Effectiveness of PSD95 Inhibitors in Permanent and Transient Focal Ischemia in the Rat

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Background and Purpose—Postsynaptic density-95 inhibitors reduce ischemic brain damage without inhibiting excitatory neurotransmission, circumventing the negative consequences of glutamatergic inhibition. However, their efficacy in permanent ischemia and in providing permanent neuroprotection and neurobehavioral improvement in a practical therapeutic window is unproven. These were tested here under conditions that included fever, which is a common occurrence in clinical stroke.

Methods—Six studies were performed in unfasted Sprague-Dawley rats. Two involved permanent pial vessel occlusion in male and female rats. Two involved permanent middle cerebral artery occlusion, which induced severe hyperthermia, and 2 involved transient middle cerebral artery occlusion. Animals were treated with a single intravenous injection of postsynaptic density-95 inhibitors (Tat-NR2B9c[SDV] or Tat-NR2B9c[TDV]) 1 hour or 3 hours after stroke. Infarct volumes and neurobehavior were assessed in a blinded manner at 24 hours (pial vessel occlusion and permanent middle cerebral artery occlusion) or at 62 days (transient middle cerebral artery occlusion).

Results—Postsynaptic density-95 inhibitors dramatically reduced infarct size in male and female animals exposed to pial vessel occlusion (>50%), in hyperthermic animals with fever exceeding 39°C exposed to permanent middle cerebral artery occlusion (approximately 50%), and at 62 days poststroke in animals exposed to transient middle cerebral artery occlusion (approximately 80%). Effectiveness of postsynaptic density-95 inhibitors was achieved without the drugs affecting body temperature. In transient middle cerebral artery occlusion, a single dose of postsynaptic density-95 inhibitor given 3 hours after stroke onset permanently maintained reduced infarct size and improved neurobehavior.

Conclusions—Postsynaptic density-95 inhibitors administrated 3 hours after stroke onset reduced infarct volumes and improved long-term neurobehavioral functions in a wide therapeutic window. This raises the possibility that they may have future clinical usefulness. (Stroke. 2008;39:2544-2553.)

Key Words: behavior | cerebral ischemia | hyperthermia | middle cerebral artery occlusion | N-methyl-D-aspartate receptor | postsynaptic density

Despite its significant socioeconomic toll, stroke has not yet been significantly impacted by neuroprotective drug therapy, and thrombolytic agents remain the mainstay of treatment for patients that qualify. Many failed neuroprotectants targeted N-methyl-D-aspartate glutamate receptors (NMDARs), which mediate excitatory neurotransmission in the central nervous system. NMDARs also mediate excitotoxicity and ischemic brain damage, but blocking them has failed as a clinical stroke therapy.1 NMDAR blockers inhibit essential excitatory neurotransmission, producing systemic and psychotropic consequences that limit their usefulness.1 These consequences may be circumvented by blocking neurotoxic NMDAR signaling such as free radical production by neuronal nitric oxide synthase (nNOS) without blocking NMDARs. We recently achieved this by perturbing the interactions of NMDARs with the submembrane scaffolding postsynaptic density-95 (PSD95) protein, which also binds nNOS.2 We then developed PSD95 inhibitors, peptides that disrupt PSD95/NMDAR and PSD95/nNOS interactions.3,4 Treatment with PSD95 inhibitors before or 1 hour after transient middle cerebral artery occlusion (MCAO) significantly reduced infarct volumes at 24 hours.3 However, it

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remains unknown whether they have efficacy in permanent ischemia, whether the neuroprotection is permanent, whether it translates to neurobehavioral improvement, or whether treatment can be instituted in a practical time window poststroke.

To test PSD95 inhibitors as putative neuroprotectants in stroke, we presently examined their efficacy in reducing stroke damage in both permanent and temporary MCAO, in posttreatment paradigms of up to 3 hours poststroke, using histological and neurobehavioral correlates. Although no single study suffices to translate a stroke treatment from rodent studies to humans, our data indicate for the first time that PSD95 inhibitors significantly reduced infarct size even in permanent MCAO compounded by hyperthermia and significantly spared sensory and motor function as well as emotional and cognitive abilities over the long term.

Materials and Methods

Postsynaptic Density-95 Inhibitors

Three synthetic peptides were fused to the cell membrane protein transduction domain of the HIV-1-Tat protein (YGRKKRRQRRR; Tat) to render them cell-permeant 3 (Advanced Protein Technology Centre, Hospital for Sick Kids, Toronto, Ontario, Canada). One comprising the C-terminal 9 residues of the NMDA NR2B subunit, KLSSIESDV, termed Tat-NR2B9c (SDV); a variant comprising KLSSIETDV, termed Tat-NR2B9c (TDV); and the inactive control comprising KLSSIEADA, termed Tat-NR2B9c (ADA). The latter is incapable of binding PSD95 and does not affect excitotoxic vulnerability or infarct size. 3 The peptides were administered intravenously in saline over 4 to 5 minutes by blinded individuals. In some experiments, the selective NR2B subunit blocker, (R,S)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (RO 25–6981) (Sigma; 6 mg/kg in saline), was used as positive control.

Animals

Adult male Sprague-Dawley rats (250 to 300 g; Charles River Laboratories, Sherbrooke, Quebec, Canada) were used according to procedures approved by the institutional animal care committees. All experiments were performed on unfasted animals. Free overnight access to both food and water resulted in higher morning blood glucose levels than in animals that had free overnight access to water but not food (5.50 versus 3.71 mmol/L; n = 12 versus 3.71 ± 0.21 mmol/L; n = 8, respectively; 1, 3 = 9.134; P < 0.001).

Surgical Preparation

For permanent pial vessel occlusion (PVO), rats were anesthetized with 100 mg/kg ketamine, 2 mg/kg acepromazine, and 50 mg/kg xylazine. For permanent and temporary middle cerebral artery occlusion (pMCAO and tMCAO, respectively), anesthesia was induced and maintained with 3.5% and 0.8% halothane, respectively, in 2:1 nitrous oxide and oxygen. Rats were intubated and ventilated (60 strokes/min, tidal volume of 30 to 35 mL). Mean arterial blood pressure, blood gases, pH, and glucose were monitored with a left femoral artery catheter. Drug delivery was through the femoral vein.

Core, Brain, and Temporalis

Temperature Measurements/Control

Core body temperature (CT) was monitored from 20 hours pre- to 24 hours post-pMCAO using intraperitoneal transmitters (Mini-Mitter/Respirotronic). To measure brain temperatures after pMCAO, the skull was exposed and 1-mm holes were drilled bilaterally at (stereotactic coordinates from bregma): anteroposterior 3.3 mm, mediolateral 4.0 mm, dorsal–ventral 3.0 mm. A 19-gauge cannula was inserted into the muscle. Cage temperatures were recorded continuously using an external probe (Tektronix). In some experiments, cage temperature was continuously adjusted based on CT using a feedback-controlled cooling fan (Igloo KoolMate18) activated whenever CT exceeded 37.1°C.

Permanent Distal Middle Cerebral Artery Pial Vessel Occlusion

The right external carotid artery was cannulated with PE10 tubing. The skull was exposed in the midline. A 6- to 8-mm cranial window was made over the right somatosensory cortex (2 mm caudal and 5 mm lateral to bregma). The pial arteries were visualized by injecting 10 to 20 μL of Patent Blue Violet (10 mmol/L; Sigma) in saline into the external carotid artery. The 3 pial arteriolar middle cerebral artery branches were cauterized and dye injections were repeated to ensure the blockage. As described, 3 this produces a reproducible infarction in the cortex underlying the coagulated pial arteries.

Middle Cerebral Artery Occlusion (permanent and transient)

The left middle cerebral artery was occluded by the intraluminal suture method of Longa et al. 8 In brief, a 3–0 monofilament nylon suture tip (0.33 to 0.36 mm diameter, 0.5 to 0.6 mm length) coated with poly-L-lysine was introduced into the internal carotid artery through the external carotid artery, effectively occluding the middle cerebral artery. For tMCAO, the suture was withdrawn after 90 minutes and the external carotid artery ligated. 3 For pMCAO, the suture was left ligated in situ. Sham animals underwent identical procedures without MCAO. All physiological parameters were similar between groups (Supplemental Table 1, available online at http://stroke.ahajournals.org). CT was maintained at 36.8°C to 37.2°C using a heat lamp.

Neurobehavioral Evaluation

After pMCAO, behavioral scoring was performed at 2 hours and 24 hours using the postural reflex and the forelimb placing tests as used previously 3 (normal score, 0; maximal score, 12).

Before term tMCAO experiments, drug effects on baseline motor function (Rotordor performance), 3 arousal/anxiety (novel object exploration), 10 and sensorimotor function (sticky label task) 11 were determined. Rotordor performance was evaluated at both 40 and 220 minutes postinjection (75 mm diameter; Stoelting) at 12 rpm/min.

Time spent exploring a novel object (5×5-cm rubber block) was measured beginning at 150 minutes and, latency to attend to a sticky substance (honey) applied to one forepaw was measured at 180 minutes after drug administration.

A strict postoperative care protocol 12 ensured the long-term survival (62 days) necessary to carry out the neurobehavioral tests described subsequently.

Tests of Physical Outcomes and Sensorimotor Function

All rats were weighed and tested for sensorimotor function over 56 days using a modified 5-task bilateral sensorimotor battery. 13 Performance on each of 11 assessments gave a cumulative deficit score for each rat (maximum unilateral deficit score of 7) scale. On postoperative day (POD) 5, 10, and 15, rats were tested for exploration and number of rotations in an open field arena (5-minute trial).

Motor agility was tested on a horizontal ladder (1.2-m ladder with equi-spaced rungs 2.5 cm apart). Latency (seconds) to reach the home cage and number/type of paw slips were recorded.

On POD 10, rats were tested in a straight alley swim task and scored for head/body angle (0: normal, 1: 45° angle, or 2: 90° angle), forelimb paddling, and escape latency. The apparatus was a 115×20×18-cm fiberglass tank.

Tests of Emotionality

Rats were tested on 3 days (5-minute trial) in an open-field arena to determine state of arousal and ability to habituate to a novel
Figure 1. Effects of ischemia and of PSD95 inhibitors on CT. Animals in A and B were implanted with intraperitoneal telemetric temperature probes and exposed to the indicated conditions. Gray bars indicate the duration of the animal surgery. Ai, Sham procedure (n=4). Aii, Sham procedure with administration of 3 nM/g Tat-NR2B9c(SDV) (n=6). Aiii, PVO (n=5). Aiv, Permanent MCAO without cage cooling (n=8). Av, Permanent MCAO with cage cooling using a feedback system (n=8). Symbols in Ai-Av indicate the means±SEM of the indicated number of animals. Avi, Effects of the indicated conditions on temporalis muscle, core, and brain temperature at the indicated times post-pMCAO (n=6/group). Core-saline, animals treated with saline 1 hour post-pMCAO. Temporalis-saline, concurrent temporalis muscle temperatures from saline-treated animals. Core-SDV, animals treated with 3 nM/g Tat-NR2B9c(SDV) 1 hour post-pMCAO. Temporalis-SDV, concurrent muscle temperature measurements from 3 nM/g Tat-NR2B9c(SDV)-treated animals. B, PSD95 inhibitors do not affect the hyperthermic response after pMCAO. Bi-Bv, CT before, during (gray bars), and after pMCAO surgery in animals treated with the indicated PSD95 inhibitor at the indicated dose. N=8/group.
environment and in the elevated plus maze to assess anxiety/emotionality. The maze consisted of 4 arms (2 open, 2 closed: 15 cm width and 60 cm length) extending from a central platform elevated 1.5 m.

Tests of Cognition
The Barnes Circular Maze is a test of spatial learning and memory. The apparatus was a white circular platform (1.2 m diameter; elevation 1.0 m) with 18 equally sized (9 cm) and spaced holes.
around the perimeter. An escape box was located under one of the holes, its location remaining constant for all trials. The animal had to locate and enter the escape box with bright light and white noise as negative reinforcement. Rats were acclimated to the maze on POD 35 and latency to find the goal box was assessed on POD 36 to 38 (maximum trial latency of 180 seconds). Between trials, the rat remained within the goal box and the maze was rotated to a preselected direction to eliminate the use of odor cues on subsequent trials. Each rat was given 4 trials per day with the goal box remaining constant on each test day. One week later, a retest was administered in which each rat received 4 additional trials with the goal box located at the same position.

On POD 56 to 59, rats were tested for spatial learning and memory in the Morris water maze. A circular tank (1.5 m diameter) filled with water (approximately 21°C) made opaque by nontoxic white paint (Reeves & Poole) was divided into 4 quadrants with a submerged clear Plexiglas escape platform in one quadrant. Extramaze cues were held constant throughout the experiment. Performance was recorded using Ethovision software (Noldus Information Technology Inc). Rats were given 4 trials per day for each of 4 test days (60 seconds per trial, 60-second intertrial interval). A 60-second probe trial was administered 24 hours after the last test day, and time spent in the target quadrant was recorded.

Infarct Volume Evaluation
Twenty-four hours after pMCAO or PVO, the animals were anesthetized, brains were removed, sliced into 8 1-mm thick coronal sections, and incubated for 30 minutes in 2% triphenyltetrazolium chloride (Sigma) in saline at 37°C. For tMCAO, animals underwent transcendocardiac perfusion with heparinized saline followed by 4% wt/vol paraformaldehyde in phosphate-buffered saline. Brains were extracted, processed, and stained with hematoxylin and eosin. Infarct areas were digitally traced on standard templates containing 8 coronal sections and thus corrected for brain edema or shrinkage. Infarct volumes were derived from templates containing 8 coronal sections and thus corrected for saline. Brains were extracted, processed, and stained with hematoxylin and eosin. Infarct areas were digitally traced on standard templates containing 8 coronal sections and thus corrected for brain edema or shrinkage. Infarct volumes were derived from templates containing 8 coronal sections and thus corrected for saline.

Data Analysis
All animal surgeries, drug infusions, behavioral assessments, and infarct volume determinations were performed by individuals blinded to the treatment group. Data are expressed in mean±SEM. Differences were analyzed using analysis of variance (ANOVA) or repeated-measures ANOVA on ranks. The Bonferroni correction was used for pairwise comparisons. In some experiments (as in Figure 5), when comparisons by analysis of variance and Bonferroni revealed no differences between the Tat-NR2B9c(SDV)-treated and saline-treated control groups, those were pooled for comparison with Tat-NR2B9c(SDV) by Student t test.

Results
Effects of Postsynaptic Density-95 Inhibitors on Core Body Temperature
Core temperature was measured in 6 animals from 20 hours pre- to 24 hours postintravenous injection of 3 nM/g of Tat-NR2B9c(SDV), the highest dose used in the present study. Controls underwent sham surgery with a saline infusion (vehicle, n=4). All animals exhibited a mild (approximately 0.5°C) transient (10 to 15 hours) CT elevation after termination of anesthesia. However, Tat-NR2B9c(SDV) had no impact on CTs at any time as compared with shams (Figure 1Ai, ii).

Effect of Permanent Middle Cerebral Artery Occlusion on Core Body, Brain, and Temporalis Muscle Temperature
Spontaneous hyperthermia is recognized in experimental MCAO. For the pMCAO experiments, the occluding fila-
Similarly, Tat-NR2B9c(TDV), a previously untested PSD95 inhibitor, also reduced hemispheric infarction volume by approximately 35% and cortical infarction by 40% to 45% (Figure 3Ai, Aii, C). To test the reproducibility of these findings a second, confirmatory study was performed by a different team blinded to the first study. Similar results were obtained, confirming that both the Tat-NR2B9c(SDV) and Tat-NR2B9c(TDV) peptides have a similar effect in reducing both hemispheric and cortical infarction volumes (Figure 3Bi–ii, C).

**Efficacy of Postsynaptic Density-95 Inhibitor Administered 3 Hours Postischemia Onset**

The pMCAO in saline-treated animals produced severe neurological deficits, which lasted for the entire observation period (composite neurological score ≥11; not shown) and
Figure 4. Effect of Tat-NR2B9c(ADA) treatment 3 hours after tMCAO (90 minutes) onset. A, Dose response study and comparison with the NR2B antagonist Ro 25–6981 (6 mg/kg) infused 30 minutes before (30 minutes PRE) or 3 hours after (3 hours POST) stroke onset. i, Infarct areas at the indicated coronal planes. ii, Infarct volumes 24 hours after ischemia onset. Each bar represents the mean±SEM of the indicated number of animals. Asterisk indicates difference from saline control (P<0.05). B, Infarcts 62 days after tMCAO. i, Infarct areas at 8 coronal planes. ii, The resulting infarct volume. Asterisk indicates different from both saline and Tat-NR2B9c(ADA) controls (ANOVA, F_{2,27}=5.72, P=0.0085). C, Representative hematoxylin and eosin histological sections from each of the treatment groups.
did not improve by 24 hours. The severity of functional impairment and high mortality rate after pMCAO is a limitation that renders long-term studies difficult as recognized by the STAIR committee.20 Because longer poststroke recovery times than 24 hours were necessary to evaluate the PSD95 inhibitors, we carried out additional experiments in a model of transient (90 minutes) MCAO.

We first tested Tat-NR2B9c(SDV) treatment administered 3 hours after ischemia onset. As a control, we used Ro 25-6981, a, selective NR2B antagonist with efficacy against excitotoxicity and oxygen–glucose deprivation in vitro and stroke in vivo.6 Ro 25-6981 (6 mg/kg) was infused intravenously 30 minutes before or 3 hours after stroke onset, whereas Tat-NR2B9c(SDV) was infused at 0.03 to 3 nMol/g over 5 minutes,
starting 3 hours after ischemia onset. Infarct volumes were evaluated at 24 hours. The dose range of Tat-NR2B9c(SDV) was based on the assumption of equal distribution throughout the animal. A dose of 3 nM/g (approximately 3 μmol/L) approximates the IC50 of Tat-NR2B9c(SDV) inhibiting NR2-PSD95 interactions and exceeds the IC50 for inhibiting PSD95-nNOS interactions.\textsuperscript{4}

Tat-NR2B9c(SDV) treatment 3 hours after ischemia onset reduced infarct volumes by 50% or more at all doses tested (ANOVA, \(F=8.087, P<0.001\)). This effect was similar to that obtained with Ro 25–6891 applied 30 minutes before ischemia, but not when Ro 25–6891 was given 3 hours after ischemia. There were no significant differences between infarct volumes obtained with the different doses of Tat-NR2B9c(SDV) (ANOVA, \(F=0.718, P=0.552\)), raising the possibility that even lower doses may have efficacy (Figure 4A\textendash ii).

**Postsynaptic Density-95 Inhibitors Offer Long-Term Neuroprotection and Improve Functional Recovery**

Next, using the 90-minute tMCAO, we determined whether PSD95 inhibitors impart permanent neuroprotection or simply delay damage and whether they improve neurological function. The inhibitors had no neurobehavioral effects of their own, because treatment of naive rats with Tat-NR2B9c(SDV) (0.5, 5, and 50 nM/g) had no effect on Rotorod function or normal excitatory neurotransmission. Consistent with this, in mutant mice lacking PSD95, long-term potentiation or normal excitatory neurotransmission occurs in normal rats,\textsuperscript{21} whereas the controls did not (\(t_{23}=3.68, P=0.001\); Figure 5F). Emotionality was also tested in the elevated plus maze on days 6, 13, and 20, and rats treated with Tat-NR2B9c(SDV), showed reduced latencies to enter the closed arm (Supplemental Table II). Thus, the behavior of Tat-NR2B9c(SDV)-treated rats is closer to that of animals without strokes than to those in the groups with strokes and those treated with vehicle or Tat-NR2B9c(ADA). Retention of memory in response to treatment was measured using the Barnes circular maze\textsuperscript{14} (days 36 to 40) and the Morris water maze\textsuperscript{15} (days 56 to 61). Shorter mean escape latencies approached significance during the acquisition phase of Barnes maze testing in rats treated with Tat-NR2B9c(SDV) (Figure 5G; \(F_{1,28}=3.80, P=0.061\)). On retest (to assess memory), Tat-NR2B9c(SDV)-treated rats performed significantly better than the controls (Figure 5H; \(t_{23}=2.77, P<0.01\)). In the Morris water maze, there were no group differences in the acquisition phase (Figure 5I), but animals injected with Tat-NR2B9c(SDV) had reduced memory deficits as evidenced by increased time in the target quadrant when compared with the combined control groups (Figure 5J; \(t_{23}=2.07, P=0.024\)).

**Discussion**

A neuroprotective agent for stroke should have a clearly determined mechanism of action, have an established molecular target, produce long-term histological and neurobehavioral neuroprotection when administered poststroke in a reasonable time window, and have limited side effects. These conditions appear to be met by PSD95 inhibitors, although the full therapeutic window still remains to be characterized, and additional neurobehavioral studies, including further controls such as sham-operated animals, need to be done. However, unlike NMDAR inhibitors, PSD95 inhibition maintains glutamatergic signaling.\textsuperscript{2,3} In previous studies, genetically disrupting PSD95 in vivo\textsuperscript{22} or with antisense oligonucleotides\textsuperscript{2} or PSD95 inhibitors\textsuperscript{3} has not affected NMDAR function or normal excitatory neurotransmission. Consistent with this, in mutant mice lacking PSD95, long-term potentiation is enhanced, but synaptic NMDA-receptor currents, subunit expression, localization, and synaptic morphology are all unaffected.\textsuperscript{22} This may be an advantage of PSD95 inhibitors over NMDAR blockers, which cause sedation, psychotropic effects, and potential dose-limiting toxicity\textsuperscript{4} in the human situation.

PSD95 inhibitors have a clear mechanism based on a specific and restricted set of molecular targets. We recently demonstrated, using a proteomic and biochemical analysis of the interactions of Tat-NR2B9c(SDV) with all known human proteins that it may bind, that this compound has high specificity, impacting excitotoxicity solely through its interactions with PSD95 and with nNOS.\textsuperscript{4} Although we cannot exclude the possibility that PSD95 inhibitors may also protect neurons through an as-yet uncharacterized off-target effect, all our previous work confirms that when the NMDAR/PSD95 interaction is unaffected, neuroprotection is not achieved.\textsuperscript{2\textendash 4}

It is recognized that presently even drugs that were apparently effective in reducing ischemic damage in animals...
in a posttreatment paradigm have failed in humans. However, past failures enhance, not diminish, the need for future neuroprotection research. To our knowledge, PSD95 inhibitors are the first pharmacological compounds that show efficacy in a posttreatment paradigm even in severe (≥39°C) hyperthermia. Also, they are the first compounds that, while addressing excitotoxic mechanisms, produce permanent neuroprotection when administered hours after the ischemic insult. This justifies a continuing evaluation of this class of compounds for the treatment of stroke.

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Disclosures
None.

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