Phosphodiesterase 5 inhibition as a therapeutic target for ischemic stroke: a systematic review of preclinical animal studies.

Running title: PDE5 inhibition and ischemic stroke.

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Abstract

Phosphodiesterase 5 inhibitors (PDE5i), such as sildenafil (Viagra®), are currently widely used for erectile dysfunction and pulmonary hypertension. Several preclinical studies suggest that PDE5i may also improve functional outcome following ischemic stroke. This systematic review aims to evaluate the effects of PDE5i in animal models of stroke. A systematic search in Medline, Embase, and The Cochrane Library was done including studies in English full text assessing the effects of selective PDE5i in animal models of stroke. Methodological quality was evaluated using the ARRIVE guidelines for animal studies. 32 publications were included describing outcome in 3649 animals. The median number of quality checklist items scored was 21 out of 38 (interquartile range: 20-24). Treatment with PDE5i demonstrated neuroprotective effects including reduced neuronal apoptosis, oxidative stress, and inflammation in the brain. Further, PDE5i increased angiogenesis and elevated cerebral blood flow in the ischemic penumbra. PDE5i improved functional recovery and inconsistent results were found regarding infarct volume. Treatment was successful when administered within the first 24 hours of ischemia, though delayed treatment up to seven days improved outcome in one study. This review demonstrates both neuroprotective and neurorestorative effects of PDE5i in animal models of stroke. Methodological quality was variable and may bias results. There is currently limited evidence on the effects of selective PDE5i in human stroke patients, hence translation of results into clinical trials in humans is warranted.

Key words: Phosphodiesterase 5 inhibitor; PDE-5 inhibitor; cerebrovascular disorder; models, animals

Introduction

Acute ischemic stroke is a major cause of mortality and morbidity worldwide (van der Worp and van Gijn, 2007, Krishnamurthi et al., 2013). While many therapeutic candidates have been developed for treatment of stroke, few have shown efficacy in humans (O'Collins et al., 2006). The only acute treatment available today, thrombolytic therapy, aims to restore blood flow to the ischemic area by clot disintegration. Due to its narrow time window of effect, thrombolysis is not a suitable treatment for the majority of patients (1995). New treatments with different mechanisms of action and wider time window of effect are required and one group of putative treatments for ischemic stroke is the phosphodiesterase 5 inhibitors.

Phosphodiesterase 5 (PDE5) is an enzyme found in multiple human tissues, including neurons (Teich et al., 2016) and vascular endothelium in the brain (Kruuse et al., 2005). It inactivates the second messenger cGMP by hydrolysis to GMP (Francis et al., 1980, Katsuki et al., 1977, Arnold et al., 1977). cGMP is known to activate PKG which further activates numerous downstream intracellular mechanisms, e.g. transcription factor CREB, involved in cell survival and synaptic plasticity (Shimizu-Albergine et al., 2003, Ciani et al., 2002, Lu et al., 1999). PDE5i, such as sildenafil (Viagra®) and tadalafil (Cialis®), inhibit PDE5 and therefore prolong the half-life and effectiveness of endogenous cGMP (Ballard et al., 1998). PDE5i are today widely prescribed on an "as required" treatment for erectile dysfunction in men, for mountain sickness (Jin et al., 2010) and as daily treatment for pulmonary hypertension (Ghofrani et al., 2006). Several studies evaluating the effects of PDE5i in different neurological disorders have been performed over the last two decades. PDE5i have shown to be neuroprotective, anti-inflammatory, and to improve cognition in animals models of Alzheimer's disease (Zhu et al., 2015, Garcia-Barroso et al., 2013), multiple sclerosis (Raposo et al., 2014, Nunes et al., 2012), and encephalopathy (Hernandez-Rabaza et al., 2015). PDE5i have also shown to ameliorate ischemic damage in animal models of myocardial infarction (Koka et al., 2013, Wang et al., 2015), renal ischemia (Kucuk et al., 2012, Medeiros et al., 2010, Zahran et al., 2015), liver ischemia (Bektas et al., 2016), testicular torsion (Zavras et al., 2014, Yildiz et al., 2012, Erol et al., 2009), and ovarian ischemia (Arikan et al., 2010) through anti-apoptotic and anti-oxidative stress mechanisms.

The principle aim of this study was to review the different molecular effects of PDE5i on neurons and cerebrovascular coupling in animal models of cerebral ischemia. In addition, we aimed to assess functional outcome, cerebral blood flow, and stroke volume.

Methods

Search strategy

We searched Medline (from 1966 to present), Embase (from 1974 to present) and the Cochrane Library on August 16, 2016, with a search strategy to identify preclinical interventional in vivo animal studies investigating the effect of selective PDE5i on experimental stroke. The review protocol followed the Cochrane Handbook for Systematic Reviews and Interventions (version 5.1.0, March 2011). The following search string was used: [((phosphodiesterase 5 OR PDE5 OR PDE-5) OR (phosphodiesterase 5 inhibitor OR phosphodiesterase 5 inhibitors OR PDE5 inhibitor OR PDE5 inhibitors OR PDE-5 inhibitor OR PDE-5 inhibitors) OR (sildenafil OR viagra OR tadalafil OR cialis OR adcirca OR vardenafil OR levitra OR staxyn OR vivanza OR avanafil OR stendra OR spedra OR lodenafil OR hydroxyhomosildenafil OR helleva OR mirodenafil OR mvix OR udenafil OR zydena OR zaprinast OR benzamidenafil)) AND (cerebrovascular disorders OR brain vascular disorders OR cerebrovascular occlusion OR brain ischemia OR experimental stroke OR neuroprotection OR animal stroke model OR stroke model OR 4-VO OR 4 vessel occlusion OR mcao OR middle cerebral artery occlusion OR cerebral hypoxia OR ischemia/reperfusion)]. Two investigators (JNEÖ and IRM) screened all titles, and abstracts for eligibility. Full texts of potentially eligible studies were obtained and the same two investigators (JNEÖ and IRM) applied inclusion and exclusion criteria to each article. Disagreement was solved through discussion or by a third investigator (CK). The reference list of all included articles was further screened for eligible studies by one investigator (JNEÖ). No effort was made to quantitatively pool data from different studies.

Inclusion and exclusion criteria

Interventional animal studies where subjects were exposed to induced ischemic stroke, brain ischemia, or brain hypoxia, and treated with a specific PDE5i were included. Outcome had to be compared with a control group. Human studies and studies using non-specific PDE5i were excluded. Only articles with full text in English were included.

Data extraction

We recorded author, publication year, type of animal (species, strain, sex, and weight), numbers of animals per treatment group, ischemia model, PDE5i used (dose, time of administration, and administration route) and outcome. Outcomes were noted under the following categories: neurogenesis/neuronal protection, synaptogenesis/synapse protection, functional outcome, stroke

volume/neurodegeneration, angiogenesis/cerebral vessel protection, cerebral blood flow, apoptosis and cell death, oxidative stress, inflammation, mortality, and other outcomes. Significance were considered when $p < 0.05$.

Quality assessment

Risk of bias was assessed using checklists derived from the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2010) and Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) (Macleod et al., 2004).

Results

Study characteristics

482 non-duplicate publications were identified after electronic search in Medline, Embase and The Cochrane Library. 405 publications were excluded after title and abstract screening and 45 publications were further excluded after full text screening. 32 studies were included in this systematic review assessing outcome in a total of 3649 animals. See supplementary material 1 for details regarding the numbers of articles using specific methods and assessing specific outcomes.

Neurogenesis and neuronal protection

Several studies show a PDE5i-dependent increase in surviving neurons and preserved neuronal structure after induced ischemic stroke (Chen et al., 2014a, Altas et al., 2014, Zhang et al., 2012, Zhang et al., 2006a). Cell proliferative markers, such as Bromdeoxiuridin stain (BrdU), demonstrate immunohistochemically that PDE5i significantly increase neurogenesis following ischemic stroke in the dentate gyrus (Ko et al., 2009, Zhang et al., 2002), subventricular zone (SVZ) (Zhang et al., 2006a, Zhang et al., 2002), and ipsilateral striatum (Zhang et al., 2006b). MRI studies applying diffusion weighted imaging (DWI) showed that sildenafil (10 mg/kg) increased axonal remodeling in the ischemic penumbra, which was histologically confirmed (Ding et al., 2011, Ding et al., 2008b).

Chen et al. (2014) showed that after middle cerebral artery occlusion (MCAO) in rats, proteins in the Nogo-receptor pathway changed, which previously have shown to influence neurite outgrowth and cell protection. Nogo-R, Ras homolog gene family, member A (RhoA) and phosphorylated phosphatase and tensin homolog (PTEN) expression significantly increased in the cortex and

striatum, while p-Akt/Akt and PI3K expression markedly reduced (Chen et al., 2014a, Chen et al., 2014b). Treatment with yonkenafil and sildenafil reversed these biochemical changes and increased the number of surviving neurons, an effect abolished when inhibitors of sGC, PKG, or PKA were co-administered with the PDE5i (Chen et al., 2014a, Chen et al., 2014b).

Synaptogenesis and synapse protection

PDE5i showed protective effects on synapse damage, including axonal swelling and demyelination that follows stroke (Chen et al., 2014b, Chen et al., 2014a). The concentration of synaptophysin, a marker for quantification of synapses, diminished in the brain following stroke (Zhang et al., 2005, Chen et al., 2014b, Chen et al., 2014a). Yonkenafil and sildenafil (8, 16, and 32 mg/kg) administered to rats 2-24 hours after stroke, reduced loss of synaptophysin and decreased ischemia-induced elevation of postsynaptic density protein 95 (PSD-95) and nNOS, effects seen together with preserved synapse structure (Chen et al., 2014b, Chen et al., 2014a, Zhang et al., 2005). In addition, ischemic stroke caused a decrease in growth factors involved in synapse functioning. Tropomyosin-related kinase B (TrkB) and brain-derived neurotrophic factor (BDNF) concentrations decreased in cortex and striatum after stroke and Tropomyosin-related kinase A (TrkA) decreased in cortex while nerve growth factor (NGF) decreased in the striatum following stroke. PDE5i significantly increased these growth factors in the brain (Chen et al., 2014b, Chen et al., 2014a).

Angiogenesis and cerebral blood flow

The density of cerebral blood vessels following stroke was histologically augmented in animals treated with PDE5i (Zhang et al., 2006a, Li et al., 2007, Zhang et al., 2005). Sildenafil (5 and 10 mg/kg) reduced the loss of capillary density and the abundance of TUNEL-labeled endothelium (Charriaut-Marlangue et al., 2014), increased BrdU-labeled endothelial cells in the ischemic penumbra (Zhang et al., 2006a, Zhang et al., 2003), and reduced extravasation of plasma IgG from cerebral vessels following stroke, expressing blood brain barrier (BBB) preservation (Charriaut-Marlangue et al., 2014). One study found no histological difference in capillary length between PDE5i treated animals and control animals with induced stroke (Wang et al., 2013). By use of MRI, sildenafil (10 mg/kg) reduced the T2*-value, representing increased angiogenesis which was histologically confirmed in the ischemic penumbra (Ding et al., 2011, Ding et al., 2008b). One study demonstrated the same effect in saline treated ischemic rats, where PDE5i could accelerate angiogenesis (Ding et al., 2008a).

By use of ultrasound-Doppler the mean blood flow velocity (mBFV) was measured in the large cerebral arteries as an estimate of cerebral blood flow (CBF). One study found no change in mBFV (Moretti et al., 2016) following sildenafil treatment, while another study observed a temporary change in mBFV in the basilary trunk and contralateral internal carotid artery (Charriaut-Marlangue et al., 2014). Laser-Doppler showed a short temporary increase in relative CBF following zaprinast treatment in ischemic rats (Gao et al., 2005).

MRI studies to asses CBF have shown conflicting results. By use of perfusion weighted MRI, sildenafil treatment (10 mg/kg subcutaneously (s.c.) starting 24 hours after MCAO and continued daily for seven days) in aged rats (18 months old) significantly increased CBF by approximately 7 % compared to controls at six weeks after stroke, but not earlier (Ding et al., 2011). One study using flow sensitive alternating inversion recovery (FAIR)-MRI showed no difference in CBF in vardenafil treated mice (10 mg/kg twice daily starting 3 h after MCAO for 14 days) compared to controls (Royl et al., 2009). Using arterial spin labeling (ASL) and dynamic contrast enhancing imaging MRI, sildenafil treatment in rats (10 mg/kg s.c. starting 24 h after MCAO and continued for six additional days) increased CBF by 16 %, respectively 36% in the ischemic penumbra at six weeks after MCAO (Ding et al., 2008b, Li et al., 2007).

Apoptosis, oxidative stress, and inflammation

4-vessel occlusion (4-VO) and bilateral common carotid artery occlusion (BCCAO), two methods often used to induce severe hypoperfusion to the brain, cause significant cell death in the hippocampal areas (Godinho et al., 2015, Ko et al., 2009) and cortex (Godinho et al., 2015). Sildenafil and tadalafil increased neuronal survival in these brain areas following severe brain hypoperfusion (Godinho et al., 2015, Dias Fiuza Ferreira et al., 2013, Romanini et al., 2010, Ko et al., 2009).

The apoptosis regulatory proteins heat shock protein 70 (hsp70), caspase-3, caspase-9, and apoptotic protease activating factor 1 (apaf-1) significantly increased in the brain following stroke (Ko et al., 2009, Chen et al., 2014a). Treatment with tadalafil significantly reduced the expression of caspase 3 (Ko et al., 2009) and yonkenafil reduced expression of apaf-1, inhibited cleavage of caspase 3 and caspase 9, and increased expression hsp70, although no information about significance were provided (Chen et al., 2014a). These changes were followed by a significant reduction in neuronal apoptosis (Chen et al., 2014a, Ko et al., 2009). Neuronal apoptosis was in two

studies, using intrastriatal malonate injections to induce focal chemical hypoxia, assessed by measuring the concentration of B-cell lymphoma 2 (bcl-2) and bcl-2-like protein 4 (Bax) ratio and activation of calpain and cyclin-dependent kinase 5 (cdk5). Pretreatment with sildenafil (1.5 mg/kg p.o) significantly increased bcl-2/Bax ratio and reduced ischemia-induced calpain and cdk5 activation. This was seen together with reduced apoptosis (Barros-Minones et al., 2013a). Pretreatment with sildenafil moreover prevented malonate induced tau hyperphosphorylation and cleavage of myocyte enhancer factor 2 (MEF2), a survival promoting transcription factor (Barros-Minones et al., 2013a). Malonate injection decreased inhibitory phosphorylation and therefore activated apoptosis signal-regulating kinase 1 (ASK1) which led to increased phosphorylation of MAPK Kinase 3/6 (MKK3/6) and MAPK Kinase 7 (MKK7), an effect significantly decreased by approximately 50 % with sildenafil 1.5 mg/kg p.o pretreatment (Barros-Minones et al., 2013b). Sildenafil reduced malonate-induced p38-MAPK activation and translocation to the neuronal nucleus and reduced c-jun N-terminal kinase (JNK) phosphorylation and activation of its downstream effector, c-Jun. The effect of sildenafil was similar to that of administering a p38-MAPK inhibitor together with malonate injection (Barros-Minones et al., 2013b).

Experimental stroke caused an increase in the oxidative stress markers AChE activity (Gulati and Singh, 2014b, Gulati and Singh, 2014a), thiobarbituric acid reactive substances (TBARS) (Gulati and Singh, 2014b, Gulati and Singh, 2014a), nitrite/nitrate levels (Gulati and Singh, 2014b, Gulati and Singh, 2014a, Gaur and Kumar, 2010a), malondialdehyde (MDA) (Gaur and Kumar, 2010a, Altas et al., 2014), and a decrease in the anti-oxidative GSH (Gulati and Singh, 2014b, Gulati and Singh, 2014a), GSH-Px (Altas et al., 2014), and SOD (Gaur and Kumar, 2010a, Altas et al., 2014). These biochemical changes were attenuated by tadalafil (5, 10, and 20 mg/kg p.o.) (Gulati and Singh, 2014b, Gulati and Singh, 2014a) and sildenafil (5 and 15 mg/kg) (Gaur and Kumar, 2010a). L-NAME, an inhibitor of NOS, abolished the PDE5i-induced positive effects on oxidative stress markers (Gulati and Singh, 2014a). Ischemia-induced elevation of NO-levels were resistant to PDE5i (Altas et al., 2014). Two studies reported that PDE5i worsened oxidative stress when administered together with antidepressants (Gaur and Kumar, 2010a, Gaur and Kumar, 2010b).

Following permanent MCAO or unilateral common carotid artery occlusion in neonatal animals, an increase in glial activation and density of microglia were seen (Moretti et al., 2016, Charriaut-Marlangue et al., 2014). Sildenafil (10 mg/kg i.p.) lowered glial activation and reduced the number of microglia in the ischemic penumbra (Moretti et al., 2016) and other areas of the brain (Charriaut-

Marlangue et al., 2014). Moreover, PDE5i had in general the ability to upregulate the neuroprotective M2-microglia and downregulate the neurotoxic M1-microglia following ischemic stroke (Moretti et al., 2016).

Functional outcome and mortality

PDE5i significantly improved functional outcome and improved memory performance measured with Morris water maze and aversive radial maze after stroke (see table 1 for details regarding PDE5i, animal model and test) (Chen et al., 2014a, Gulati and Singh, 2014b, Charriaut-Marlangue et al., 2014, Gulati and Singh, 2014a, Menniti et al., 2012, Ding et al., 2011, Menniti et al., 2009, Li et al., 2007, Zhang et al., 2006b, Zhang et al., 2006a, Zhang et al., 2002, Ko et al., 2009). The positive effects of PDE5i on functional outcome were abolished when inhibitors of NOS (Gulati and Singh, 2014b, Gulati and Singh, 2014a), PKG or sGC (Chen et al., 2014b, Chen et al., 2014a), were co-administered with tadalafil. Five studies found no improvement of functional outcome and memory performance after PDE5 inhibition (Godinho et al., 2015, Dias Fiuza Ferreira et al., 2013, Wang et al., 2013, Gaur and Kumar, 2010a, Royl et al., 2009). One study found a positive effect on young rats (8-12 weeks) but not aged rats (18 months) following embolic MCAO (Zhang et al., 2005). PDE5 inhibition reduced mortality in two studies (Novitzky et al., 2016, Romanini et al., 2010) and had no effect in four studies (Godinho et al., 2015, Dias Fiuza Ferreira et al., 2013, Royl et al., 2009, Zhang et al., 2005).

Stroke size

Seven studies showed a significant reduction in stroke size after PDE5 inhibition (Chen et al., 2014a, Gulati and Singh, 2014b, Charriaut-Marlangue et al., 2014, Chen et al., 2014b, Gulati and Singh, 2014a, Barros-Minones et al., 2013b, Gao et al., 2005) whilst ten studies demonstrated no significant effect (Novitzky et al., 2016, Moretti et al., 2016, Zhang et al., 2012, Wang et al., 2013, Ding et al., 2011, Li et al., 2007, Zhang et al., 2006b, Zhang et al., 2006a, Zhang et al., 2005, Zhang et al., 2002). By applying a NOS inhibitor (L-NAME 1.5 or 3.0 mg/kg i.p.) 30 to 90 minutes prior to BCCAO, the positive results of tadalafil (10 mg/kg) on stroke size were abolished (Gulati and Singh, 2014a, Gulati and Singh, 2014b). Inhibitors of PKA (IP-20, 0.34 mg/kg), PKG (DT-3, 1 mg/kg) or sGC (ODQ, 3.5 mg/kg) administered intraperitoneal immediately after MCAO, likewise eradicated the reduced lesion size established by sildenafil and yonkenafil (16 mg/kg) (Chen et al., 2014b, Chen et al., 2014a). Two studies demonstrated, by use of MRI, that the stroke size was reduced in PDE5i treated animals, though this could not be verified histologically (Royl et al., 2009,

Ding et al., 2011).

Study quality

The median number of ARRIVE criteria scored was 21 out of 38 (interquartile range: 20-24), and CAMARADES score four out of ten (interquartile range: 3.5-5). Some of the most commonly reported ARRIVE criteria, except for title and abstract, were sufficient scientific background, details of how the procedure was carried out, and interpretation of results. 47% of the studies reported randomization and only 21% reported blinded assessment of outcome. None of the studies reported a sample size calculation and only 62% provided a statement of conflict of interest. See supplementary material 2 regarding ARRIVE and CAMRADES score for each article.

Discussion

In this systematic review the effects of PDE5i, as a potential therapeutic target in stroke, have been evaluated. The neuroprotective effect is multi-diverse involving reduced neuronal apoptosis, oxidative stress and inflammation in the brain. Increased neurogenesis is seen in different areas of the brain together with preserved synapse function, increased angiogenesis and cerebral blood flow to the ischemic penumbra. These results demonstrate that PDE5i not only elicit its effects in the blood vessels, but pass the blood brain barrier (BBB) and directly affect brain tissue, in accordance with previous findings (Gomez-Vallejo et al., 2016, Garcia-Barroso et al., 2013). The neuroprotective effects manifest as improved functional outcome and, in some studies, as reduced stroke size, even in doses relevant for human use. PDE5i were in general successful when administered within the first 24 hours of stroke, although one study showed that delayed treatment up to seven days post-ischemia was effective in promoting neuroprotection and improving functional outcome.

Several of the included studies assessed whether the neurorestorative effects seen by PDE5i were abolished when inhibitors of downstream or upstream targets of cGMP were co-administered. Biochemical measurements revealed increased brain cGMP concentration following PDE5i administration (Moretti et al., 2016, Kim et al., 2013, Ko et al., 2009, Zhang et al., 2006a, Zhang et al., 2005, Zhang et al., 2002) and that simultaneous treatment with inhibitors of NOS, GC, PKG, or PKA eliminated the neurorestorative effect seen by PDE5i alone (Gulati and Singh, 2014a, Gulati and Singh, 2014b, Chen et al., 2014b, Chen et al., 2014a). This indicates that the effects seen by PDE5i are dependent on the NO-cGMP-PKG-pathway and PKA is further involved.

In ischemic stroke blood flow is reduced to the brain area supplied by the occluded artery or arteriole. In the ischemic core neurons are lost, however the surrounding ischemic penumbra can be restored with sufficient blood flow. Studies show an increase in blood vessel formation and reduced endothelial cell death following PDE5i treatment in stroke animals (Li et al., 2007, Ding et al., 2008a, Wang et al., 2013, Zhang et al., 2005, Ding et al., 2011, Ding et al., 2008b, Zhang et al., 2003, Zhang et al., 2006a). MRI show an increase in CBF following PDE5i in the penumbra zone (Li et al., 2007, Ding et al., 2011, Ding et al., 2008b), related to the increased angiogenesis and vessel protection, thus improving cerebral perfusion. This effect, in addition to the anti-apoptotic and neuron protective effect, is most likely involved in the reduced stroke size shown by some studies after PDE5 inhibition.

Activation of the Nogo-R receptor complex is known to negatively influence the Akt pathway, important for neurite outgrowth and cell survival (Song et al., 2005, Tonges et al., 2011). Chen et al. (2014) revealed that stroke increased activation of the Nogo-R receptor complex leading to cell death and reduced axonal regeneration. PDE5i blocked the stroke induced increase of the Nogo-R pathway, consequently increasing the brain-concentration of Akt further leading to increased cell survival and neurite outgrowth (Chen et al., 2014b, Chen et al., 2014a). They further revealed that stroke caused a reduction in TrkB-BDNF and TrkA-NGF, four growth factors implicated in neuronal death prevention, neurotransmitter synthesis, and synapse functioning (Greenberg et al., 2009, Cui et al., 2010, Jang et al., 2007, Chen et al., 2014b, Chen et al., 2014a). PDE5i blocked the stroke-induced decrease of these signaling pathways and increased synapse preservation (Chen et al., 2014a, Chen et al., 2014b).

Oxidative stress is one of the most prominent contributors to the range of tissue damage after stroke. It oxidize and cause dysfunction of cellular macromolecules, leading to apoptosis (Loh et al., 2006). PDE5i significantly reversed the ischemia induced abundance of oxidative stress molecules and increased important antioxidants, such as SOD and GSH (Gaur and Kumar, 2010a, Altas et al., 2014, Gulati and Singh, 2014a, Gulati and Singh, 2014b). SOD has shown to be involved in preventing activation of the apoptotic p38-pathway (described below) (Nito et al., 2008) and endorse the cell promoting Akt pathway (Noshita et al., 2003).

Hsp70 is a protein involved in preventing cellular apoptosis (Matsumori et al., 2006). It halts mitochondrial cytochrome c release thus reduce expression of apaf-1 and prevent cleavage of caspase-9 and caspase-3, three key molecules in apoptosis (Chelluboina et al., 2014, Matsumori et

al., 2006). Following acute ischemic stroke, an increase in hsp70, apaf-1, and cleavage of caspase-9 and caspase-3 were detected (Ko et al., 2009, Chen et al., 2014a). PDE5i further increased hsp70, lowered apaf-1, and reduced cleavage of caspase-9 and caspase-3 which was featured together with increased neuronal cell survival (Ko et al., 2009, Chen et al., 2014a). In animals with chemically induced hypoxia, sildenafil increased concentration of the anti-apoptotic protein bcl-2 and lower the pro-apoptotic Bax (Barros-Minones et al., 2013a), a protein capable of triggering apoptosis (Korsmeyer et al., 2000, Levine et al., 2008). Sildenafil could further reduce pro-apoptotic P-ERK1/2 and P-p38, two molecules known to involve bcl-2 as a downstream effector, however the exact mechanism is still not fully mapped (Zhuang and Schnellmann, 2006, De Chiara et al., 2006).

Intra-striatal malonate injections inhibit mitochondrial succinate dehydrogenase and induce focal hypoxia, imitating neuropathological features seen in stroke (Schulz et al., 1998). It activates calpain which cleaves p35-cdk5 to p25-cdk5 (Patrick et al., 1999, Lee et al., 2000). P25-cdk5 induces tau hyperphosphorylation and inactivation of transcription factor MEF2, known to be involved in increased cell survival (Wen et al., 2007, Tang et al., 2005). Barros-Miñones et al. (2013) showed that pretreatment with sildenafil before malonate induced stroke, reduced calpain activity and therefore p25-cdk5 activation resulting in increased cell survival. Interestingly, inhibitors of calpain or cdk5 mimicked the effect seen by sildenafil, implicating their crucial role in neuronal apoptosis after malonate induced stroke (Barros-Minones et al., 2013a). In another study, malonate injection increased Ask-1 activation leading to phosphorylation and activation of MKK7 and MKK3/6 (Hattori et al., 2009, Enslen et al., 1998, Barros-Minones et al., 2013b). These MAP-kinases are responsible for phosphorylation of p38 and its translocation to the neuronal nucleus, JNK phosphorylation and activation of its downstream effector, c-Jun (Enslen et al., 1998, Bogoyevitch and Kobe, 2006). P-p38, as previously described, and c-Jun have been demonstrated to induce neuronal cell death (Behrens et al., 1999, Zhao et al., 2011).

There are several methodological limitations to the included studies. The studies often lack randomization and blinding and even when randomization is stated, the method for it is seldom described. Moreover, sample size calculations were not provided in any of the articles, study limitations were rarely reported and, conflicts of interest were only declared in 62 % of the articles. While the exclusion of non-English articles is a potential risk of bias, only one publication in Chinese was found using the search string described. Also, to minimize bias, the search string used free text search, not limited to MeSH terms.

Few trials have studied the effects of selective PDE5i in human stroke patients (Lorberboym et al., 2010, Lorberboym et al., 2014, Silver et al., 2009, Di Cesare et al., 2016). Ischemic animal stroke models do not entirely represent the pathophysiology of stroke in humans, as included animals are young and male, without comorbidities. Further, pretreatment of PDE5i was applied in many studies, a treatment regimen not relevant for human ischemic stroke.

Conclusion

In animal models of stroke, PDE5i demonstrate neuroprotective effects dependent on cGMP downstream mechanisms. PDE5i improved functional outcome and in some studies reduced stroke size. Outcomes were related to reduced inflammation, oxidative stress, and apoptosis in the brain and formation of new neurons and blood vessels in the lesioned area. There is limited evidence today on PDE5i effect in human stroke, though all suggest it safe to use in stroke patients. New supplementary treatment targets are warranted not only restoring the reduced perfusion but also aiming at neurorestoration post-ischemia in stroke. We propose PDE5 to be such a treatment target and specific PDE5i is the tool to test this.

Author contributions

JNEÖ is the first reviewer and first author who screened all articles, organized data extraction and scored all included publications for quality. He prepared the outline for the article and wrote it. IRM is the second reviewer who screened articles for eligibility and had valuable inputs to the writing process. AH provided valued input to the design and writing process and made language corrections. CK is the last author, who came up with the idea of the study, helped with the design and the writing.

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Conflict of interest

The authors declare no conflict of interest.

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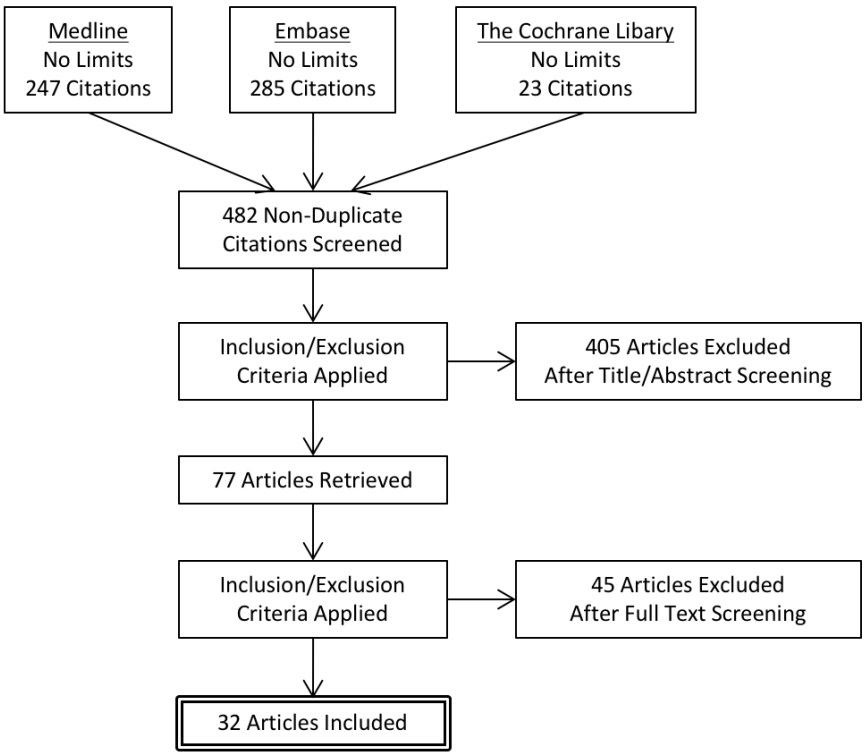

Figure 1: Literature search and study selection.

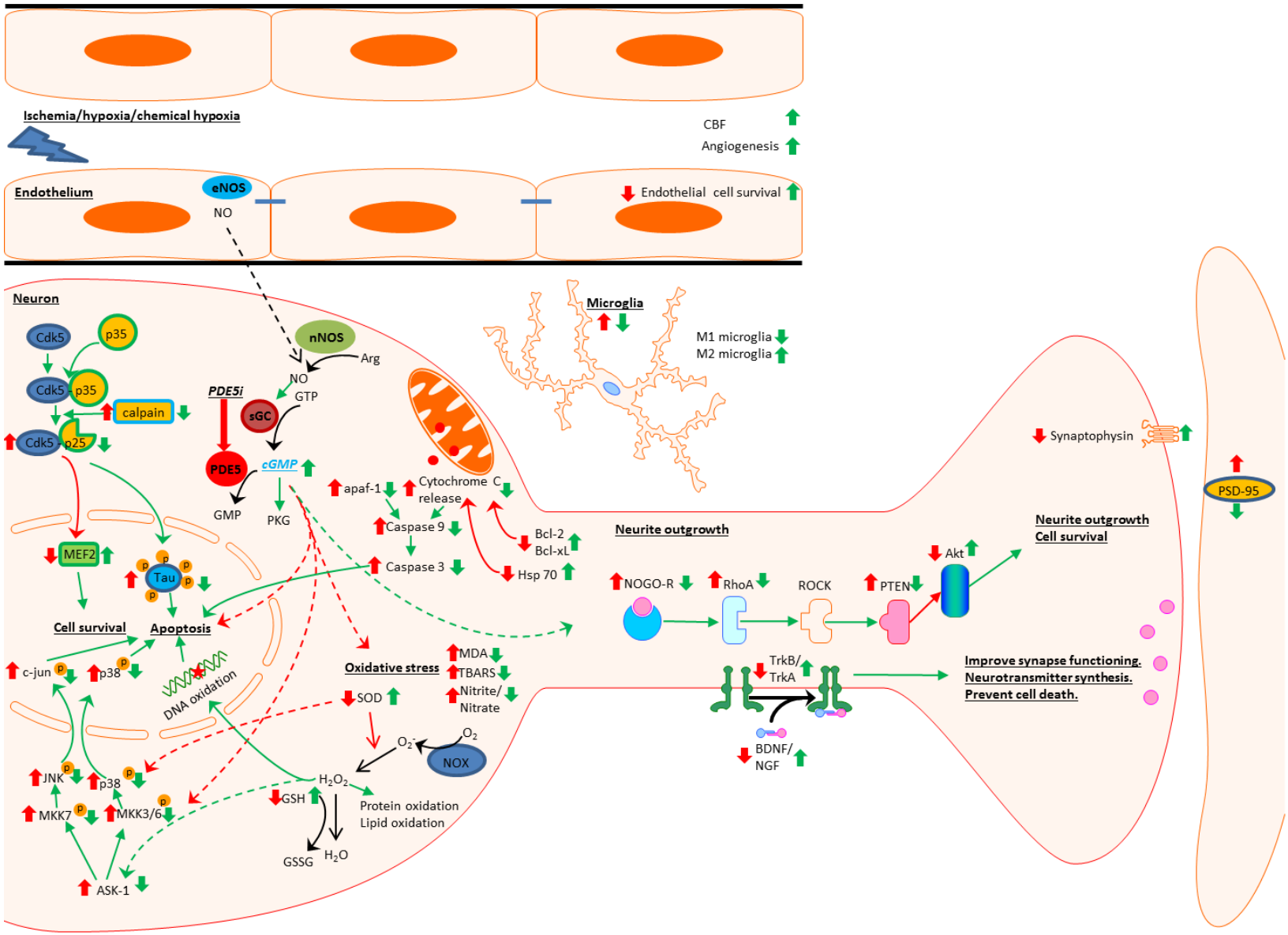

Figure 2: Proposed cellular mechanisms of PDE5i in stroke models. ⬆= increased concentration/activity after cerebral ischemia. ⬇= decreased concentration/activity after cerebral ischemia. ⬆= increased concentration/activity after PDE5i following cerebral ischemia. ⬇= decreased concentration/activity after PDE5i following cerebral ischemia. → = positively regulates. ⟶ = negatively regulates/blocks. ⇢ = indirectly positively regulates. ⇢ = indirectly negatively regulates/blocks. See table 1 text for abbreviations.

Table 1: Overview of the included studies and their results of selective PDE5 inhibitors in animal models of stroke.

Reference	Species	Ischemia method	Treatment	Effects of PDE5i	Functional outcome and stroke size
(Royl et al., 2009)	C57BL/6 mice, 7 weeks old, male.	tMCAO, 45 min.	Vardenafil 10 mg/kg p.o. twice daily starting 3 h post ischemia and continued for 14 days.	→ CBF (FAIR-MRI)	→ Modified Benderson score, wire hanging test score, pole test score → Stroke volume (↓T2 lesion – MRI, → HE lesion – histology) → Mortality
(Novitzky et al., 2016)	C57Bl/6 mice, adult, male.	tMCAO, 60 min.	Sildenafil, 24 µg i.p., single-dose immediately after reperfusion.		→ Infarct volume ↓ Mortality (no information about significance)
(Wang et al., 2013)	Mice (wild-type C57 black/6, adult (2-3 months), male.	tMCAO, 60 min.	Sildenafil 0.3 mg/kg/day p.o. starting 24 h after MCAO and continued for 6 days.	→ Capillary length	→ Adhesive removal test score, cylinder test score → Infarct volume → Body weight
(Chen et al., 2014a)	Sprague-Dawley rats, 8 weeks old, male.	tMCAO, 2 h.	Yonkenafil 4, 8, 16, 32 mg/kg i.v. starting 2, 4, or 6 h after after reperfusion.	↓ Loss of neurons (↓ Nogo-R, ↓ RhoA, ↓ p-PTEN, ↑ p-Akt, ↑ PI3K) ↑ Synaptic preservation (↑ synaptophysin, ↓ PSD-95, ↓ nNOS levels, ↑ TrkB-BDNF, ↑ TrkA-NGF) ↓ Apoptosis biomarkers (↑ hsp70, ↓ apaf-1, ↓ caspase-9, ↓caspase-3)	↓ Infarct volume ↑ Beam walking score, Rotarod test score ↓ Neurological deficits
(Chen et al., 2014b)	Sprague-Dawley rats, unknown age, male.	tMCAO, 2 h.	Sildenafil 4, 8, 16, 32 mg/kg i.p. or i.v. 2, 4, or 6 h after reperfusion.	↓ Loss of neurons (↓ Nogo-R, ↓ RhoA, ↓ p-PTEN, ↑ p-Akt, ↑ PI3K) ↑ Synaptic preservation (↑ synaptophysin, ↓ PSD-95, ↓ nNOS levels, ↑ TrkB-BDNF, ↑ TrkA-NGF)	↑ Beam walking score, rotarod test score ↓ Infarct volume
(Moretti et al., 2016)	C57Bl/6 mice pups.	pMACO.	Sildeanfil 0.5, 2.5, 10, 15 mg/kg i.p. 5 min after MCAO induction.	↓ Inflammation ↑ cGMP brain concentration → CBF	↓ Infarct volume

(Zhang et al., 2012)	Nestin-CreERT2; R26R-stop-YFP mice, 14 months old, male.	pMCAO.	Sildenafil 10 mg/kg/day s.c. for 7 days starting one day after MCAO.	↑ Neurogenesis (↑ YFP,↑ DCX,↑ NeuN) ↑ Oligodendrogenesis (↑ YFP/CNPase)	→ Infarct volume
(Menniti et al., 2012)	Sprague-Dawley rats, unknown age, male.	pMCAO.	PF-5 (1.0 mg/kg) or PF-489,791 (10 mg/kg) s.c. starting 24 h after MCAO and continued for 7 days.	PF-5 crosses BBB. PF-489,791 does not cross BBB.	↑ Limb placement scores, body swing (effect by both PDE5i).
(Ding et al., 2011)	Wistar rats, 18 months old, male.	pMCAO.	Sildenafil 10 mg/kg/day s.c. starting 24 h after MCAO and continued for 7 days.	↑ Axonal remodeling (↑ DA value - MRI, ↑ B&LFB stain – histology) ↓ Ventricle volume (T2 – MRI) ↑ Angiogenesis (↓ T2* value - MRI, ↑EBA – histologically) ↑ CBF (perfusion weighted imaging – MRI)	↓ Neurological severity score ↑ Adhesive removal test score, foot-fault test score. → Infarct volume - histologically
(Menniti et al., 2009)	Sprague-Dawley rats, unknown age, male.	pMCAO.	Sildenafil (10 mg/kg) or PF-5 (0.1, 1, 10 mg/kg) s.c. Administration at different time-points.		↑ Fore limp placement score, body swing test score PDE5i was effective when administration was given 72 h after MCAO. No effect of PDE5i when treatment was delayed to 14 days after MCAO. No difference in effectiveness between 7 or 28 days treatment.
(Ding et al., 2008b)	Wistar rats, adult (8-12 weeks old), male.	pMCAO.	Sildenafil 10 mg/kg s.c. starting 24 h after MCAO and continued for 7 days.	↑ Axonal remodeling/density (MRI and B&LFB – histology) ↑ Angiogenesis (histology) ↑ CBF (ASL-MRI)	
(Ding et al., 2008a)	Wistar rats, adult (8-12 weeks old), male.	pMCAO.	Sildenafil 10 mg/kg s.c. starting 24 h after MCAO and continued for 7 days.	↑ Angiogenesis (T2*/SWI - MRI, Ki value – MRI, EBA stain – histology)	
(Li et al., 2007)	Wistar rats, adult (8-12 weeks old), male.	pMCAO.	Sildenafil 10 mg/kg s.c. starting 24 h after MCAO and continued for 7 days.	↑ Angiogenesis ↑ CBF (dynamic contrast enhancing imaging MRI)	↓ Neurological severity score, foot fault test score ↓ Infarct volume (non-significant).

(Zhang et al., 2006a)	Wistar rats, unknown age, male.	pMCAO.	Tadalafil 2, 10 mg/kg p.o. starting 24 h after MCAO and continued every other day for 6 days (3 administrations).	↑ Neurogenesis (BrdU staining) ↑ cGMP brain concentration	↑ Foot-fault test score, neurological severity score (NSS), adhesive removal test score. → Infarct volume
(Zhang et al., 2006b)	Wistar rats, 3-4 months (young adults) and 18 months (aged), male.	pMCAO.	Sildenafil 3 mg/kg i.p. starting 7 days after MCAO and continued for 7 days.	↑ Neurogenesis (MCM-2, Ki67, HH3, BrdU, NeuN)	↑ Neurological severity score, adhesive removal test score, foot-fault test score → Infarct volume
(Gao et al., 2005)	Wistar rats, unknown age, male.	pMCAO.	Zaprinast 10 mg/kg i.v. infusion for 2 min starting 10 min before MCAO. Second i.v. infusion in infarct volume evaluation after 110 min from first i.v. infusion.	↑ rCBF (laser-Doppler) ↑ cGMP brain concentration	↓ Infarct volume
(Zhang et al., 2005)	Wistar rats, 8-12 weeks (young) and 18 months (aged), male.	pMCAO.	Sildenafil 2 mg/kg p.o. or 10 mg/kg s.c. Administration started 24 h after MCAO and continued daily for additionally 6 days.	↑ Synaptic preservation (synaptophysin) ↑ Angiogenesis (anti – vWF) ↑ cGMP brain concentration	↑ Neurological severity score, adhesive removal test score, foot-fault test score, corner test score. → Infarct volume
(Zhang et al., 2003)	Wistar rats, unknown age, male.	pMCAO.	Sildenafil 2 mg/kg p.o. started 24 h after MCAO and continued for an additional 6 days.	↑ Angiogenesis (BrdU staining) ↑ VEGF brain concentration	
(Zhang et al., 2002)	Wistar rats, unknown age, male.	pMCAO.	Sildenafil 2 or 5 mg/kg p.o. starting either 2 h or 24 h after MCAO and continued for an additional 6 days.	↑ Neurogenesis (BrdU staining and Tuj1 staining) ↑ cGMP brain concentration	↑ Foot-Fault test score, adhesive removal test score. ↓ Weight loss → Infarct volume
(Kim et al., 2013)	Mongolian Gerbils, 15 weeks old, male.	BCCAO, 5 min.	Tadalafil 0.1, 1, 10 mg/kg/day p.o. once daily for 7 days, starting 1 day after BCCAO.	↑ cGMP brain concentration ↓ Loss of D2 receptor concentration in striatum and substantia nigra ↓ Increase in thyrosin hydroxylase expression in striatum and substantia nigra.	
(Gaur and Kumar, 2010b)	Laca mice, unknown age, unknown sex.	BCCAO, 5 min.	Sildenafil 5 mg/kg i.p. starting on day of surgery and continued for 4 days post-surgery.	→ Oxidative stress (PDE5i reversed oxidative stress reduction by antidepressants).	

(Gaur and Kumar, 2010a)	Laca mice, unknown age, unknown sex.	BCCAO, 5 min twice with 10 min in between.	Sildenafil 5 mg/kg starting 2 h after BCCAO and continued to day 4. No information about route of administration.	→ Oxidative stress (PDE5i reversed oxidative stress reduction by sertralin).	→ Lateral push, forced swim test.
(Ko et al., 2009)	Monglian gerbils, 12-14 weeks old, male.	BCCAO, 7 min.	Tadalafil, 0.1, 1, 10 mg/kg p.o. daily for 7 consecutive days, starting one day after surgery.	PDE5i suppressed the ischemia-induced neurogenesis in the dentate gyrus (BrdU staining). ↓ Loss of neurons (↓ TUNEL staining) ↓ Apoptosis (↓ caspase 3) ↑ cGMP brain concentration	↑ Memory (↑ Step-down latency time)
(Gulati and Singh, 2014b)	Swiss mice, unknown age, male.	BCCAO, 12 min.	Tadalafil 5, 10, 20 mg/kg p.o. starting 60 min before BCCAO.	↓ Oxidative stress (↓ AChE, ↓ TBARS, → Nitrite/Nitrate, ↑ reduced GSH)	↑ Memory (AvRM) ↑ Rotarod test score, Beam walking score, Lateral push score ↓ Infarct volume
(Gulati and Singh, 2014a)	Swiss mice, unknown age, male	BCCAO, 12 min.	Tadalafil 5, 10, 20 mg/kg p.o. 60 min prior to BCCAO.	↓ Oxidative stress (↓ AChE, ↓ TBARS, → Nitrite/Nitrate, ↑ reduced GSH)	↑ Morris Water Maze score ↑ Rotarod test score, beam walking score, lateral push test score ↓ Infarct volume
(Altas et al., 2014)	Wistar rats, unknown age, male.	BCCAO, 20 min.	Tadalafil 2 mg/kg/day, administered once daily for 4 days before BCCAO. No information about route of administration.	↓ Loss of neurons ↓ Oxidative stress (↓ MDA, ↑ NO, ↑ SOD, ↑ GSH-Px)	
(Godinho et al., 2015)	Wistar rats, middle-aged, male.	4-VO/ICA, 2 stages.	Sildenafil, 3.0 mg/kg p.o. Start 45 min after first occlusion stage.	↑ surviving neurons in hippocampus	→ Memory
(Dias Fiuza Ferreira et al., 2013)	Wistar rats, middle aged (12-15 months old), male.	4-VO/ICA, 3 stages.	Sildenafil, 3.0 mg/kg/day p.o. starting soon after first step in 4-VO/ICA and continued for 9 days.	↓ Loss of hippocampal neurons	Memory → Mortality →
(Romanini et al., 2010)	Wistar rats, 3-4 months old, male.	4-VO/ICA, 2 stages.	Sildenafil 0.75, 1.5, 3.0 mg/kg/day p.o. starting after first occlusion stage (3-VO) and continued for 7 days.	↓ Loss of neurons	↓ Mortality

(Charriaut-Marlangue et al., 2014)	Sprauge-Dawley rat pups, both sexes.	Right common carotid artery occlusion followed by 120 min of hypoxia (FiO2 = 8%).	Sildenafil 5 or 10 mg/kg i.p. after hypoxia-ischemia.	↑ Blood vessel preservation (↑ Glut1 labeled vessels, ↓ TUNEL labeled endothelium) ↓ BBB leakiness (↓ IgG extravasation) ↑ mBFV in basilary trunk ↓ Inflammation (↓ GFAP, ↓ TL)	↑ Motor behavior ↓ Infarct volume
(Barros-Minones et al., 2013a)	Wistar rats, unknown age, male.	Chemical hypoxia via intrastriatal malonate injection.	Sildenafil 1.5 mg/kg p.o. Administration at many different time points.	↓ Loss of neurons (↓ calpain-cdk5 pathway) ↓ Apoptosis (↑ Bcl-2, ↑ Bcl-xL)	↓ Infarct volume
(Barros-Minones et al., 2013b)	Wistar rats, unknown age, male.	Chemical hypoxia via intrastriatal malonate injection.	Sildenafil 1.5 mg/kg p.o. Administration at many different time points.	↓ Loss of neurons (↓ ASK1-MKK3/6-p38 pathway, ↓ ASK1-MKK7-JNK-c-jun pathway)	↓ Infarct volume

Publications are sorted after ischemia model. tMACO = temporary middle cerebral artery occlusion; pMCAO = permanent middle cerebral artery occlusion; BCCAO = bilateral common carotid artery occlusion; 4-VO/ICA = four vessel occlusion/internal carotid artery; p.o. = per os; s.c. = subcutaneous; i.p. = intra peritoneal; PCR = Polymerase Chain Reaction; TEM = Transmission electron microscopy; ELISA = enzyme-linked immunosorbent assay; MRI = magnetic resonance imaging; cGMP = cyclic guanosine monophosphate; CBF = cerebral blood flow; Nogo-R = Reticulon 4 receptor; RhoA = Ras homolog gene family, member A; PTEN = Phosphatase and tensin homolog; Akt = Protein kinase B; PI3K = phosphatidylinositol-3-kinases*;* synaptophysin major synaptic vesicle protein p38*;* PSD-95 postsynaptic density protein 95= ; nNOS = neuronal nitric oxide synthase; TrkB = Tropomyosin receptor kinase B; BDNF = Brain-derived neurotrophic factor; TrkA = Tropomyosin receptor kinase A; NGF = Nerve growth factor; hsp70 = 70 kilodalton heat shock proteins; apaf-1 = Apoptotic protease activating factor 1; AChE = Acetylcholinesterase; TBARS = Thiobarbituric acid reactive substances; GSH = Glutathione; GFAP = Glial fibrillary acidic protein; TL = Tomato Lectin; Glut1 = Glucose transporter 1; TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling; IgG = Immunoglobulin G; mBFV = menarblood flow velocity; MDA = Malondialdehyde; NO = nitric oxide; SOD = Superoxide dismutase; GSH-Px = Glutathione peroxidase; D2 receptor = Dopamine receptor D_2; cdk5 = Cyclin-dependent kinase 5; ASK1 = Apoptosis signal-regulating kinase 1; MKK3/6 = mitogen-activated protein kinase kinase 3/6; p38 = P38 mitogen-activated protein kinases; MKK7 = mitogen-activated protein kinase kinase 7; JNK = c-Jun N-terminal kinases; YFP = Yellow fluorescent protein; DCX = Doublecortin; NeuN = Neuronal Nuclei ; CNPase = 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; DA value = Diffusion anisotropy value; B&LFB stain = Bielschowsky's silver stain and Luxol fast blue; EBA = endothelial barrier antigen; PDE5i = Phosphodieseterase 5 inhibitor; FAIR-MRI = flow sensitive alternating inversion recovery magnetic resonance imaging; Bcl-2 = B-cell lymphoma 2 protein; Bax = Apoptosis regulator BAX; ERK1/2 = extracellular signal–regulated kinases 1/2; TdT = Terminal deoxynucleotidyl transferase; HIF-1a = Hypoxia-inducible factor 1-alpha; ASL = arterial spin labeling; SWI = Susceptibility weighted imaging; Ki = inhibitory constant; MCM-2 = DNA replication licensing factor MCM2; HH3 = Phosphohistone-H3; vWF = Von Willebrand factor; VEGF = Vascular endothelial growth factor; Tuj1 = Neuron-specific Class III β-tubulin, ↓ = decreased, ↑ = increased, and → = no change.