Trans-activator of Transcription-mediated Delivery of NEP1-40 Protein into Brain Has a Neuroprotective Effect against Focal Cerebral Ischemic Injury via Inhibition of Neuronal Apoptosis

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Background: The Nogo-66 antagonistic peptide (NEP1-40) is a potential candidate for therapeutic intervention of neuronal injury. However, delivery of the proteins across the blood–brain barrier is severely limited by its size and biochemical properties. The current study was designed to evaluate the transducible effects of the trans-activator of transcription (TAT) transduction system for NEP1-40 to cross the blood–brain barrier and to clarify whether intraperitoneal administration of TAT-NEP1-40 could protect cerebral neurons from ischemic injury.

Methods: Adult male Sprague-Dawley rats were submitted to a 120-min focal ischemia and received an intraperitoneal injection of No-TAT-NEP1-40, TAT-NEP1-40, TAT-β-galactosidase, or vehicle. The existence of the proteins in the brain was analyzed with immunofluorescence and Western blot techniques at 6 h after injection. Brain ischemic injury was evaluated by neurologic deficit scores, infarction volumes, terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling staining, and assay of caspase-3 activity.

Results: Western blot analysis and immunofluorescence staining confirmed the presence of TAT-NEP1-40 protein in the brains 6 h after injection. Intraperitoneal injection of TAT-NEP1-40 could attenuate the numbers of terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling–positive cells and activated caspase-3 positive cells, and increase the viability of the cells in the ischemic bounder zone, compared with that treated with No-TAT-NEP1-40, TAT-β-Gal, or vehicle. Furthermore, treatment with TAT-NEP1-40 significantly improved the neurologic outcomes and reduced the size of the infarction in rats.

Conclusions: The results demonstrate that the TAT-NEP1-40 could be efficiently delivered into the rat brains and improve ischemia-induced neurologic outcomes through attenuating cell apoptosis in ischemic brains.

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ACUTE cerebral ischemia–reperfusion injury results in severe neurologic deficit and even leads to death. Finding effective neuroprotective medicines and therapeutic measures that are able to improve prognosis of the patients with stroke has long been a hot field in medical research.[1] Although some of treatment regimens, primarily based on physical therapy, have partly improved the recovery of these patients, there are no drugs available that could effectively repair the brain tissue damage resulting from ischemia or mechanical injury.[2]

Axonal damage is a key pathologic manifestation in a variety of injuries of the central nervous system (CNS), such as stroke and traumatic brain injury. An attractive drug discovery strategy to treat brain injuries is to search for agents that can promote CNS axonal regeneration.[1,3] CNS myelin is a primary source for axonal growth inhibitor in the adult brain, and three proteins, Nogo-A,[4] myelin-associated glycoprotein,[5] and oligodendrocyte myelin glycoprotein,[6] seem to be the main constituents responsible for the inhibition of axonal growth. The identification of the Nogo-66 receptor (NgR),[7] which interacts with one of two Nogo-A inhibitory domains as well as with myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein, has established its crucial role in mediating the inhibitory effects of CNS myelin elements. This discovery provides an unprecedented opportunity to manipulate adult CNS axonal regeneration. Therefore, it is possible that blockage of NgR could promote the functional recovery and reverse the devastating consequences of CNS injuries.[8]

Nogo-A has two inhibitory domains: 66–amino acid region (Nogo-66) and N-terminal region (Amino-Nogo). Further molecular analyses reveal that a 40-residue peptide (Nogo extracellular peptide 1-40 [NEP1-40]) behaves as an antagonist of NgR. The blockage of the Nogo-66 interaction with NgR using NEP1-40 increases functional recovery and axonal regeneration after spinal cord injury.[9,10] Recently, some studies have demonstrated that the interruption of the Nogo-NgR pathway might enhance axonal sprouting and thereby promote the re-

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covery after focal brain injury.[11-13] Although NEP1-40 peptide seems to be a potential candidate for therapeutic intervention after CNS injury, for many years delivery of these therapeutic proteins into the brain parenchyma has been limited because of the obstacle of the blood–brain barrier (BBB). Kilic *et al.*[14] have demonstrated that systemic delivery of neuroprotective protein Bcl-xL does not lead to protection after focal cerebral ischemia. However, systemic administration of recombinant fusion protein of Bcl-xL containing the trans-activator of transcription (TAT) protein transduction domain (PTD) attenuated ischemic brain injury and neuronal apoptosis.[15,16] Therefore, a primary obstacle to clinical use of NEP1-40 is the lack of an efficient, noninvasive method for transporting a biologically relevant amount of the respective protein across the BBB.

It is now known that PTD can serve as a transporter for macro molecular-like full-length proteins to cross cell membrane to cytoplasm or cross BBB to brain parenchyma. PTD-mediated protein transduction technique represents a novel and promising strategy for the therapy of a majority of neurologic diseases, including ischemic stroke.[17,18] It has been demonstrated that fusion proteins containing TAT domain enable them to cross cell membranes and the BBB after systemic administration.[14,17] Intraperitoneal injection of the 120-kd β-galactosidase (β-Gal) protein fused to the TAT protein results in a successful delivery of a biologically active fusion protein in various kinds of tissues, including the mouse brain.[19,20] In fact, several examples of TAT fusion proteins, such as Bcl-xL, glial cell line–derived neurotrophic factor, superoxide dismutase, and X chromosome–linked inhibitor of apoptosis, have been tried and shown evident neuroprotective effects in adult models of cerebral ischemia.[15,21-23]

Because of the potential neuroprotective effects of NEP1-40 protein, the current study was designed to assess the possible beneficial effect of TAT-NEP1-40 fusion protein in focal cerebral ischemic injury. First, to evaluate the transducible effects of the TAT transduction system, the coding sequence of NEP1-40 was cloned into the pTAT-HA vector, and TAT-NEP1-40 fusion protein was expressed in *Escherichia coli*. We then tested the ability of transduction of TAT-NEP1-40 across the BBB. Finally, we investigated whether an intraperitoneal administration of TAT-NEP1-40 fusion protein may functionally alleviate brain injury after transient focal ischemia in rats.

Materials and Methods

Materials

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University in Xi'an, Shaanxi, China, and was conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University. The animals were provided by the Experimental Animal Center of the Fourth Military Medical University and housed under controlled condition with a 12-h light–dark cycle, temperature at $21° \pm 2°C$, and humidity in 60–70% for at least 1 week before drug treatment or surgery. The rats were allowed free access to standard rodent diet and tap water. The pTAT-HA and pTAT-HA-β-galactosidase vectors were kindly provided by Steven F. Dowdy, Ph.D. (Professor, Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, La Jolla, CA). The pTAT-HA vector harbors a T7 polymerase promoter with downstream multiple cloning sites fused with an N-terminal 6-histidine (purified tag) leader followed by the 11–amino acid PTD, minimal transduction domain (residues 47–57 of HIV Tat, -YGRKKRRQRRR-) termed TAT, and a hemagglutinin tag.[24] The bicinchoninic acid protein assay kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). A terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling (TUNEL) kit (*in situ* cell apoptosis detection kit I) and 3,3-diaminobenzidine tetrahydrochloride kit were purchased from Boster Bioengineering Ltd. (Wuhan, China). Anti-6 histidine (6-His) mouse monoclonal antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-cleaved caspase-3 (Asp175) rabbit polyclonal antibody was obtained from Merck KGaA (Darmstadt, Germany). Fluorescein isothiocyanate (FITC)–labeled goat anti-mouse immunoglobulin G (IgG), FITC-labeled goat anti-rabbit IgG, and horseradish peroxidase–labeled goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR). 2,3,5-Triphenyltetrazolium chloride, *N*-dimethylformamide, dimethylsulfoxide, and sodium dodecylsulfate were purchased from Sigma-Aldrich Co. (St. Louis, MO). All of the solvents used for the isolation and purification were of the highest purity available from Tianjin Kemio Co. (Tianjin, China).

Experimental Grouping

To determine the transducible effect of TAT-NEP1-40 in the brain, nine adult male Sprague-Dawley rats weighing 280–320 g were submitted to a 120-min focal ischemia induced by intraluminal thread occlusion of the middle cerebral artery and received intraperitoneal injections of 40 nmol No-TAT-NEP1-40, TAT-NEP1-40, or TAT-β-Gal at the onset of reperfusion. These animals were killed 6 h after injection. Brains were used for the immunofluorescence and Western blot analysis of TAT-β-Gal, No-TAT-NEP1-40, TAT-NEP1-40 transduction (n = 3 per group).

To assess the protective effect of TAT-NEP1-40 fusion against focal cerebral injury, additional animals were randomly assigned to four groups. Intraperitoneal injections of either 4.0 ml phosphate-buffered saline (PBS; 0.1 M) or 40 nmol TAT-NEP1-40, No-TAT-NEP1-40, or TAT-β-Gal dissolved in 4.0 ml PBS (0.1 M) were performed imme-

diately after a 120-min episode of ischemic injuries induced by middle cerebral artery occlusion (MCAO) followed by different durations of reperfusion, according to the indicators to be observed.

In a separate experiment, the physiologic variables in five additional rats weighing 280–320 g were measured during ischemia and up to 30 min after the onset of reperfusion. The animals were anesthetized with 2% isoflurane delivered by a facemask at a flow rate of 2 l/min oxygen. The right femoral artery was cannulated for continuous monitoring of blood pressure and arterial blood sampling. A rectal probe was inserted to monitor core temperature. Arterial blood gases and plasma glucose were measured at the onset of ischemia, 60 min after ischemia, and 30 min after reperfusion.

Expression and Purification of Recombinant Fusion Proteins

The NEP1-40 coding sequence (amino acid residues: -RIYKGVIQAIQKSDEGHPFRAYLESEVAISEELVQKYSNS-) was amplified from the Nogo-A complementary DNA (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) by polymerase chain reaction with the primer pairs 5′-CTCCATGGTTAGGATATACAAGGGTGTGATC-3′ and 5′-ACCTCGAGTCAAGAATTACTGTACTTCTGAAC-3′ (the underlined were *Nco*I and *Xho*I sites, respectively; the polymerase chain reaction cycling program was 94°C for 5 min for degeneration in advance, 25 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 30 s, and finally, 72°C for 10 min for elongation). Amplified fragments were digested with *Nco*I/*Xho*I and cloned into the corresponding sites of pTAT-HA vector (kindly provided by Steven F. Dowdy, Ph.D.). Therefore, the final expression plasmid pTAT-NEP1-40 was generated. To prove the transduction of TAT fusion protein, the pNo-TAT-NEP1-40 plasmid as a control protein was obtained from pTAT-NEP1-40 by cutting down TAT sequence with BamH1 digestion (due to the presence of two *BamH*I restriction sites at both ends of TAT sequence). The plasmids pTAT-NEP1-40 and pNo-TAT-NEP1-40 were identified by automated DNA sequencing. To prove the function of TAT-NEP1-40, TAT-β-Gal was expressed as a control protein (the pTAT-β-Gal vector was also kindly provided by Steven F. Dowdy, Ph.D.). All expression cassettes contain a sequence encoding six consecutive histidine residues as purification tag. The fusion proteins were expressed and purified following the protocol in literature.[25] Briefly, TAT-NEP1-40, No-TAT-NEP1-40, and TAT-β-Gal were expressed in *E. coli* BL21(DE3)pLysS (Novagen, Merck KGaA, Darmstadt, Germany) bacteria strain with 1 mM isopropylthiogalactoside induction. The harvested bacteria were lysed by sonication. The inclusion bodies containing TAT-NEP1-40 and No-TAT-NEP1-40 in the *E. coli* lysates were denatured in 6 M guanidine hydrochloride before being purified with the Ni^{2+}-nitrilotriacetic acid affinity column (Qiagen, Hilden, Germany). The fusion proteins TAT-

NEP1-40 and No-TAT-NEP1-40 were refolded by dilution–dialysis method.[24,25] TAT-β-Gal present in the supernatant of bacterial lysate was purified by the Ni^{2+}-nitrilotriacetic acid affinity column.[24,25] The concentrations of proteins were determined by the bicinchoninic acid protein assay kit and stored in 10% glycerol–PBS at −80°C until use.

Establishment of Transient Focal Cerebral Ischemia

Transient focal cerebral ischemia was induced by MCAO in the rats as described in our previous study.[26,27] Briefly, the rats were fasted for 12 h but were allowed free access to water before surgery. Anesthesia was induced with 4% isoflurane and was maintained with 2% isoflurane delivered by a mask. The right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were ligated proximally. A 3-0 nylon monofilament suture (Ethicon Nylon Suture; Ethicon Inc., Osaka, Japan) with its tip rounded by heating near a flame was inserted through an arteriectomy in the common carotid artery just below the carotid bifurcation and then advanced into the internal carotid artery approximately 17–18 mm distal to the carotid bifurcation until a mild resistance was felt, thereby occluding the origins of the anterior cerebral artery, the middle cerebral artery, and the posterior communicating artery. Reperfusion was accomplished by withdrawing the suture after 120 min of ischemia. The incision sites were infiltrated with 0.25% bupivacaine hydrochloride for postoperative analgesia. Rectal temperature was monitored with a probe (Spacelabs Medical Inc., Redmond, WA) and maintained at 37.0°–37.5°C by surface heating and cooling during surgery, which usually lasts for 10–15 min.

Western Blotting Analysis of TAT-NEP1-40 Transduction into Brain

Brains were dissected, complemented with lysis buffer, homogenized, and centrifuged, and supernatants were used for sodium dodecylsulfate–polyacrylamide gel electrophoresis. Equal amounts of protein were diluted in 7× sample buffer, boiled, and loaded onto 15% polyacrylamide gels. Proteins were transferred onto polyvinyl diflouride membranes. Membrane was incubated in blocking solution containing 0.05% Tween-20 and 5% nonfat dry milk for 1 h, and immersed with monoclonal mouse antibody against 6-His (1:2,000) in antibody dilution buffer (Tween-20 containing 1% nonfat dry milk) for 2 h in room temperature. The membrane was rinsed, incubated in horseradish peroxidase–labeled goat anti-mouse IgG (1:12,500) for 1 h in room temperature, washed, immersed in electrochemiluminescent reagent, and exposed to Electrochemiluminescence-Hyperfilm (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence Analysis of TAT-NEP1-40 Transduction into Brain

For analysis of TAT-NEP1-40 transduction, after 6-h injection intervals, the rats were perfusion fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) during deep anesthesia. The brains were removed and were postfixed in the same fixatives for 3 h and immersed in 20% sucrose in 0.1 M phosphate buffer at 4°C overnight. Sagittal sections thickened in 14 μm were cut with a Leica CM1800 Cryostat (Leica Microsystems, Wetzlar, Germany). The sections were washed three times for 5 min for each in PBS and immersed in PBS containing 0.3% triton and 10% normal goat serum for 30 min. After three further risings in PBS, sections were incubated overnight at 4°C with monoclonal mouse antibody against 6-His (1:1,000) dissolved in PBS containing 0.3% triton and 2% normal goat serum. On the next day, sections were washed 3 times 5 min for each in PBS and incubated for 2 h with FITC-conjugated anti-mouse IgG (1:500) diluted with PBS. After 3 further rinses in PBS, the distribution of fluorescence was analyzed under a BX-60 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Neurobehavioral Evaluation

After withdrawing the suture and recovery from anesthesia, the rats were put back to their cages with free access to food and water. Twenty-four hours after reperfusion, the animals were neurologically assessed by an investigator who was unaware of the animal grouping. A six-point scale modified from that described by Longa *et al.*[28] was used for neurologic assessment: 0 = no deficit; 1 = failure to extend left forepaw fully; 2 = circling to the left; 3 = falling to the left; 4 = no spontaneous walking with a depressed level of consciousness; 5 = dead.

Measuring Infarction Volume by 2,3,5-Triphenyltetrazolium Chloride Staining

Twenty-four hours after reperfusion, the rats were reanesthetized with 4% isoflurane in oxygen and decapitated. The brains were rapidly removed and cooled in iced saline for 10 min. Six 2-mm-thick coronal sections were cut with the aid of a brain matrix. Sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min Unstained areas were defined as infarct and measured by using Adobe Photoshop 7.0.1 (Adobe Systems Incorporated, San Jose, CA). The demarcation between infarcted and noninfarcted tissue was outlined, and the area of infarction in the section was calculated by subtracting the area of the nonlesioned ipsilateral hemisphere from the total area of the contralateral hemisphere. The total volume of infarction was calculated by integration of the lesion areas from all six sections measured.

TUNEL Staining

After 12 h of reperfusion, nuclear damage after ischemia was assessed *in situ* by TUNEL staining. The protocol for detecting DNA fragmentation was based on the method by Wang *et al.*[29] with slight modification for use in frozen sections. Briefly, air-dried sections were postfixed in 4% paraformaldehyde at room temperature for 15 min and washed twice in 50 mM Tris-Cl/NaCl on ice. After the sections were exposed to 2% H_2O_2 in methanol to block the endogenous peroxidase activity and washed three times for 2 min with TBS, the enzyme reaction was conducted at 37°C for 2 h with the following reaction mixture: 1 μl terminal deoxynucleotidyl transferase, 1 μl Digit-d-UTP, and 18 μl terminal deoxynucleotidyl transferase labeling buffer using a total volume of 20 μl for each section. After blocking with 2% bovine serum albumin for 30 min, sections were incubated with biotinylation-labeled Digit Ab (1:100) at room temperature for 30 min and then washed with TBS three times. Sections were subsequently incubated with streptavidin–biotin complex (1:100) at 37°C for 1 h and then washed with TBS five times, 4 min for each. Finally, the sections were incubated in 3,3-diaminobenzidine and counterstained with hematoxylin. In the TUNEL staining studies, the results were quantitatively evaluated with the method described by Wang *et al.*[29] Briefly, 32 pixels of 0.10 mm^2 were placed by light microscope with 100× magnification; 16 pixels were from the boundary zone of middle cerebral artery and anterior cerebral artery, and the others were from that of middle cerebral artery and posterior cerebral artery. Then, the total number of positively stained cells in these pixels was counted and expressed as cells/mm^2.

Immunofluorescence Analysis of Caspase-3 Activation

For analysis of activated caspase-3, after 12 h of reperfusion, the rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) during deep anesthesia. The brains were removed and were postfixed for 4 h in 4% paraformaldehyde in 0.1 M PBS and immersed in 20% sucrose in 0.1 M PBS at 4°C overnight. Sagittal 14-μm sections were cut with a Leica CM1100 Cryostat. The sections were washed three times for 5 min in 0.1 M PBS and immersed for 30 min in 0.1 M PBS containing 0.3% triton and 10% normal goat serum. After three further rinses in 0.1 M PBS, sections were incubated overnight at 4°C in PBS containing 0.3% triton, 2% normal goat serum, and rabbit anti-cleaved caspase-3 (Asp175) antibody (1:1,000). Subsequently the sections were washed three times for 5 min in 0.1 M PBS and incubated for 2 h in 0.1 M PBS containing 2% normal goat serum and FITC-conjugated anti-rabbit second antibody, diluted to 1:500. After three further rinses in PBS, caspase-3 activation was evaluated under a BX-60 fluorescence microscope. The number of activated caspase-3–positive cells
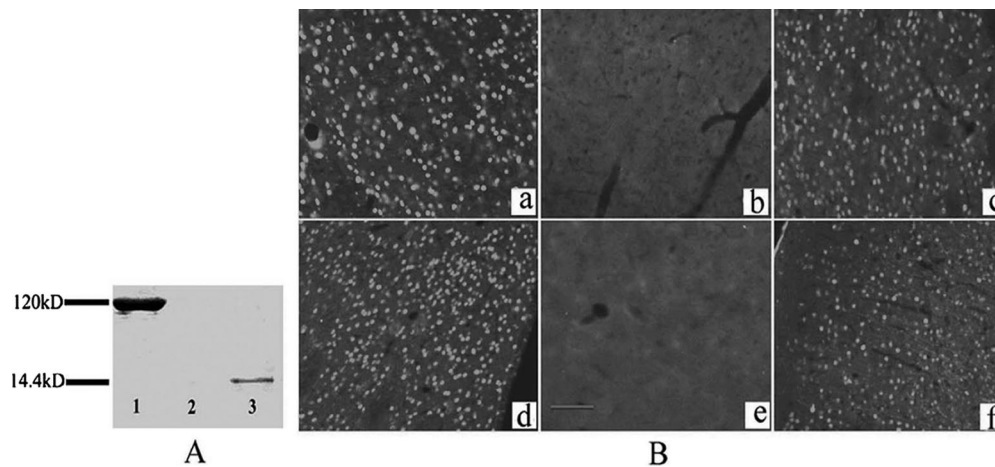
Fig. 1. Western blotting and immunofluorescence analysis of NEP1-40 transduction in the brains of TAT-β-Gal–, No-TAT-NEP1-40–, and TAT-NEP1-40–treated animals (n = 3). (*A*) Western blotting shows the high levels of β-Gal protein at approximately 120-kd position in the TAT-β-Gal–treated animals (*lane 1*) and NEP1-40 protein at 14.4-kd position in the TAT-NEP1-40–treated animals (*lane 3*). *Line 2* is the blotting result of No-TAT-NEP1-40–treated animals. (*B*) Representative microphotographs showing the immunofluorescence staining in the brains of TAT-β-Gal (*a* and *d*)–, TAT-NEP1-40 (*c* and *f*)–, and No-TAT-NEP1-40 (*b* and *e*)–treated animals. *Scale bar* = 100 μm; same magnification is used in all parts of *B*. *a*, *b*, and *c* = the ipsilateral hemisphere to ischemia; *d*, *e*, and *f* = the contralateral hemisphere to ischemia. β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.

was quantified as described by Wang *et al.*[29] and expressed as cells/mm^2.

Statistical Analysis

Brain sections were examined by two independent and blinded investigators. The software, SPSS 11.0 for Windows (SPSS Inc., Chicago, IL), was used to conduct statistical analyses. All values, except for neurologic scores, are presented as mean ± SD. The physiologic data were analyzed using one-way analysis of variance with repeated measures. Differences in physiologic variables between groups were analyzed using analysis of variance with *post hoc* Student–Newman–Keuls test. The infarct volumes and the numbers of TUNEL-positive cells, viable cells, and activated caspase-3–positive cells were analyzed by one-way analysis of variance, and between-group differences were detected with *post hoc* Student–Newman–Keuls test. The neurologic deficit scores were expressed as median (range) and were analyzed with Kruskal–Wallis test followed by Mann–Whitney *U* test with Bonferroni correction. Values of $P < 0.05$ were considered as statistically significant.

Results

Assessment of In Vivo Transduction of TAT-NEP1-40 Fusion Protein into Rat Brains

As shown in figure 1A, Western blotting showed a visible band at 14.4-kd in TAT-NEP1-40–treated animals 6 h after reperfusion. Similarly, TAT-β-Gal was detected as well in TAT-β-Gal–treated animals. However, no protein transduction was detectable in the animals treated with No-TAT-NEP1-40. Concomitantly, existence of TAT-NEP1-40 in the brain was also demonstrated by using immunofluorescence. As shown in figure 1B, immunofluorescent staining revealed that TAT-β-Gal and TAT-NEP1-40 fusion proteins were seen in the brain, whereas no fluorescence signals were detected in the brain of rats treated with No-TAT-NEP1-40. These results indicate that TAT-mediated NEP1-40 fusion was transduced into brains.

Physiologic Variables

Physiologic variables are summarized in table 1. There were no significant differences for the variables among different time points (at the onset of ischemia, 60 min

Table 1. Physiologic Variables (n = 5)

	T, °C	MAP, kPa	Arterial Blood Gases			
			pH	Pao$_2$, kPa	Paco$_2$, kPa	Glucose, mm
Baseline	37.3 ± 0.38	14.7 ± 1.1	7.39 ± 0.06	32.3 ± 5.9	5.2 ± 0.6	6.32 ± 0.63
60 min ischemia	37.2 ± 0.36	14.5 ± 0.9	7.36 ± 0.05	31.1 ± 7.5	5.7 ± 0.8	6.81 ± 0.70
30 min reperfusion	37.2 ± 0.32	14.6 ± 1.1	7.34 ± 0.04	30.5 ± 8.1	6.1 ± 1.0	6.87 ± 0.75

Physiologic data were obtained from five animals at each time point. Variables are presented as mean ± SD. Between–time point analysis did not detect significant differences.

MAP = mean arterial pressure; Paco$_2$ = arterial carbon dioxide partial pressure; Pao$_2$ = arterial oxygen partial pressure; T = rectal temperature.

Table 2. Neurologic Deficit Scores in Four Groups (n = 8)

Group	Neurologic Deficit Score						Median (Range)
	0	1	2	3	4	5	
Control	0	1	5	2	0	0	2 (1–3)
TAT-NEP1-40	2	5	1	0	0	0	1 (0–2)*
No-TAT-NEP1-40	0	2	4	2	0	0	2 (1–3)
TAT-β-Gal	0	1	6	1	0	0	2 (1–3)

Values of neurologic score are presented as median (range). Note that treatment with TAT-NEP1-40 significantly improved the neurologic deficit score, as compared with that in control group ($P < 0.01$).

* $P < 0.01$ *vs.* control group.

β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.

after ischemia, and 30 min after reperfusion). Arterial blood gases (partial pressure of oxygen, partial pressure of carbon dioxide, pH), mean arterial pressure, body temperature, and plasma glucose remained in the normal range during the experimental period observed.

Neurologic Assessment

The neurologic deficit scores of four groups are shown in table 2. Twenty-four hours of treatment with TAT-NEP1-40 improved the neurologic deficit scores to 1 (0–2), significantly different from 2 (1–3) in the control, No-TAT-NEP1-40, and TAT-β-Gal groups ($P = 0.004$, 0.010, and 0.004, respectively). There were no statistical differences in the neurologic deficit scores among latter three groups.
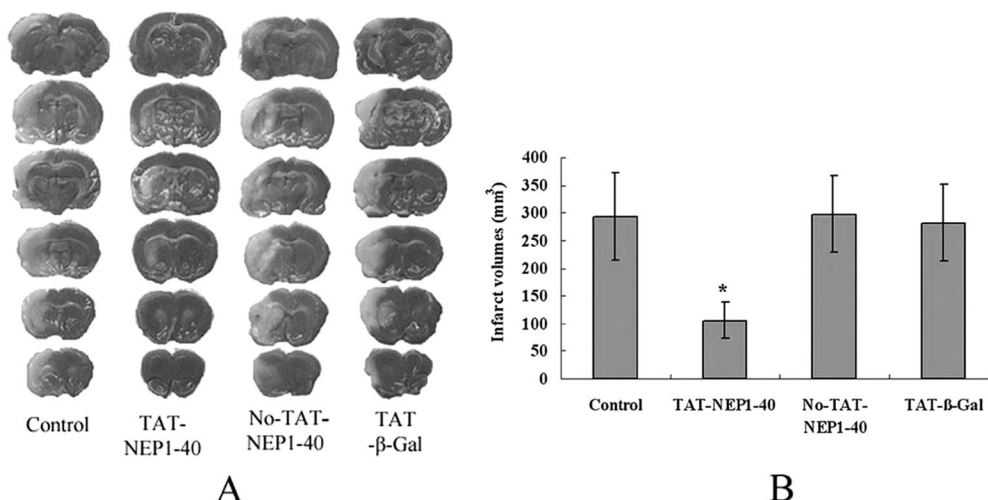
Infarction Volume

Focal cerebral ischemia was produced in 24 rats by transient MCAO. All animals were subjected to 120 min of ischemia and 24 h of reperfusion. The areas of MCAO-induced ipsilateral cerebral infarction were determined by loss of triphenyltetrazolium chloride staining (fig. 2A). The infarct volumes of four groups are shown in figure 2B. There was a significant reduction in infarction size in the rats that received TAT-NEP1-40 treatment in comparison with the PBS–, No-TAT-NEP1-40–, and TAT-β-Gal–treated rats ($P < 0.01$ for each comparison). There were no differences in infarction volume among the control, No-TAT-NEP1-40, and TAT-β-Gal groups.

TUNEL Staining

Ischemia-induced brain injury and the neuroprotective effect of TAT-NEP1-40 treatment were also assessed by observation of the level of cell apoptosis in the brain with TUNEL staining and hematoxylin counterstaining. As shown in figure 3, TUNEL staining was negative in the brain sections at the contralateral hemisphere of ischemic groups and in the whole brain of sham animals at 12 h after reperfusion (data no shown). However, the TUNEL staining was strong in the ipsilateral hemisphere of control group 12 h after reperfusion (figs. 3A and E). TUNEL-positive cells were distributed in the cerebral cortex and dorsal caudate. A large number of stained cells in the ischemic bounder zone of rat brain were seen in the No-TAT-NEP1-40 (figs. 3C and G) and TAT-β-Gal groups (figs. 3D and H), whereas in contrast, only small TUNEL-positive cells in the TAT-NEP1-40–treated group were observed (figs. 3B and F). The treatment with TAT-NEP1-40 significantly reduced the number of TUNEL–positive cells at the time 12 h after reperfusion, compared with PBS–, No-TAT-NEP1-40–, and TAT-β-Gal–treated animals ($P < 0.05$). There was no difference among the latter three groups. On the other hand, the number of viable cells was significantly increased in

Fig. 2. Reduction of infarct volumes by TAT-NEP1-40 in middle cerebral artery occlusion (MCAO)–induced focal cerebral ischemic injury brain injury was assessed by 2,3,5-triphenyltetrazolium chloride staining. (*A*) Representative 2,3,5-triphenyltetrazolium chloride–stained sections outlining the infarcted areas in rats of four groups. Unstained areas were defined as infarct. (*B*) The bar graph showing the statistical analysis for infarct volumes in four groups (n = 8). Note that TAT-NEP1-40 significantly reduces the infarct volume. Values given are mean ± SD. * $P < 0.05$ *versus* control group. β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.
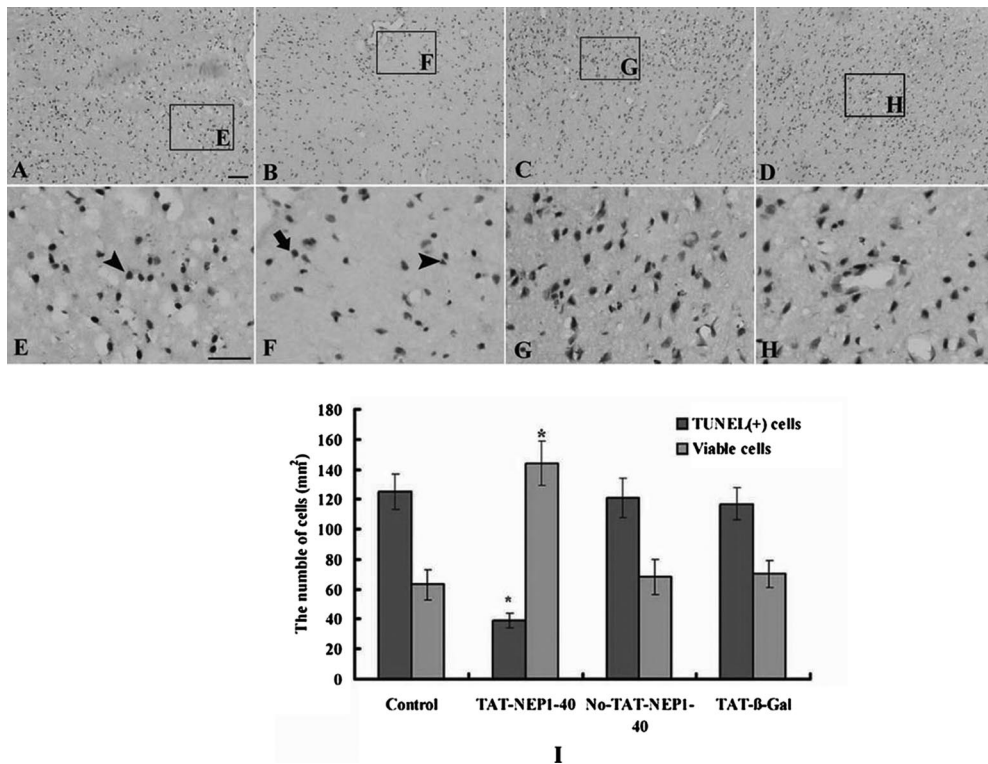
Fig. 3. Attenuation of neuronal apoptosis by TAT-NEP1-40 in middle cerebral artery occlusion (MCAO)–induced brain injury was assessed by terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling (TUNEL) staining and hematoxylin counterstaining (n = 5). (*A–H*) Representative photomicrographs of TUNEL staining in the ischemic boundary zone. Many TUNEL-positive cells are shown in the ischemic boundary zone of the control group at 12 h of reperfusion (*A* and *E*). A large number of stained cells in the ischemic bounder zone of rat brain were seen in the No-TAT-NEP1-40 (*C* and *G*) and TAT-β-Gal groups (*D* and *H*), whereas only a small number of TUNEL-positive cells (*B* and *F*) in the TAT-NEP1-40–treated group was observed. *E*, *F*, *G*, and *H* are higher magnification for part of *A*, *B*, *C*, and *D*, respectively. The *arrow* indicates viable cells, and the *arrowhead* indicates TUNEL-positive cells. *Scale bars* = 100 μm; same magnification is used in *A–D*; same magnification is used in *E–H*. (*I*) The bar graph showing quantitative analysis of the number of TUNEL-positive cells and viable cells in the ischemic area of rats in four groups. Note that TAT-NEP1-40 significantly reduces the number of TUNEL-positive cells and increases the number of surviving cells. Values are given as mean ± SD. * $P < 0.05$ *versus* control group. β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.

TAT-NEP1-40 in comparison with PBS–, No-TAT-NEP1-40–, and TAT-β-Gal–treated animals ($P < 0.05$), as revealed by hematoxylin staining, but there was no difference among the latter three groups (fig. 3I).
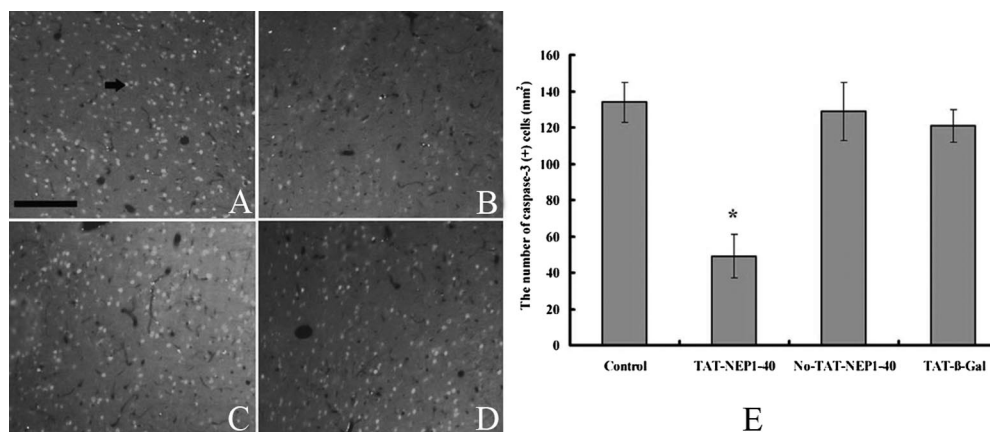
Activity of Caspase-3

As shown in figure 4, immunohistochemistry analysis revealed that caspase-3 immunofluorescent cells were distributed primarily in the border zone of cortical infarction at 12 h after reperfusion. In ischemic animals treated with the vehicle (fig. 4A), No-TAT-NEP1-40 (fig. 4C), and TAT-β-Gal (fig. 4D), a considerable number cells immunoreactive to activated caspase-3 were found in the border zone of cortical infarction 12 h after reperfusion. In contrast, the amounts of caspase-3 immunofluorescent cells greatly decreased in animals treated with TAT-NEP1-40 ($P < 0.01$; fig. 4B).

Discussion

In the current study, by using a recombinant technique, we produced a biologically active NEP1-40 fusion protein containing the TAT-PTD that allows an *in vivo* delivery of the fusion protein across the BBB. We found that when TAT-NEP1-40 was injected intraperitoneally, the protein was efficiently transduced into brain parenchyma within 6 h. Furthermore, the entrance of TAT-NEP1-40 to brain significantly decreased the infarct sizes and improved the neurologic outcomes 24 h after reperfusion followed by 120 min of focal ischemia. The ischemia-induced neuronal apoptosis and activity of caspase-3 in the brain were also markedly attenuated by treatment with TAT-NEP1-40. These results provide the first evidence that the engineered TAT-NEP1-40 protein transduced into the brain still keeps biologic activity and protects the brain against ischemia–reperfusion injury through inhibition of neuronal apoptosis.

The NgR is thought to be the primary target of Nogo-66 action, though additional receptor molecules might interact with the other Nogo-A domains. Studies have demonstrated that NgR is also a functional receptor for two other known myelin-derived axonal outgrowth inhibitors, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein. Therefore, NgR is a central

Fig. 4. Inhibition of caspase-3 activation by TAT-NEP1-40 in middle cerebral artery occlusion (MCAO)–induced brain injury was determined by immunofluorescence staining (n = 5). (*A–D*) Representative immunofluorescent images for demonstrating the activated caspase-3–positive cells in the border of the infarct area of rats in each group. Many activated caspase-3–positive cells are seen in the control group (*A*). A large number of activated caspase-3–positive cells were found in the No-TAT-NEP1-40 (*C*) and TAT-β-Gal groups (*D*), whereas only small number of activated caspase-3–positive cells was seen in the TAT-NEP1-40–treated group (*B*). *Scale bar* = 100 μm; same magnification is used in *A–D*. The *black arrow* indicates activated caspase-3–positive cells. (*E*) The bar graph showing the quantitative analysis of the number of activated caspase-3–positive cells in the border of the infarct area of rats in four groups. Note that the number of activated caspase-3–positive cells after MCAO increased remarkably in the control group, whereas the number of positive cells of the activated caspase-3 in the same brain areas was reduced by TAT-NEP1-40 treatment ($P < 0.01$). Values are given as mean ± SD. * $P < 0.05$ *versus* control group. β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.

point for the convergence of three myelin inhibitors. Research on NEP1-40 peptide has provided evidence that CNS axon regrowth can be promoted by manipulating the inhibitory components of myelin. Because the NEP1-40 peptide specifically blocks the Nogo-NgR interaction, it seems that the peptide might be a potential candidate for therapeutic intervention after CNS injury.[8,30] However, a primary obstacle for clinical use of NEP1-40 is the lack of an efficient, noninvasive method for delivery of this protein across the BBB in biologically relevant amounts. It would be of great importance to find out specific measures that transfer this biologically active protein across the BBB into the brain.

It is well known that the delivery of therapeutic proteins into tissues and across the BBB is severely limited by the size and biochemical properties of the respective proteins. Therefore, therapeutic compounds, peptidyl mimetics, and proteins may pass cell membranes only when the molecules are small, *i.e.*, less than 600 Da. Diffusion of bioactive peptides across the BBB is also restricted to short ($\leq$ 6 amino acids) and highly lipophilic peptides. As mentioned previously, cerebral ischemia induces disruption of the BBB, allowing large molecules including proteins to enter the brain. However, this disruption occurs after several hours of ischemia, whereas the therapeutic time window for most neuroprotective agents is less than 6 h. Therefore, to be more effective to achieve its therapeutic action, such neuroprotective agents must cross the BBB when it is still "intact." Interestingly, it has been demonstrated that the PTD of TAT protein is a promising technique for disease therapy because of its ability to deliver a variety of full-length protein–peptides through the plasma mem-

brane into cells and, most importantly, across the BBB into brain tissue *in vivo*.[20,30,31] In this study, the transduction capability of TAT-NEP1-40 to the brain was evaluated in a rat model of focal cerebral ischemia by using Western blotting and immunohistochemical techniques. Our results showed that a translocation of TAT proteins into brain tissue including the ischemic and the contralateral hemisphere within 6 h after an intraperitoneal injection of TAT-NEP1-40 or TAT-β-Gal. Similar results were reported by other researchers, in whose studies 120-kd β-Gal protein and FITC peptide fused to TAT protein were injected intraperitoneally.[19,20] They found a successful delivery of TAT-FITC peptide and TAT-β-Gal into various tissues in mice, including the brain. Taken together, these studies suggest that these TAT fusion proteins not only rapidly enter the brain parenchyma, but also keep their biologic activity, confirming that this method is effective for the therapy of brain diseases.

Recently, TAT-mediated delivery of proteins with therapeutic potential has been achieved in several models of nerve trauma and ischemia. Several groups have published positive results using PTD for the delivery of therapeutic proteins in relevant animal models of human ischemic stroke. Intravenous delivery of several TAT fusion proteins, such as Bcl-xL, glial cell line–derived neurotrophic factor, superoxide dismutase, and X chromosome–linked inhibitor of apoptosis and intraperitoneal administration of PTD-Bcl-xL fusion protein, efficaciously reduced brain injury in adult models of cerebral ischemia.[16,21,32] Moreover, some studies have demonstrated that the blockade of the Nogo-NgR pathway can enhance axonal sprouting and thereby promote recovery after focal brain infarction.[11–13,33,34] In rats with

MCAO, both motor skills and corticofugal axonal plasticity are promoted by intracerebroventricular administration of a functional NgR blocking fragment. Behavioral improvement is achieved when the therapy is initiated 1 week after arterial occlusion.[12] Recently, after distal MCAO in stroke-prone renovascular hypertensive rats, the expression of Nogo-A in oligodendrocytes persistently increased, and its localization became redistributed around damaged axons and dendrites. Intracerebroventricular administration of NEP1-40 down-regulated the expression of Nogo-A, reduced axonal injury, and enhanced axonal regeneration, suggesting that inhibition of Nogo-A/NgR signal using NEP1-40 can reduce neuronal damage in the thalamus after distal MCAO.[35] Similarly, NEP1-40 promoted regrowth of lesioned entorhinohippocampal axons *in vitro* by blocking of Nogo-66/NgR signaling.[36] Summarized from above, Nogo-66 and NgR have a central role in limiting axonal regeneration after CNS injury, and interruption of the Nogo-NgR pathway can enhance functional recovery and axonal regeneration. As a matter of course, the NEP1-40 is a potential therapeutic agent for neuronal damage.

The death-suppressing effect of TAT-NEP1-40 fusion proteins was initially confirmed in cultures of PC12 cells in our previous study, in which TAT-NEP1-40 but not TAT-β-Gal inhibited oxygen and glucose deprivation-induced apoptotic death (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org). Because of its cytoprotective effects, we chose to study the effects of TAT-NEP1-40 fusion protein *in vivo* in the current study. In a highly reproducible focal cerebral ischemia model induced by MCAO, we demonstrated that intraperitoneal administration of TAT-NEP1-40 at a dose of 40 nmol resulted in reduction of infarct sizes and improvement of neurologic function 24 h after focal ischemia and reperfusion. This efficacy likely results from the direct protection by TAT-NEP1-40, because the protection occurred without altering body temperature or other physiologic parameters during or after ischemia. At 12 h after reperfusion, the TUNEL labeling appeared intense and abundant, whereas the intensity of hematoxylin staining was significantly reduced in these damaged structures as visualized by TUNEL staining and counterstained with hematoxylin staining, indicating a decreased viability of the ischemic cells as a result of the ischemic injury. Intraperitoneal administration of TAT-NEP1-40 at the onset of reperfusion significantly reduced the number of TUNEL-positive cells and increased the viability of the ischemic cells in ischemic areas after 12 h reperfusion, indicating that the protective effect of TAT-NEP1-40 against ischemic injury may be mediated by an antiapoptotic mechanism.

Activation of caspase-3 is a key step in the apoptosis pathway.[37] Decreased apoptosis was observed in caspase-3 knockout mouse or treatment with caspase-3 inhibitors (Z-VAD-FMK),[38] whereas increased caspase-3 activity has been demonstrated in transient and permanent focal ischemia. In a rat focal ischemia model, caspase-3 enzymatic activity in the penumbral cortex peaked at 6–12 h after ischemia.[39] Consistent with other reports, the results from the current study showed that ischemia induced an increase in caspase-3 activity, whereas treatment with TAT-NEP1-40 reduced the caspase-3 activity at 12 h after reperfusion. The reduction of caspase-3 activity could be attributed to an overall reduction of neuronal injury, thereby reducing the amount of toxic agent that induces caspase-3 activation, but not direct inhibition of the enzyme. Therefore, we deduce that TAT-NEP1-40 transferred into brain parenchyma exerts its neuroprotective effect through reduction of brain damage and as a consequence, caspase-3 activity is decreased.

Previous studies have demonstrated that cellular expression of Nogo-A and NgR are significantly up-regulated in the ischemic cortex where neuronal damage occurs, suggesting a role of Nogo-A and NgR in cerebral pathologic damage.[40] When Nogo-A binds and activates NgR, NgR is then coupled with P75 neurotrophin receptor. Activation of the NgR–P75 leads to activation of the Rho and Rho kinase pathway, which induces caspase-3 activation and results in neurons apoptosis, suggesting an important role of the complex signaling of NgR pathway in ischemia-induced neuronal apoptosis.[41] Although we did not explore the detailed mechanism underlying the protective effect of TAT-NEP1-40 observed, we speculate that blockage of the interaction of Nogo-A with NgR using TAT-NEP1-40 might inhibit the activation of the NgR–P75 complex, suppress the downstream effecter RhoA activation, and reduce caspase-3 activity and neuronal apoptosis through a variety of downstream effectors.

In the current experiment, protection of brain tissue was seen when NEP1-40 was conjugated with TAT and administered after ischemia. The result demonstrates that the TAT system as a carrier is a powerful tool for delivery of curative protein into the brain. Because the manipulation of the method is simple and the result is elegant, no surgical interventions (such as trephination of the skull) are required, and the proteins reliably reach their site of action. Therefore, fusion proteins may be a useful tool in transporting large molecules across the BBB.

In conclusion, the current study shows that intraperitoneal delivery of TAT-NEP1-40 has a neuroprotective effect on focal cerebral ischemic injury. The mechanism might be associated with attenuation of caspase-3 activity and neuronal apoptosis. Our experimental results also indicate that the TAT-mediated protein transduction can be used to deliver biologically active neuroprotective agents across the BBB, providing a potential application in treatment of stroke, traumatic brain injury, and other neurologic diseases. However, the long-term effect of TAT-NEP1-40 on brain plasticity after focal ischemia

deserves further investigation, and the associated molecular mechanisms underlying the TAT-NEP1-40 neuroprotective effect need to be further explored.

References

1. Green JB: Brain reorganization after stroke. Top Stroke Rehabil 2003; 10:1–20

2. Ziemann U, Meintzschel F, Korchounov A, Ilic TV: Pharmacological modulation of plasticity in the human motor cortex. Neurorehabil Neural Repair 2006; 20:243–51

3. Wieloch T, Nikolich K: Mechanisms of neural plasticity following brain injury. Curr Opin Neurobiol 2006; 16:258–64

4. GrandPre T, Nakamura F, Vartanian T, Strittmatter SM: Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 2000; 403:439–44

5. Bartsch U, Bandtlow CE, Schnell L, Bartsch S, Spillmann AA, Rubin BP, Hillenbrand R, Montag D, Schwab ME, Schachner M: Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. Neuron 1995; 15:1375–81

6. Wang JM, Hayashi T, Zhang WR, Sakai K, Shiro Y, Abe K: Reduction of ischemic brain injury by topical application of insulin-like growth factor-I after transient middle cerebral artery occlusion in rats. Brain Res 2000; 859:381–5

7. Fournier AE, GrandPre T, Strittmatter SM: Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 2001; 409:341–6

8. Lee DH, Strittmatter SM, Sah DW: Targeting the Nogo receptor to treat central nervous system injuries. Nat Rev Drug Discov 2003; 2:872–8

9. GrandPre T, Li S, Strittmatter SM: Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 2002; 417:547–51

10. Li S, Strittmatter SM: Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. J Neurosci 2003; 23:4219–27

11. Seymour AB, Andrews EM, Tsai SY, Markus TM, Bollnow MR, Brenneman MM, O'Brien TE, Castro AJ, Schwab ME, Kartje GL: Delayed treatment with monoclonal antibody IN-1 1 week after stroke results in recovery of function and corticorubral plasticity in adult rats. J Cereb Blood Flow Metab 2005; 25:1366–75

12. Lee JK, Kim JE, Sivula M, Strittmatter SM: Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. J Neurosci 2004; 24:6209–17

13. Wiessner C, Bareyre FM, Allegrini PR, Mir AK, Frentzel S, Zurini M, Schnell L, Oertle T, Schwab ME: Anti-Nogo-A antibody infusion 24 h after experimental stroke improved behavioral outcome and corticospinal plasticity in normotensive and spontaneously hypertensive rats. J Cereb Blood Flow Metab 2003; 23:154–65

14. Kilic E, Kilic U, Hermann DM: TAT fusion proteins against ischemic stroke: Current status and future perspectives. Front Biosci 2006; 11:1716–21

15. Yin W, Cao G, Johnnides MJ, Signore AP, Luo Y, Hickey RW, Chen J: TAT-mediated delivery of Bcl-xL protein is neuroprotective against neonatal hypoxic-ischemic brain injury *via* inhibition of caspases and AIF. Neurobiol Dis 2006; 21:358–71

16. Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH, Chen J: In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. J Neurosci 2002; 22:5423–31

17. Denicourt C, Dowdy SF: Protein transduction technology offers novel therapeutic approach for brain ischemia. Trends Pharmacol Sci 2003; 24:216–8

18. Misra A, Ganesh S, Shahiwala A, Shah SP: Drug delivery to the central nervous system: A review. J Pharm Pharm Sci 2003; 6:252–73

19. Cai SR, Xu G, Becker-Hapak M, Ma M, Dowdy SF, McLeod HL: The kinetics and tissue distribution of protein transduction in mice. Eur J Pharm Sci 2006; 27:311–9

20. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF: *In vivo* protein transduction: Delivery of a biologically active protein into the mouse. Science 1999; 285:1569–72

21. Kilic U, Kilic E, Dietz GP, Bahr M: Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice. Stroke 2003; 34:1304–10

22. Guegan C, Braudeau J, Couriaud C, Dietz GP, Lacombe P, Bahr M, Nosten-Bertrand M, Onteniente B: PTD-XIAP protects against cerebral ischemia by anti-apoptotic and transcriptional regulatory mechanisms. Neurobiol Dis 2006; 22:177–86

23. Kim DW, Eum WS, Jang SH, Kim SY, Choi HS, Choi SH, An JJ, Lee SH, Lee KS, Han K, Kang TC, Won MH, Kang JH, Kwon OS, Cho SW, Kim TY, Park J, Choi SY: Transduced Tat-SOD fusion protein protects against ischemic brain injury. Mol Cells 2005; 19:88–96

24. Becker-Hapak M, McAilister SS, Dowdy SF: TAT-mediated protein transduction into mammalian cells. Methods 2001; 24:247–56

25. Zhuo Q, Piao JH, Wang R, Yang XG: Refolding and purification of nonfusion HPT protein expressed in *Escherichia coli* as inclusion bodies. Protein Expr Purif 2005; 41:53–60

26. Wang Q, Xiong L, Chen S, Liu Y, Zhu X: Rapid tolerance to focal cerebral ischemia in rats is induced by preconditioning with electroacupuncture: Window of protection and the role of adenosine. Neurosci Lett 2005; 381:158–62

27. Xiong L, Zheng Y, Wu M, Hou L, Zhu Z, Zhang X, Lu Z: Preconditioning with isoflurane produces dose-dependent neuroprotection *via* activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. Anesth Analg 2003; 96:233–7

28. Longa EZ, Weinstein PR, Carlson S, Cummins R: Reversible middle cerebral artery occlusion without craniotomy in rats. Stroke 1989; 20:84–91

29. Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z: Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 2002; 417:941–4

30. Dietz GP, Kilic E, Bahr M: Inhibition of neuronal apoptosis *in vitro* and *in vivo* using TAT-mediated protein transduction. Mol Cell Neurosci 2002; 21:29–37

31. Kilic E, Kilic U, Hermann DM: TAT fusion proteins against ischemic stroke: current status and future perspectives. Front Biosci 2006; 11:1716–21

32. Kilic E, Kilic U, Hermann DM: TAT-GDNF in neurodegeneration and ischemic stroke. CNS Drug Rev 2005; 11:369–78

33. Markus TM, Tsai SY, Bollnow MR, Farrer RG, O'Brien TE, Kindler-Baumann DR, Rausch M, Rudin M, Wiessner C, Mir AK, Schwab ME, Kartje GL: Recovery and brain reorganization after stroke in adult and aged rats. Ann Neurol 2005; 58:950–3

34. Zhou C, Li Y, Nanda A, Zhang JH: HBO suppresses Nogo-A, Ng-R, or RhoA expression in the cerebral cortex after global ischemia. Biochem Biophys Res Commun 2003; 309:368–76

35. Wang F, Liang Z, Hou Q, Xing S, Ling L, He M, Pei Z, Zeng J: Nogo-A is involved in secondary axonal degeneration of thalamus in hypertensive rats with focal cortical infarction. Neurosci Lett 2007; 417:255–60

36. Mingorance A, Sole M, Muneton V, Martinez A, Nieto-Sampedro M, Soriano E, del Rio JA: Regeneration of lesioned entorhino-hippocampal axons *in vitro* by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NgR signaling. FASEB J 2006; 20:491–3

37. Denault JB, Salvesen GS: Caspases: Keys in the ignition of cell death. Chem Rev 2002; 102:4489–500

38. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW: Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 1998; 12:806–19

39. Cho S, Liu D, Gonzales C, Zaleska MM, Wood A: Temporal assessment of caspase activation in experimental models of focal and global ischemia. Brain Res 2003; 982:146–55

40. Wang H, Yao Y, Jiang X, Chen D, Xiong Y, Mu D: Expression of Nogo-A and NgR in the developing rat brain after hypoxia-ischemia. Brain Res 2006; 1114:212–20

41. Zhang Y, Gu X, Yuan X: Phenylalanine activates the mitochondria-mediated apoptosis through the RhoA/Rho-associated kinase pathway in cortical neurons. Eur J Neurosci 2007; 25:1341–8