Sildenafil Mediates Blood-Flow Redistribution and Neuroprotection After Neonatal Hypoxia-Ischemia

Christian Charriaut-Marlangue, PhD; Thao Nguyen, MS; Philippe Bonnin, MD, PhD; An Phan Duy, MD; Pierre-Louis Leger, MD, PhD; Zsolt Csaba, PhD; Julien Pansiot, MS; Thomas Bourgeois, MS; Sylvain Renolleau, MD, PhD; Olivier Baud, MD, PhD

Background and Purpose—The best conceivable treatment for hypoxia-ischemia (HI) is the restoration of blood flow to the hypoxic-ischemic region(s). Our objective was to examine whether boosting NO-cGMP signaling using sildenafil citrate, a phosphodiesterase-type 5 inhibitor, could modify cerebral blood flow and reduce lesions in the developing brain.

Methods—HI was induced in P7 Sprague–Dawley rats by unilateral carotid artery occlusion and hypoxia, and followed by either PBS or sildenafil. Blood-flow velocities were measured by ultrasound imaging with sequential Doppler recordings to evaluate collateral recruitment. Cell death, blood–brain barrier integrity, and glial activation were analyzed by immunohistochemistry. Motor behavior was evaluated using an open-field device adapted to neonatal animals.

Results—Sildenafil citrate (10 mg/kg) induced collateral patency, reduced terminal dUTP nick-end labeling–positive cells, reactive astrogliosis, and macrophage/microglial activation at 72 hours and 7 days post-HI. Sildenafil also reduced the number of terminal dUTP nick-end labeling–positive endothelial cells within lesion site. Seven days after HI and sildenafil treatment, tissue loss was significantly reduced, and animals recovered motor coordination.

Conclusions—Our findings strongly indicate that sildenafil citrate treatment, associated with a significant increase in cerebral blood flow, reduces HI damage and improves motor locomotion in neonatal rats. Sildenafil may represent an interesting therapeutic strategy for neonatal neuroprotection. (Stroke. 2014;45:850-856.)

Key Words: neonatal stroke ■ nitric oxide ■ sildenafil

Neonatal hypoxic-ischemic (HI) brain injury is one of the most common causes of severe neurological handicap (cerebral palsy, mental retardation, and epilepsy) in children. No therapeutic option is available for HI, apart from the initiation of hypothermia within 6 hours after birth, which benefits only 1 infant in 6.1 The best conceivable treatment for HI is the restoration of blood flow to the affected region(s) as soon as possible because decreased cerebral blood flow (CBF) during reperfusion in the first 12 to 24 hours indicates poor prognosis in term newborns with perinatal asphyxia and HI encephalopathy.2,3

Decreased regional CBF is the principal factor determining the topography of tissue injury after HI in the immature rodent brain although metabolic factors (ie, intrinsic vulnerability) may influence injury in some brain structures.4,5 Recently, the inhalation of nitric oxide (iNO), a well-known vasoactive molecule, has been found to be beneficial after ischemia in adult mice and sheep,6 and after cardiac arrest and cardiopulmonary resuscitation in mice.7 In contrast, in neonatal or juvenile rats, iNO seems to be beneficial only when given during ischemia but is deleterious when administered during reperfusion.8 When the Rice-Vannucci model is applied to P9 mouse pups, iNO (50 ppm) reduces neuronal damage when given during hypoxia.6 Conversely, iNO at 10 or 40 ppm has been reported to increase lesion size when given after the HI insult.9 Together, these data suggest that NO-dosage and time period of exposure are crucial and vary depending on the vascular responses specific to each model of ischemic and HI injury.

Another option that enhances the effects of endogenous NO is to manipulate the NO-cyclic guanosine monophosphate (cGMP) pathway by blocking cGMP degradation by phosphodiesterases (PDEs). In particular, sildenafil and tadalafil, 2 potent selective PDE-5 inhibitors, prolong the action of cGMP in multiple vascular territories.10–12 For instance, there is substantial evidence supporting the cardioprotective role of various PDE-5 inhibitors after ischemia-reperfusion injury in the heart.11–13

The goal of this study was to test the hypothesis that boosting NO-cGMP signaling through PDE-5 inhibition with sildenafil would enhance microcirculatory CBF, reduce ipsilateral...
HI-induced brain damage including neuroinflammation, and finally, improve motor performance.

Materials and Methods
An expanded version of the methods is available in the online-only Data Supplement.

Ethics Statement
All experiments complied with the ethical guidelines of the Robert Debre Hospital Research Council Review Board (A75-19-01), INSERM and ARRIVE (http://www.nc3rs.org/ARRIVE), which approved this study.

Hypoxia-Ischemia
Surgical procedures were performed in P7 Sprague–Dawley rat pups (Janvier, Le Genest-St Isle, France; 16.1±1.5 g, n=88, both sexes). Rat pups underwent occlusion of the right common carotid artery under isoflurane anesthesia followed by 120 minutes of hypoxia (FiO2=8%). Pups were allowed to recover for 5 minutes in room air before Doppler ultrasound measurements and then returned to their mothers. Animals were euthanized 72 hours and 7 days (at P14) after HI. The mortality rate at the end of the hypoxic period was <10% in all groups of animals. Sham controls underwent anesthesia and incision only. All analyses were performed in a blind setup. Tissue loss at P14 was quantified using the validated method of calculating the ratio of ipsilateral to contralateral areas measured on cresyl-violet-stained sections.

Ultrasonographic Brain Imaging
Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Voluson i; GE Medical Systems ultrasound, Horten, Norway) equipped with a 12-MHz linear transducer (12 L).²

Drug Treatment
Included animals were randomly assigned to 2 groups and treated with either PBS or a single dose of sildenafil citrate (10 or 5 mg/kg; Pfizer, France) given intraperitoneally (IP) after hypoxia-ischemia and ultrasonographic measurements.

Physiological Parameters
Blood gases (P O2, P CO2) and pH were measured in PBS- and sildenafil-treated rat pups (n=6, each group) using a clinical blood gas analyzer (ABL 80, Radiometer SAS, Copenhagen, DK).

Immunohistochemistry and Terminal dUTP Nick-End Labeling Assay
Coronal 15-μm thick paraffin sections at the hippocampal level (corresponding to −3.20 mm from the bregma in the adult brain) from animals killed at 72 hours and 7 days were stained with rat anti-IgG (1:200, Dako, France), mouse antiamyelin basic protein (MBP; 1:500, Chemicon, Millipore, France), mouse antiambillary fibrillary acidic protein (GFAP; 1:500, Dako), and rabbit anti-Glut1 (54 kDa form, 1:500; Millipore, Molshem, France) antibodies and tomato lectin (1:500, AbCys, Paris, France). Sections were then processed for terminal dUTP nick-end labeling (TUNEL) according to manufacturer instructions (In situ Cell Death Detection Kit, AbCys, Paris, France). For colocalization, double-stained sections were analyzed using a Leica TCS SP8 confocal-laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with 488-nm Argon and 561-nm DPSS lasers.

MBP Quantification
The optical density of MBP (myelin sheath) labeling was expressed as the ratio of ipsilateral to contralateral measurements (IL:CL)MBP²⁵, for each brain sample, the (IL:CL)MBP of pixels was calculated.

Motor Performance
Because quadruped locomotion is only apparent from postnatal day 10 (P10), pups were evaluated at P14 using an open-field test to assess overall activity.²⁶ We used trajectories to calculate the total distance traveled and the longest distance (remoteness) traveled along straight-line segments during a 1-minute test.

Statistical Analysis
All results are expressed as means±SD. One- or 2-way ANOVAs and post hoc Newman–Keuls tests (for variations in tissue loss) and paired or unpaired Student t tests were used to analyze differences between groups.

Results
Setup of the Animal Model and Effect of Sildenafil on CBF in P7 Rat Pups
A preliminary study demonstrated that animals (n=28, 2 animals died during the first 24 hours) subjected to the HI procedure at P7 exhibited the first 24 range of brain tissue loss at P14, with some animals showing no or little tissue loss (≤5% of the volume of the contralateral hemisphere; Figure 1A). The latter animals displayed a significant increase in mean blood-flow velocity (mBFV, ≥160%) in the contralateral internal carotid artery (ICA; measured by color-coded pulsed Doppler ultrasound imaging) 5 to 10 minutes after HI, as compared with brain-lesioned animals (Figure 1B and Figure 1 in the online-only Data Supplement). In contrast, no difference was observed in the basilar trunk (BT; Figure II A in the online-only Data Supplement) between unlesioned and lesioned pups. This increased in mBFVs was still detected and remained significant in the contralateral ICA (Figure IID in the online-only Data Supplement) 24 hours after HI. However, at that later time point, mBFVs were also significantly increased in the BT in both unlesioned and lesioned animals (Figure IIC in the online-only Data Supplement). Heart rates remained stable throughout the 24 hours evaluation (basal=361±25 bpm; post-HI=349±31 bpm; 24 h after HI=361±32 bpm).

We first observed that sildenafil did not change physiological parameters (pH, P O2, and P CO2) as compared with PBS (Table I in the online-only Data Supplement). We then assessed 29 animals by Doppler ultrasound immediately after HI and excluded those that exhibited mBFVs >160% of basal values (n=5). Included animals (n=24) then received either PBS (n=12) or a single dose of sildenafil citrate (10 mg/kg, IP, n=12). One hour after sildenafil injection (10 mg/kg), mBFV was significantly increased in the BT compared with basal values (P<0.05; Figure 1C), and significantly increased in the contralateral ICA (CL-ICA, P<0.001 versus basal values, and versus PBS-treated HI animals; Figure 1D). Heart rates were also increased in sildenafil-treated animals (390±27 bpm) compared with PBS-treated animals (358±26 bpm; P<0.05). In contrast, mean BFVs in the BT and CL-ICA were not different between the 2 groups at 24 hours after treatment (Figure III in the online-only Data Supplement), and heart rate as well (361±32 bpm in PBS versus 320±23 bpm in sildenafil, not significant). Arterial blood flow in the BT and CL-ICA 1 hour after injection of 5 mg/kg sildenafil was unchanged (Figure IV in the online-only Data Supplement).
Effect of Sildenafil on Glial Activation and Cell Death After HI

In the ipsilateral hemisphere of PBS-treated HI animals, reactive astrocytes were present, showing a marked increase in GFAP intensity, hypertrophy, and increased process thickness in the corpus callosum at 72 h post-HI. Reactive astrocytes were also observed in the damaged hippocampus, thalamus, and caudate-putamen. Sildenafil treatment after HI significantly reduced astrogliosis (leading to a more fibrous aspect with thinner processes) and GFAP-positive cell density in both the cingulum and the external capsule of the white matter (Figure 2A). Seven days after HI, a marked increase in GFAP intensity was still observed in the cortex, hippocampus, and white matter, which was significantly reduced after sildenafil treatment (Figure 2C).

At this time point of recovery, the HI insult was associated with an increase in the density of tomato lectin–positive microglial cells in the ipsilateral cortex and the hippocampus, as well as macrophage recruitment in the cortex and subcortical white matter. Sildenafil treatment after HI was associated with a significant decrease in the density of tomato lectin–positive cells in the cortex and hippocampus (P<0.001) as well as in the white matter (P<0.005), to levels similar to those in the contralateral hemisphere (Figure 2B). Seven days after HI, a marked increase in GFAP intensity was still observed in the cortex, hippocampus, and white matter, which was significantly reduced after sildenafil treatment (Figure 2C).

At this time point of recovery, the HI insult was associated with an increase in the density of tomato lectin–positive microglial cells in the ipsilateral cortex and the hippocampus, as well as macrophage recruitment in the cortex and subcortical white matter. Sildenafil treatment after HI was associated with a significant decrease in the density of tomato lectin–positive cells in the cortex and hippocampus (P<0.001) as well as in the white matter (P<0.005), to levels similar to those in the contralateral hemisphere (Figure 2B). Seven days after HI, a marked increase in GFAP intensity was still observed in the cortex, hippocampus, and white matter, which was significantly reduced after sildenafil treatment (Figure 2C).
HI, macrophages were mainly detected in the white matter, whereas ameboid microglial cells were present in the cortex, and in a small number in the hippocampus. Sildenafil significantly reduced their number in all the regions (Figure 2D).

The exposure of P7 rats to HI induced unilateral brain damage in the hippocampus (CA1 and CA3), cortex (as columnar patches), subcortical white matter, and thalamus. Patchy cell death was particularly well observed in the cortex and subcortical white matter, as shown by TUNEL at 72 hours (Figure VA in the online-only Data Supplement). Sildenafil treatment (10 mg/kg) was associated with a lower density of TUNEL-positive nuclei (Figure VB in the online-only Data Supplement). Seven days after HI, animals displayed either a cystic (Figure VC in the online-only Data Supplement) or a noncystic cortex with numerous TUNEL-positive nuclei, most of them being present within macrophages (inset in Figure VC in the online-only Data Supplement). Sildenafil significantly reduced their number (Figure VD in the online-only Data Supplement).

**Effect of Sildenafil on Capillary Cell Death and Blood–Brain Barrier Disruption**

All capillaries are highly labeled for the glucose transporter Glut1, which is thus used as a marker for changes in blood–brain barrier capillary density. Glut1 immunoreactivity demonstrated a paucity of vessels and the rarefaction of branching in the ipsilateral cortex of PBS-treated animals at 72 hours (Figure VI in the online-only Data Supplement). In contrast, both capillary branching and the number of labeled vessels were closer to the contralateral cortex in sildenafil-treated animals (Figure 3A and 3B). Three-μm-confocal analysis (Figure 3C and 3D) demonstrated colocalization of Glut1 (red) and TUNEL (green), highly suggesting endothelial cell death. In Glut1-TUNEL-stained sections, the number of TUNEL-positive endothelial cells was reduced in sections from sildenafil-treated animals when compared with PBS-treated animals (1.6±0.9 versus 5.0±1.8 per vessel; P<0.01; inset in Figure 3B).

Extravasation of endogenous IgG was observed in the ipsilateral cortex, CA1, and CA3 of the hippocampus and the stria medularis of the thalamus in all PBS-treated HI animals at 72 hours (Figure 4A), and revealed a larger area of blood–brain barrier leakiness than that indicated by TUNEL. In contrast, sildenafil significantly reduced the density of IgG labeling, mostly remaining in the microvessels and in all the regions analyzed in contrast to PBS sections (Figure 4B–4D).

**Effect of Sildenafil on Lesion Size and Motor Behavior**

At P14, that is, 7 days after HI, body weight was not different between PBS-treated and sildenafil-treated pups (24.1±2.4 versus 24.0±2.2 g, respectively). A dose of 10 mg/kg of sildenafil significantly reduced brain tissue loss (by 42.9±15.2%; P<0.01), whereas a half-dose of 5 mg/kg did not prevent tissue loss (7.3±5.1% reduction; not significant) when compared with PBS (Figure 5A and 5B). All sildenafil-treated (using 10 mg/kg) animals almost displayed cortex recovery, whereas only half of these animals displayed hippocampal recovery. In addition, the index of myelinated fiber density (ratio of the ipsilateral to the contralateral hemisphere, [IL:CL]MBP) was found to be increased in sildenafil-treated animals (10 mg/kg) in both the cingulum (0.84±0.1 versus 0.57±0.19; P<0.01) and the external capsule (0.66±0.09 versus 0.39±0.24; P<0.05) when compared with PBS-treated P14 animals (Figure 5C and 5D).

PBS-treated and sildenafil-treated (10 mg/kg) rat pups were assessed for motor behavior at P14, and compared with age-matched sham-operated animals (Figure 6A). By P14, sham pups (PBS and sildenafil treated) traveled along straight-line segments all around the acrylic plate. In contrast, PBS-treated HI pups demonstrated pivoting and predominantly onsite body motion in the center of the plate. Sildenafil-treated HI pups presented more straight-line segments than PBS-treated HI animals. The total distance traveled (Figure 6B) and remoteness or the longest distance traveled (Figure 6C), both reduced in PBS-treated animals, partially improved in sildenafil-treated animals to reach levels similar to those of sham animals.

**Discussion**

In this study, we show for the first time that the selective inhibition of PDE-5 by sildenafil citrate has neuroprotective effects and improves locomotion in a model of mild/moderate neonatal HI brain damage. These effects were associated with increased arterial BFV both in the BT and the contralateral ICA after HI injury.
As previously reported for a neonatal stroke model based on an ischemia-reperfusion sequence, the Rice-Vannucci model also results in variable lesion volumes both in rat and mouse pups. In our hands, up to 20% of animals never developed detectable brain lesions after HI. Interestingly, animals displaying no or small lesions (<5% of hemispheric volume) were associated with a significant increase in mBFV in the contralateral ICA just after HI. This increased mBFV demonstrates (1) the effectiveness of collateral recruitment through the circle of Willis, and (2) the opening of arterial anastomoses between the territories of the anterior, middle, and posterior cerebral arteries. In contrast, rat pups with significant lesions after HI do not present these compensatory mechanisms. Therefore, we used mBFV as a reliable marker to select animals that would develop significant and consistent brain lesions in response to HI to evaluate the effect of sildenafil on histological features and motor locomotion.

In animals without vascular compensation after HI, sildenafil at a dose of 10 mg/kg was able to increase mBFV to levels similar to those measured in unlesioned HI animals, strongly supporting the occurrence of pharmacological vasodilation and collateral recruitment/patency through the NO-cGMP pathway, as previously reported with inhaled NO in a neonatal stroke model. Sildenafil has been shown to similarly increase CBF at the ischemic boundary in adult rats with embolic stroke. Tadalafil, another specific PDE-5 inhibitor, alleviates microvascular ischemia and fully restores the blood-flow regulation after a single dose (10 mg/kg per os) in Becker muscular dystrophy, an effect that is both marked and immediate. The preserved density and branching of Glut1-immunolabeled

![Figure 4](image)

**Figure 4.** Status of the blood–brain barrier (BBB) 72 hours after hypoxia-ischemia (HI) in PBS-treated and sildenafil-treated animals. Representative images of cells immunolabeled for rat IgG (as an indicator of extravasation attributable to BBB leakiness) within the ipsilateral cortex and the hippocampus (HPC) in PBS-treated (A) and sildenafil-treated (B) animals (n=5 each group). C, Representative images of IgG-labeled microvessels within the ipsilateral cortex in PBS-treated and sildenafil (sil)-treated animals. D, Quantification of IgG-positive areas. Bar represents 100 μm (A and B) and 30 μm (C).

![Figure 5](image)

**Figure 5.** Effect of sildenafil on lesion size and myelinated fiber density 7 days after hypoxia-ischemia (HI). A, Representative cresyl-violet–stained sections at the level of the dorsal (upper) and ventral (lower) hippocampus in PBS-treated (n=12) and sildenafil-treated (Sil, n=12) animals. B, Variation in tissue loss 7 days after injury after treatment with 10 or 5 mg/kg sildenafil. C, Representative mouse antimyelin basic protein (MBP) immunolabeling in the cingulum and fragmentation of the myelin sheath in PBS and sildenafil-treated animals. EC: external capsule. Scale bars: 50 μm (20 μm in enlarged panels). D, Quantification of the optical density of MBP immunoreactivity expressed as the ratio of ipsilateral (IL) to contralateral (CL) labeling, (IL/CL)MBP. *P<0.01, **P<0.001, sildenafil vs PBS treatment.
vessels in our study suggests that the increased mBFV we observed in sildenafil-treated animals may be because of a similar enhancement of perfusion and consequently, improved CBF, throughout the ipsilateral hemisphere.

In the present study, sildenafil (10 mg/kg) reduced cell death and astrocyte/microglial activation, which are hallmarks of HI-induced injury.22 Sildenafil (1 μmol/L) treatment has been shown to decrease TUNEL-positive cells in isolated adult23 and neonatal24 cardiomyocytes in culture by increasing protein kinase G activity, as well as in ischemic cardiomyopathy in mice.11 Our data are also consistent with a recent work demonstrating that sildenafil leads to a decrease in GFAP expression and astrocyte activation as well as microglial/macrophage activation and recruitment, in association with the downregulation of cytokine production in a mouse model of cuprizone-induced multiple sclerosis.25 More recently, Zhang et al26 have shown that sildenafil can reduce the cognitive deficits associated with β-amyloid peptide–induced neuroinflammation in mice. In the present study, the transient improvement in the cerebral hemodynamic status by sildenafil, either by increasing plasma cGMP and NO metabolites27 or by improving endothelial function after HI (impaired at P7),28,29 leads to reduce injury and consequently to reduce inflammatory responses.

Seven days after HI, sildenafil significantly reduced tissue loss and improved motor locomotion in association with increased myelination, as observed in a mouse model of cuprizone-induced multiple sclerosis.25 This observation confirms the functional impact of the histological benefits observed earlier. Other studies have investigated the ability of sildenafil to promote endogenous repair and neurogenesis in aged animals. For instance, the elevation of cGMP by sildenafil (10 mg/kg) enhances neurogenesis, angiogenesis, and synaptic plasticity, closely associated with improvements in neurological outcome and memory in animal models of adult stroke30,31 and Alzheimer’s disease.32 Recently, Zhang et al33 have also demonstrated that sildenafil enhances nestin expression in neural stem cells and the generation of neuronal and oligodendroglial progeny in the ischemic brain of the middle-aged mouse. Whether sildenafil could induce similar changes in the developing brain remains to be elucidated.

Boosting NO-cGMP signaling through PDE-5 inhibition seems to be a promising therapeutic strategy against cerebral ischemia and HI. However, in the developing brain, the dosage and time period of exposure to NO (or NO-pathway–related molecules such as sildenafil) remain to be clearly defined for each model, depending on interactions between NO and its derivatives, its effectors, and the local redox environment.34,35 Indeed, exposure to 20 ppm of iNO is beneficial when given during ischemia but not after reperfusion in a neonatal stroke model.8 Similarly, iNO at 50 ppm reduces neuronal damage when given during HI, whereas lower doses increase lesion size after HI. Together, these data suggest that excess NO during reperfusion exacerbates oxidative stress4 and is detrimental to the immature brain.36 Sildenafil does not act by increasing endogenous NO but prevents the rapid degradation of its main vasoactive mediator, cGMP, and therefore improves CBF in hypoxic microvessels.

Emerging therapies aimed at augmenting brain repair processes using NO donors and PDE-5 inhibitors are currently being evaluated in preclinical studies of stroke in adulthood and show histological improvements and functional recovery. This study demonstrates the occurrence of similar neuroprotective processes in the developing brain subjected to HI, providing new insights into the therapeutic effects of sildenafil on brain repair.

Acknowledgments

We are indebted to Justine Dupanloup and Assia Mouri for technical assistance.

Disclosures

None.

References


Sildenafil Mediates Blood-Flow Redistribution and Neuroprotection After Neonatal Hypoxia-Ischemia
Christianie Charriaut-Marlangue, Thao Nguyen, Philippe Bonnin, An Phan Duy, Pierre-Louis Leger, Zsolt Csaba, Julien Pansiot, Thomas Bourgeois, Sylvain Renolleau and Olivier Baud

Stroke. 2014;45:850-856; originally published online January 28, 2014;
doi: 10.1161/STROKEAHA.113.003606
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/45/3/850

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2014/01/28/STROKEAHA.113.003606.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL - Charriaut-Marlangue et al.

Sildenafil mediates blood-flow redistribution and neuroprotection after neonatal hypoxia-ischemia.

Supplemental Methods

**Hypoxia-ischemia.** Surgical procedures were performed in P7 Sprague-Dawley rat pups (Janvier, Le Genest-St Isle, France; 16.1±1.5 g, n=32, both sexes). Rat pups underwent occlusion of the right common carotid artery under isoflurane anesthesia followed by 120 min of hypoxia (FiO₂=8%). Animals were sacrificed 72 hours and 7 days (at P14) after HI.

**Ultrasonographic (US) Brain Imaging.** Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Voluson i, GE Medical Systems ultrasound®, Horten, Norway) equipped with a 12-MHz linear transducer (12L)¹. Time-average mean blood-flow velocities (mBFVs) were measured in both intracranial internal carotid arteries (ICA) and basilar trunk (BT) before surgery (basal condition), during the first min after the hypoxic-ischemic procedure, 1 hour after treatment, and finally 24 hours after HI. Measurements take ~10 min for 1 litter (10 pups). Heart rates were measured and reflected changes in cardiac output, as ventricular stroke volume is quite invariable in newborns.

**Blood gas analysis.** Blood gases were analyzed in PBS- and sildenafil-treated rat pups using a clinical blood gas analyzer (ABL 80, Radiometer SAS, Copenhagen, DK). Naïve pups were treated by PBS and/or sildenafil (10 mg/kg), and 1 hour after treatment, were decapitated and blood samples collected from the neck in heparinized capillary tubes, and gases measured immediately.

**Drug treatment.** Included animals (n=24, 3 litters) were randomly assigned to two groups (for each litter), and treated with either PBS or a single dose of sildenafil citrate (10 or 5 mg/kg; n=12 per group; Pfizer, France) given intraperitoneally (i.p.) after hypoxia-ischemia and US measurements.

**Confocal analysis.** For confocal imaging, sections were analyzed using a Leica TCS SP8 confocal scanning system (Leica Microsystems, Wetzlar, Germany) equipped with 488-nm Argon and 561-nm DPSS lasers. A series of optical sections separated by 0.3 µm was collected using the 63x HC PL APO CS2 objective (numerical aperture 1.40). For each
optical section, double-fluorescence images were acquired in sequential mode to avoid potential contamination by fluorescence emission cross-talk. The orthogonal sectioning and 3D view were produced by the Leica LAS AF software (Leica Microsystems). Composite illustrations were built in Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

**Motor performance.** As quadruped locomotion is only apparent from postnatal day 10 (P10), pups were evaluated at P14 using an open-field test to assess overall activity. Each P14 pup was placed in the center on a translucent acrylic plate (50 cm × 50 cm) covered with a silicone gel. Two cameras were placed below the plate. One acquired the pup’s paw contacts with the floor, which appeared as high-contrast areas due to frustrated total internal reflection, and were identified using custom-made software. The second camera acquired the trajectory of the pup. We used trajectories to calculate the total distance traveled, and the longest distance (remoteness) traveled along straight-line segments during a 1-min test.

**Supplemental Results and Discussion**

*Setup of the animal model of hypoxia-ischemia (HI) and CBF modulation*

In a pilot study we determined that lesion volume was 20.4±12.6 (n=18) and 17.7±13.6% (n=14, NS) at 3 and 7 days, respectively, suggesting that the area “at risk” is well delineated as early as 72 hours post HI. No significant difference was observed between males and females. All animals displayed stable heart rates (HR) during all the procedure. We found an inverse exponential regression between infarct volume (at 7 days after HI) and the ratio of mBFV (5-10 min post-HI versus basal) (r=0.75, see supplemental Fig. IA). This wide variation in damage (supplemental Fig. IB) was similar to the one found in the HI model induced in P12 mouse pups. Blood-flow velocities indicate that more mBFV increased in the contralateral (CL) internal carotid artery (CL-ICA), less was the lesion size. As the ipsilateral CCA remained permanently occluded, the delta increase in mBFVs in the CL-ICA represents the blood-flow volume, which anterogradelly perfuses the CL anterior carotid artery (ACA), and then retrogradelly the ipsilateral (IL) ACA to support the IL middle cerebral artery (MCA). Furthermore, the delta increase in mBFVs in the BT represents the supplemental blood-flow volume, which supports the IL posterior carotid artery (PCA) through the IL posterior communicant artery.
Supplemental Figure I: Regression analysis (A) of the infarct volume measured at 7 days after hypoxia-ischemia versus the ratio of mBFV (between basal and 1 hour after HI) in the contralateral ICA (CL-ICA). B: Representative cresyl violet-stained sections (at the level of dorsal hippocampus) showing variability in lesion size (from up to down: 14, 22 and 33 %).

Effect of Sildenafil on physiological parameters and blood flow after hypoxia-ischemia

Sildenafil (10 mg/kg, n=12) did not modify pH and blood gases, but significantly increased heart rates (see supplemental Table 1) one hour after the treatment. In contrast, sildenafil (5 mg/kg) did not increase heart rates (335±26 bpm, n=10) as compared to PBS (342±33bpm, NS; n=10).

Supplemental Table I: Physiological parameters in P7 rat pups.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>pO₂, mm Hg</th>
<th>pCO₂, mm Hg</th>
<th>Heart rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-PBS (n=5)</td>
<td>7.365 ± 0.038</td>
<td>53.1 ± 7.2</td>
<td>49.2 ± 6.6</td>
<td>358 ± 26</td>
</tr>
<tr>
<td>Control-Sildenafil 10 mg/kg (n=6)</td>
<td>7.312 ± 0.075</td>
<td>56.2 ± 9.4</td>
<td>51.4 ± 3.7</td>
<td>390 ± 27*</td>
</tr>
</tbody>
</table>

bpm indicates beat per min.

Whereas sildenafil (10 mg/kg, n=10) increased mBFVs and heart rates, a half dose did not (supplemental Fig. II).
Supplemental Figure II: Redistribution of arterial blood flow 1 hour (A, B) and 24 hours (C, D) after neonatal hypoxia-ischemia (HI) in P7 rat pups. Mean blood-flow velocities (mBFVs) were measured in the contralateral ICA (CL-ICA) and basilar trunk (BT). Note a significant increase in the CL-ICA, but not in the basilar trunk (BT) in un-lesioned (no lesion) as compared to lesioned rat pups, 1 hour after HI. In contrast, a significant increase in mBFVs in the BT was found both in un-lesioned and lesioned pups as compared to the basal condition (*p<0.05 versus basal) 24 hours after HI. In the CL-ICA, mBFVs remained very significantly increased (***p<0.001 vs basal; ###p<0.001 vs lesion) in the un-lesioned pups compared to lesioned rat pups.

Supplemental Figure III: Redistribution of arterial blood flow in BT and CL-ICA. Mean BFVs were measured in PBS and Sil groups. No significant differences were observed (NS).
Supplemental Figure III: Arterial blood flow in the basilar trunk (BT) and contralateral ICA (CL-ICA) 24 hours after neonatal hypoxia-ischemia (HI) and PBS and/or Sildenafil (Sil, 10 mg/kg) treatment. Note that mBFVs are similar in the two groups.

Supplemental Figure IV

Supplemental Figure IV: Arterial blood flow in the BT and CL-ICA 1 hour after neonatal hypoxia-ischemia (HI) and PBS and/or Sildenafil (Sil, 5 mg/kg) treatment. Note that treatment maintained mBFVs as measured in basal (Bas) condition.

Supplemental Figure V

Supplemental Figure V: TUNEL-positive nuclei/mm² in cortex and WM treated with PBS and Sil. Significance: *** p < 0.001.
Supplemental Figure V: Effect of sildenafil on cell death 72 hours (A-B) and 7 days (C-D) after HI. DNA fragmentation was determined by TUNEL assay in PBS- (n=5) and sildenafil-treated (n=6) animals. A-C: Representative images of TUNEL* nuclei in the cortex (Cx) and white matter (WM) of a PBS-treated animal (A, C), and in the WM of a sildenafil-treated animal (Sil, A). Note the presence of TUNEL* nuclei around a cavity in a PBS-treated animal and a TUNEL* nucleus engulfed in a macrophage (stained with tomato-lectin, inset in C) 7 days after HI. B-D: Quantification of TUNEL-positive nuclei in the cortex and white matter. ***: p<0.001. Scale bar: 100 µm (40 µm in the inset).

Glut-1 immunoreactivity (supplemental Fig. VI) demonstrated a paucity of vessels and the rarefaction of branching in the ipsilateral cortex of PBS-treated animals at 72 hours. In contrast, both capillary branching and the number of labeled vessels were closer to the contralateral cortex in sildenafil-treated animals.

Supplemental Figure VI

Supplemental Figure VI: Effect of sildenafil on the number of microvessels in the cortex 72 hours after hypoxia-ischemia and treatment. A-B: Glut-1 protein immunoreactivity in PBS (A, n=5) and sildenafil (B, n=6) treated animals. Note the paucity of vessels in PBS-treated animals. Bar represents 100 µm.
Supplemental References
