Neuroprotection by Freezing Ischemic Penumbra Evolution Without Cerebral Blood Flow Augmentation With a Postsynaptic Density-95 Protein Inhibitor

Bernt T. Bråtane, MSc; Hong Cui, PhD; Douglas J. Cook, MD; James Bouley, BSc; Michael Tymianski, MD; Marc Fisher, MD

Background and Purpose—The purpose of this study was to determine whether neuroprotection is feasible without cerebral blood flow augmentation in experimental permanent middle cerebral artery occlusion.

Methods—Rats were subjected to permanent middle cerebral artery occlusion by the suture occlusion method and were treated 1 hour thereafter with a single 5-minute intravenous infusion of the postsynaptic density-95 protein inhibitor Tat-NR2B9c (7.5 mg/kg) or saline (n=8/group). Arterial spin-labeled perfusion-weighted MRI and diffusion weighted MRI were obtained with a 4.7-T Bruker system at 30, 45, 70, 90, 120, 150, and 180 minutes postmiddle cerebral artery occlusion to determine cerebral blood flow and apparent diffusion coefficient maps, respectively. At 24 hours, animals were neurologically scored (0 to 5), euthanized, and the brains stained with 2–3-5-triphenyl tetrazolium chloride to ascertain infarct volumes corrected for edema. Additionally, the effects of Tat-NR2B9c on adenosine 5′-triphosphate levels were measured in vitro in neurons subjected to oxygen–glucose deprivation.

Results—Final infarct volume was decreased by 30.3% in the Tat-NR2B9c-treated animals compared with controls (P=0.028). There was a significant improvement in 24 hours neurological scores in the Tat-NR2B9c group compared with controls, 1.8±0.5 and 2.8±1.0, respectively (P=0.021). Relative to controls, Tat-NR2B9c significantly attenuated diffusion-weighted imaging lesion growth and preserved the diffusion-weighted imaging/perfusion-weighted imaging mismatch (ischemic penumbra) without affecting cerebral blood flow in the ischemic core or penumbra. Tat-NR2B9c treatment of primary neuronal cultures resulted in 26% increase in cell viability and 34% greater adenosine 5′-triphosphate levels after oxygen–glucose deprivation.

Conclusions—Preservation of adenosine 5′-triphosphate levels in vitro and neuroprotection in permanent middle cerebral artery occlusion in rats is achievable without cerebral blood flow augmentation using a postsynaptic density-95 protein inhibitor. (Stroke. 2011;42:00-00.)

Key Words: cerebral ischemia ■ DWI ■ mismatch ■ MR imaging ■ penumbra ■ PWI ■ stroke ■ Tat-NR2B9c

An effective neuroprotective treatment for stroke has yet to be translated to humans despite many promising strategies identified in preclinical studies.1 By contrast, reperfusion of occluded vessels through thrombolytic and mechanical strategies is an established and effective means of salvaging tissue at risk after stroke.2 However, reperfusion therapies are limited in practice by the effective therapeutic window and specific contraindications aimed at reducing the risk of hemorrhage.3 Neuroprotection (defined as a strategy that increases the resiliency of the brain to ischemia) is theoretically achievable by reducing metabolic demand, increasing energy reserves, and/or preventing neurotoxic signaling in tissues at risk. However, if a putative neuroprotectant were to improve cerebral blood flow, improved outcome in an experimental setting may be mistakenly attributed to increased tissue resiliency as opposed to blood flow augmentation. This occurred previously with some N-methyl-D-aspartate receptor antagonists.4,5 Peri-infarct blood flow augmentation is effective in rodents but may not occur in higher order animals with larger brains due to unfavorable surface/volume ratios. Thus, neuroprotective agents have not been effective in human trials,6 and it is uncertain if experimental stroke treatment without reperfusion can improve outcome through neuroprotection, independently of blood flow, or whether infarct volume and outcome improvements can only occur if there is peri-infarct blood flow augmentation.

To examine this question, we selected Tat-NR2B9c, a promising neuroprotectant that has been shown to reduce infarct size in experimental permanent middle cerebral artery occlusion by freezing ischemic penumbra evolution without cerebral blood flow augmentation.
occlusion (pMCAO) in rats. Tat-NR2B9c blocks the interactions of postsynaptic density-95 protein, an N-methyl-D-aspartate receptor-associated postsynaptic density protein, with other downstream signaling proteins (a postsynaptic density-95 protein inhibitor). This uncouples N-methyl-D-aspartate receptors from neurotoxic signaling proteins such as nitric oxide synthase (NOS)6 and reduces stroke damage in rats.7,9 We used MRI perfusion-weighted imaging (PWI) to measure the effects of Tat-NR2B9c on cerebral blood flow after pMCAO and combined this with diffusion-weighted imaging (DWI) to characterize the evolution of the ischemic penumbra in vivo.10 Additionally, we used in vitro measurements of the effect of Tat-NR2B9c on adenosine 5'-triphosphate (ATP) levels of neurons exposed to oxygen–glucose deprivation (OGD), because this setting is dissociated from blood flow effects. From this we determined whether stroke reduction in pMCAO is achievable by neuroprotection or whether it requires blood flow augmentation.

Methods
All procedures used in this study were performed in accordance with institutional guidelines for animal studies set forth by the Animal Medicine Review Committee, University of Massachusetts Medical School, Worcester, MA, and the Animal Care Committee, University Health Network, Toronto, Ontario, Canada.

Middle Cerebral Artery Occlusion
Spontaneously breathing male Wistar rats (n=16; Taconic Farms, Hudson, NY) weighing 331±27 g were anesthetized with isoflurane (5% for induction, 2% for surgery, 1.2% for maintenance) in room air. PE-50 polyethylene tubing was inserted into the femoral artery for continuous monitoring of mean arterial blood pressure and for obtaining blood samples to measure blood gases (pH, PaO2, PaCO2), electrolytes (Na+, K+, Ca2+), and plasma glucose at before, as well as 50 and 180 minutes after stroke. PE-50 tubing was placed into the femoral vein to allow for intravenous infusion of sodium cromolyn (to inhibit possible mast cell degranulation and possible hypotension related to the administration of a cationic peptide) followed by Tat-NR2B9c or placebo. Body temperature was monitored continuously with a rectal probe and maintained at 37.1±0.2°C with a thermostatically controlled heating blanket.
PwMAO was performed in 16 animals blindly randomized to either placebo (n=8) or drug (n=8) using 4-0 silicone-coated nylon filament sutures as previously described in detail.11 Neurological scoring was performed at 4 and 24 hours as previously described.12

Study Design
The rats either received Tat-NR2B9c (7.5 mg/kg) or placebo (saline). The infusion was administered intravenously over 5 minutes starting at 60 minutes after MCAO. This timeframe of drug administration was selected because of our prior MRI experiments in this pMCAO model that demonstrated an optimal ischemic penumbra at 1 hour postischemia onset.13 All animals also received an infusion of sodium cromolyn (5 mg/kg) over 5 minutes starting at 50 minutes after MCAO to ameliorate possible blood pressure-lowering effects of Tat-NR2B9c. Animals remained anesthetized until 3 hours after MCAO. The animals were then euthanized at 24 hours and infarct volumes were measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining with edema correction.13 One placebo-treated animal that died prematurely between 16 and 24 hours after stroke onset was included in this data analysis.14

MRI Protocol
MRI experiments were performed on a 4.7-T/40-cm horizontal magnet equipped with a Biospec Bruker console (Billerica, MA) and a 20-G/cm gradient insert (internal diameter=120 mm, 120-µs rise time). A surface coil (internal diameter=23 mm) was used for brain imaging and an actively decoupled neck coil for perfusion labeling.13 Animals were imaged at 25, 45, 70, 90, 120, 150, and 180 minutes after MCAO. DWI and PWI were acquired as previously described in detail.13 Calculation of the quantitative apparent diffusion coefficient (ADC) and cerebral blood flow (CBF) maps allows for rater independent lesion volume assessment derived from the DWI and PWI, respectively.13 Using previously validated thresholds for ischemia, effects of Tat-NR2B9c on ADC- and CBF-derived spatiotemporal lesion evolution were evaluated.13

Calculation of In Vivo Lesion Size
Images were analyzed using QuickVol II (www.quickvol.com).15 Quantitative CBF and ADC maps and their corresponding threshold-derived lesion volumes were calculated as described previously.13 The thresholds used to define, respective, normal ADC and CBF regions in which a reduction to 0.53×10−3 mm/s and 0.3 mL/g/min were previously validated.13

Region of Interest Analysis of CBF
Four regions of interest were defined in each rat, 2 in the ischemic side (ischemic core and cortical penumbra) and 2 in the same location on the contralateral side. DWI and PWI at 30 minutes defined the locations of the regions of interest. The regions of interest were applied to all imaging time points and the percentage reduction in blood flow in the core and cortical penumbra were calculated.

Primary Neuronal Cell Culture
Primary murine cortical neurons were isolated from E16 CD1 Swiss mice (Charles River, Ontario, Canada) as previously described.16,17 Cells were plated on poly-L-ornithine-coated 96-well plates (Corning, Corning, NY) at a density of 1×105 cells/well (cell culture reagents were from Invitrogen, Burlington, Ontario, Canada). After 4 days in vitro, growth of nonneuronal cells was halted by exposure to 10 µmol/L FDU solution (5 µmol/L uridine and 5 µmol/L (+)-5-fluoro-2-deoxyuridine) for 48 hours. Previous studies have shown that these cultures contain 85% neurons.18

Oxygen–Glucose Deprivation
Cultures maintained for 13 to 14 days were transferred to an anaerobic chamber (Model 1025; Thermo Forma, Marietta, OH) containing 5% CO2, 10% H2, and 85% N2 (O2<0.2%).19 They were washed 3× with 500 µL of deoxygenated glucose-free Hanks balanced salt solution (0 mmol/L glucose, 121 mmol/L NaCl, 10 mmol/L HEPES acid, 7 mmol/L HEPES-Na Salt, 1 mmol/L Na-pyruvate, 1.8 mmol/L CaCl2, 3 mmol/L NaHCO3, 0.01 mmol/L NaCl, 12.5 mmol/L glucose, 10 µmol/L CNQX, 2 µmol/L Nimodipine, pH 7.4) and maintained under anoxic conditions for 2 hours at 37°C. Normoxic control cells were incubated for 2 hours in 5% CO2 and atmospheric air in a normoxic buffer identical to the OGD solution except 20 mmol/L glucose was added. OGD was terminated by washing the cultures with oxygenated glucose-containing (20 mmol/L) Hanks balanced salt solution. The cultures were maintained for a further 1 hour or 20 hours at 37°C in a humidified 5% CO2 atmosphere until determination of ATP levels or cellular viability.

Quantification of Cell Death In Vitro
Cell death was determined in OGD and control wells by quantitative measurements of propidium iodide fluorescence using a multiwell plate fluorescence scanner (Fluoroskan Ascent FL; Thermolab Systems, Burlington, Ontario, Canada) with excitation/emission wavelengths at 530/620 accordingly 20 hours after OGD. The fraction of dead cells in culture was calculated as follows: fraction dead=(Ft-F0) where Ft is propidium iodide fluorescence at time t and F0 is initial propidium iodide fluorescence at t=0 hours.
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Table. Neuroscores*

<table>
<thead>
<tr>
<th>Group</th>
<th>4-H Menzies</th>
<th>24-H Menzies</th>
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</thead>
<tbody>
<tr>
<td>Placebo (n=8)</td>
<td>2.9±0.4</td>
<td>2.8±1.0</td>
</tr>
<tr>
<td>Tat-NR2B9c (n=8)</td>
<td>2.6±0.7</td>
<td>1.8±0.5†</td>
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*There was no significant difference between placebo and Tat-NR2B9c at 4 h but a significant improvement in neuroscores was observed in the Tat-NR2B9c-treated group at 24 h poststroke (†P<0.05).

Measurement of Intracellular ATP Levels In Vitro

Intracellular ATP content was measured using a CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer’s instructions (Promega, Madison, WI). Cultures were recovered under normoxia for 1 hour after OGD. Equal-volume Cell-Titer-Glo Reagent was added into wells containing cortical neurons and incubated in the dark for 10 minutes and then measured with a VICTOR multilabel counter microplate reader (PerkinElmer; Turku, Finland). An ATP standard curve was generated for each measurement to calculate the ATP content of each well. Protein content in cell lysates was determined by the bicinchoninic acid method. Intracellular ATP content was expressed as nanomoles per milligram protein.

Drug Treatment and Experimental Design

One hour before OGD experiments, cell culture wells were treated with either 100 nmol/L Tat-NR2B9c or an equivalent volume of 0.9% NaCl solution. In vitro experiments were conducted with Tat-NR2B9c and saline treatment under normoxic and OGD conditions. Cell death was reported as a fraction of normoxic, saline-treated controls and ATP level is reported as a percentage of normoxic saline-treated controls.

Statistical Analysis

The MRI experiment was performed in a blinded, randomized manner. A power calculation before starting the experiment demonstrated that 8 animals per group had an α of 0.05 and a β of 0.8 for detecting a difference in lesion volume of 30% between the 2 treatment groups. Data are presented as mean±SD unless stated otherwise. Statistical comparisons of in vivo data were performed using repeated-measures analysis of variance with post hoc Tukey-Kramer test for multiple comparisons and 2-tailed Student t test, where appropriate. In vitro data were collected from paired experiments, performed in triplicate, and repeated 4 times. These data were compared using 1-way analysis of variance following Tukey multiple comparison test. Calculations were performed using NCSS 2007 (NCSS, Kaysville, UT) or SPSS (Version 15.0; SPSS Inc, Chicago, IL). P<0.05 was considered significant.

Results

Blood gases, electrolytes, and blood glucose did not differ between the 2 groups and were within physiological range (data not shown). There was no statistically significant difference in mean arterial blood pressure at all imaging time points except at 70 minutes after MCAO when the Tat-NR2B9c group exhibited a statistically significant reduction in mean arterial blood pressure as compared with placebo (98±5 versus 107±8 mm Hg). Neuroscores at 4 hours did not show differences between groups but at 24 hours, the Tat-NR2B9c group had a significantly improved neuroscore as compared with placebo (Table).

The spatiotemporal evolution of the ischemic stroke described by threshold-derived ADC and CBF lesion volumes, as compared with the TTC-derived infarct volumes, are plotted in Figure 1. The CBF-derived lesion volumes did not change significantly over time and were similar between the 2 treatment groups (placebo and Tat-NR2B9c). The ADC-derived lesion in placebo-treated animals evolved over 120 minutes and was not significantly smaller than CBF-derived lesion volumes starting at 90 minutes after MCAO. The final infarct volume determined at 24 hours by TTC staining was well correlated with both the MRI-derived DWI and PWI lesion volumes at the final imaging time point (180 minutes) in the placebo group. Figure 2 shows representative DWI, PWI, and TTC images for both treatment groups.

In Tat-NR2B9c-treated animals, the ADC lesion volume increased from 25 minutes to 45 minutes after MCAO as in the placebo group. However, at the 70 minutes time point, just after initiation of Tat-NR2B9c, the growth of ADC lesion volume was attenuated in the drug-treated animals. At 120 minutes, the ADC lesion volume in Tat-NR2B9c-treated animals was significantly smaller than in placebo-treated rats. At subsequent time points, the ADC lesion volume remained significantly smaller in the Tat-NR2B9c group as compared with placebo (153.8±108.1 mm³ versus 249.1±45.1 mm³ at 180 minutes). The ADC-derived lesion volume was significantly smaller than the CBF-derived lesion volume at all time points in the Tat-NR2B9c group. TTC-defined infarct volumes were significantly smaller in Tat-NR2B9c-treated animals as compared with placebo-treated animals (183.9±78.7 mm³ versus 263.9±48.7 mm³, P=0.028).

Figure 1. Temporal evolution of DWI- and PWI-derived ischemic lesion volumes and 24 hours TTC-derived final infarct volume. Data are mean±SD. *P<0.05 between placebo- and Tat-NR2B9c-treated animals. †P<0.05 between CBF- and ADC-derived ischemic lesion volumes. DWI indicates diffusion-weighted imaging; PWI, perfusion-weighted imaging; TTC, 2,3,5-triphenyl tetrazolium chloride; CBF, cerebral blood flow; ADC, apparent diffusion coefficient.
Figure 3 indicates the absolute mismatch between CBF- and ADC-derived lesion volumes. Relative to placebo animals, the ADC/CBF mismatch lesion volumes were significantly larger starting at 90 minutes after occlusion in the Tat-NR2B9c group. The region of interest analysis of the relative CBF values in the core and cortical penumbra regions showed no significant change in relative CBF between time points in either treatment group (Supplemental Figure I; http://stroke.ahajournals.org), indicating no effect of treatment on CBF.

Primary neuronal cultures treated with 100 nmol/L Tat-NR2B9c demonstrated no significant difference in cell death or ATP levels compared with saline controls under normoxic conditions (1.08%±0.077% [Tat-NR2B9c] versus 1.00%±0.11% [saline] and 104.0%±6.1% [Tat-NR2B9c] versus 100.0%±5.7% [saline], respectively; Figures 4 and 5). However, pretreatment with 100 nmol/L Tat-NR2B9c resulted in a 26% reduction in cell death as compared with saline treated controls 20 hours after OGD (1.19±0.079 [Tat-NR2B9c] versus 1.61%±0.125% [saline], P=0.0086; Figures 4A and 5). ATP levels were 34% greater in Tat-NR2B9c-treated cultures 1 hour after OGD compared with saline-treated cultures (96.3%±4.9% [Tat-NR2B9c] versus 71.9%±12.8% [saline], P=0.013; Figure 4B).

Discussion

Tat-NR2B9c Is Neuroprotective in pMCAO

Administration of Tat-NR2B9c 60 minutes after MCAO in the rat resulted in a statistically significant reduction in ADC lesion volume of 38% at 180 minutes after MCAO. This
ADC lesion volume. Had Tat-NR2B9c worked by augment-ischemic penumbra evolution and prevented progression of
umbra volume in saline-treated controls. Tat-NR2B9c “froze” of ADC lesion volume and shrinkage of the ischemic pen-
minutes in Tat-NR2B9c-treated animals versus progression stabilization in ADC and ischemic penumbra volume to 180
NR2B9c- and placebo-treated animals to 70 minutes with a
sion of ADC and ischemic penumbra volume in Tat-
and ischemic penumbra volume revealed matched progres-
Tat-NR2B9c-treated animals. Serial measurements of ADC
core and cortical penumbra after MCAO in placebo- or
of tissue at risk or relative CBF value in regions of interest of
Serial measurements CBF demonstrated no change in volume
hypotension would be expected to worsen CBF and stroke
this brief period of hypotension would bias our conclusions as
after the completion of drug administration. It is unlikely that
except for a statistically significant drop in mean arterial
demonstrated no differences in temperature or blood pressure
Physiological measurements in each experimental group
demonstrated no differences in temperature or blood pressure
except for a statistically significant drop in mean arterial
pressure in Tat-NR2B9c-treated animals for 5 minutes
after the completion of drug administration. It is unlikely that
this brief period of hypotension would bias our conclusions as
hypotension would be expected to worsen CBF and stroke
outcome.

Tat-NR2B9c Does Not Affect CBF, Slows Evolution of the Ischemic Penumbra In Vivo, and Decreases ATP Depletion In Vitro
Serial measurements CBF demonstrated no change in volume
tissue at risk or relative CBF value in regions of interest of
core and cortical penumbra after MCAO in placebo- or
Tat-NR2B9c-treated animals. Serial measurements of ADC
and ischemic penumbra volume after revealed matched progres-
sion of ADC and ischemic penumbra volume in Tat-
NR2B9c- and placebo-treated animals to 70 minutes with a
stabilization in ADC and ischemic penumbra volume to 180
minutes in Tat-NR2B9c-treated animals versus progression of
ADC lesion volume and shrinkage of the ischemic pen-
umbra volume in saline-treated controls. Tat-NR2B9c “froze” ischemic penumbra evolution and prevented progression of
ADC lesion volume. Had Tat-NR2B9c worked by augment-
ing blood flow, the penumbra would have shrunk toward the ischemic core.

In addition, Tat-NR2B9c treatment prevented ATP depletion and improved neuronal survival in primary neuronal
cultures exposed to OGD. This supports our hypothesis that Tat-NR2B9c has a neuroprotective rather than a flow aug-
mentation mechanism of action. NOS activation has been implicated in ATP depletion after neuronal ischemia; there-
fore, it is plausible that prevention of NOS activation by Tat-NR2B9c resulted in decreased ATP depletion in these
cultures. An alternate explanation for this observation in-
cludes a mechanism that results in a decrease in ATP consumption in Tat-NR2B9c-treated cells that would artifi-
cially elevate ATP levels; however, a mechanism of this
nature has not been previously observed with Tat-NR2B9c.
Furthermore, if Tat-NR2B9c enhanced cell survival through an alternative mechanism, elevated ATP measurements may
have been the result, rather than the cause of, increased cell
viability with more cells available to recuperate aerobic
respiration and ATP production during the 1-hour normoxia
period after OGD. This is an unlikely explanation because
ATP levels were normalized to intrawell protein content and
cell death in the acute phase of OGD is necrotic in nature and
protein from lyzed cells would have been cleared during a
wash step.

After the onset of cerebral ischemia, there is a rapid drop in
intracellular ATP in neurons and glia that is associated with
disruption of homeostatic mechanisms, failure of cellular
function, and cell death. Regions of diffusion restriction
visualized as areas of hypointensity on ADC maps correlate
spatially and temporally with cerebral ATP depletion after
ischemia onset and can be reversed by restoring blood flow,
oxygen, and glucose. The mechanisms underlying diffu-
sion restriction are not completely characterized; however,
the failure of Na+/K+/2Cl− ATPase pumps, compartmental-
ization of water in astrocytes and endothelial cells, and
neuronal shrinkage have been demonstrated to play a role in
this phenomenon.

ATP depletion results from multiple cellular mechanisms,
including direct effects of nitric oxide and NOS-derived
reactive oxygen species on mitochondrial respiration. In
cerebral ischemia, nitric oxide is produced by NOS after
activation of the neuronal N-methyl-d-aspartate receptor that
is coupled to NOS through the postsynaptic density-95
protein. Disrupting the interaction of N-methyl-d-aspartate
receptor and postsynaptic density-95 protein confers neuro-
protection in models of ischemic neuronal death and in
animal models of stroke by preventing NOS activation and
neurotoxic signaling. Tat-NR2B9c acts by interfering with this
protein–protein interaction and is neuroprotective in
models of excitotoxicity and stroke.

If the observation that Tat-NR2B9c reduces ATP depletion
in vitro translates to in vivo cerebral ischemia, it is likely that
slowed progression of ADC lesion volume and preservation
of the ischemic penumbra is a result of decreased ATP
depletion in the ischemic tissue. The correlation between
ATP level and DWI lesion has previously been documented in
rat brains. Direct measurement of ATP in vivo is
technically difficult; hence, we have not directly tested for

Figure 5. Propidium iodide fluoresce images of cortical cultures 20 hours post-OGD or normoxic control. A, normoxic control with saline; (B) normoxic control with Tat-NR2B9c; (C) 2 hours OGD with saline; and (D) 2 hours OGD with Tat-NR2B9c. Note: Hoechst 33324 (1 µ g per ml) was used for nuclear stain. OGD indicates oxygen–glucose deprivation.
this effect. A strategy to preserve the ischemic penumbra is a potentially viable approach to extend the therapeutic window of reperfusion therapies that requires further study before translation to clinical trials.30

Based on the results of the current study, improvements in experimental stroke outcomes are achievable through neuroprotection even in pMCAO. Given that reperfusion is also an important therapeutic clinical goal, agents such as Tat-NR2B9c may be useful to extend the therapeutic window for reperfusion by slowing the evolution of the ischemic penumbra.

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Disclosures
M.T. is the president and CEO of NoNO Inc, a biotechnology company developed to translate neuroprotectants discovered through cellular and molecular research in his laboratory to patients.

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Supplemental figure 1: Temporal evolution of relative CBF values in (a) core and (b) cortical penumbra in placebo and Tat-NR2B9C treated animals.