Inhaled NO improves brain damage by collateral recruitment in a neonatal stroke model.

Supplemental Methods

Neonatal ischemia-reperfusion. All experiments complied with ethical guidelines of Robert Debré Hospital Research Council Review Board (A75-19-01), INSERM and the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Ischemia was induced in Wistar P7 rat pups (16-18 g; Janvier, Le Genest St-Isle, France; both sexes) and was adapted to isoflurane anesthesia. Briefly, thermoregulated (37.0±0.5°C) and anesthetized pups [1% isoflurane in O₂/N₂O (1:3)] were exposed to left middle cerebral artery electocoagulation (MCAo) combined with a transient (50 min) and concomitant occlusion of both common carotid arteries (CCA) by using vascular clips (18055-04, Fine Science Tools, Heidelberg, Germany). Carotid blood-flow restoration was verified with the aid of a microscope. During ischemia and recovery the pups were placed in a humidified incubator at 32-34°C. After recovery, pups were transferred to their mothers.

Ultrasound brain imaging. Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Vivid 7, GE Medical Systems ultrasound®, Horten, Norway) equipped with a 12-MHz linear transducer (12L). Time-average mean blood-flow velocities (mBFVs) were measured in both intracranial internal carotid arteries (ICA) and basilar trunk (BT) (supplemental Fig. 1) before surgery, during ischemia (at 40 min) and at 15 min after removal of the CCA occlusion. Heart rates were measured and reflected changes in cardiac output, as ventricular volume is quite invariable in newborns.
Regional cerebral blood-flow monitoring. Cortical regional cerebral blood flow (rCBF) was monitored in the MCA territory by laser Doppler flowmetry (Moor Instruments Ltd, Axminster, UK) in thermoregulated rats under isoflurane anesthesia [1% in O$_2$/N$_2$O (1:3), n=6, each condition]. Doppler probe was placed on the skull (~2 mm posterior and ~3 mm lateral to the bregma). Relative changes in rCBF were recorded in 3 regions of interest (penumbra) over a period of 5 min in basal and at the end of ischemia, and averaged; rCBF measurements were normalized to baseline in each animal.

Cortical NO detection. Rat pups were premedicated with buprenorphine (0.1mg/kg i.p.), pentobarbital sodium (25 mg/kg i.p.) and supplemented by additional injections of pentobarbital sodium (15 mg/kg) every 90 min. Three-electrode potentiostatic system allowing, by use of differential normal pulse voltametry, detection and quantification of an oxidation current (+ 650 mV) specific for NO. Reference (Ag/AgCl wire, 250 µm in diameter) and auxiliary (Tungsten wire, 250 µm in diameter) electrodes were implanted in contact with the dura. The NO sensor was prepared on the basis of a carbon fiber (diameter: 30 µm, length: 500 µm) coated with porphyrin-nickel (Interchim, Montluc, on, France) and Nafion as previously described $^3$,$^4$. The NO sensor was stereotaxically implanted into the frontal cortex (P: 2 mm, L: 1 mm/bregma, and 500 µm in depth) according to Paxinos and Watson’s atlas. Variations occurring in the NO peak height (oxidation current) were continuously monitored (1 measurement/2 min) with a Biopulse (Tacussel, France) and computerized (analogical/digital interface, National Instrument, USA) throughout 40-60 min consecutive baselines condition for each animal. The changes occurring in the oxidation current in all other conditions (iNO 20-ppm given using a nasal mask, injection of a NO donor, diethylamine NONoate, 50 mg/kg) were expressed in percentage versus the basal mean value of the NO release considered as reference.

Immunohistochemistry, immunofluorescence and TUNEL staining. Sections (n=6-8 each condition) from air- and iNO 20 ppm-exposed rat pups subjected to ischemia and sacrificed
at 48 hours were processed as previously described and incubated with a primary antibody against nitrotyrosine (NT, AB5411, 1:100, Millipore, St-Quentin-en-Yvelines, France). Sections were then incubated with a biotinylated anti-rabbit secondary antibody and immunolabeling was visualized using the streptavidin-biotin-peroxidase method. The specificity of the primary antibodies that we used was tested by their omission. Antibodies against tomatolectin (TL, 1:500, AbCys, Paris, France) to stain resident, activated macrophage/microglia were revealed using streptavidin coupled to the red fluorescent marker Cy3 (Jackson ImmunoRes laboratories, Interchim, Asnieres, France). These sections were then processed for TUNEL staining according to the manufacturer’s instructions (In situ Cell Death Detection Kit, AbCys, Paris, France). TUNEL-, NT- and TL-positive cells were counted (in a blind manner) in three to four coronal sections in the fronto-parietal cortex (at Bregma 0.24 mm) under an X20 objective using a 0.065 mm²-grid.

Physiological parameters. Arterial blood was drawn by intracardiac puncture and gases (pH, \( \text{paO}_2 \), \( \text{paCO}_2 \)) were measured under air (n=4) and 20-ppm iNO exposure (n=6) by the means of blood-gas analyzer (Ciba-Corning 248).

Supplemental Results

Supplemental Table

Table 1: Physiological parameters in P7 rat pups.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>( \text{paO}_2 ), mm Hg</th>
<th>( \text{paCO}_2 ), mm Hg</th>
<th>Heart rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia (n=4)</td>
<td>7.377 ± 0.047</td>
<td>33.3 ± 5.9</td>
<td>51.9 ± 4.5</td>
<td>322 ± 29</td>
</tr>
<tr>
<td>Normoxia + iNO 20-ppm (n=6)</td>
<td>7.364 ± 0.041</td>
<td>41.5 ± 7.3*</td>
<td>51.3 ± 3.2</td>
<td>363 ± 31**</td>
</tr>
</tbody>
</table>

bpm indicates beat per min. Values are mean ± SD. *p<0.05, **p<0.01 versus normoxia.
Supplemental Figure 1: Representation of the collateral arterial recruitment during ischemia. **Left:** Circle of Willis is supplied by the two ICA and the BT. In rodents, in contrast with humans, the three cerebral arteries are branches of each ICA; each Pcom is developed between the BT and each PCA. Thus, circle of Willis is constituted, from behind to forward, by BT, the two Pcoms, the proximal segment of each PCA (P1), the two intracranial ICAs, the ACAs which flow into the median azygos ACA. **Right:** During ischemia (left MCAo and transient right and left CCAo, 50 min), only the BT supplied two hemispheres through the Pcoms and cortical anastomoses from PCA and ACA to MCA territories. These blood-flow redistributions constitute the collateral arterial recruitment evidenced using US imaging and showed to be protective towards lesion extent in developing brain. Less effective is collateral blood flow supply more extensive is the brain lesion in the left MCA territory (Bonnin et al., 2011).
Supplemental Figure 2: Detrimental inhaled NO (iNO) during reperfusion. a: Effect of iNO at high concentration (80 ppm) during ischemia on rat pups submitted to ischemia. Time-average mBFVs (cm.s⁻¹) in basilar trunk (BT) were increased during ischemia (to 203±55%, n=5, **p<0.01 vs untreated rat pups) and remained elevated after reperfusion (to 138±47%, *p<0.05 vs untreated (n=13), whereas untreated ischemic rat pups returned to basal values. b: Infarct volumes at 48 hours was higher in iNO per-ischemia treated compared to untreated rat pups (21.4±6.2%, n=5 and 14.6±5.8%, n=12, respectively, *p<0.05 iNO vs untreated animals).

Supplemental Figure 3: Effect of iNO on oxidative stress at 48 hours after ischemia-reperfusion. Quantification of the number of 3 NT- positive cells in air- and iNO-exposed animals (n=5 each condition) with iNO given either during ischemia (iNO per) or after reperfusion (iNO post). Data are given in mean ± SD positive cells/per 0.065 mm². **p<0.01 versus air.