainly in chronic treatments, whereas it has to be more fully investigated. Despite the safety and benefits of this class of drugs administrated chronically to patients and the success in preclinical studies, there are no Phase I and Phase II clinical trials, which need to be developed to move forward the repositioning of PDE5-Is as therapy to treat neurological diseases.

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