Neuroprotective Effect of Orexin-A Is Mediated by an Increase of Hypoxia-inducible Factor-1 Activity in Rat

Li-bang Yuan, M.D.,* Hai-long Dong, M.D., Ph.D.,† Hao-Peng Zhang, M.D.,* Rui-ni Zhao, M.S.,‡ Gu Gong, M.D.,§ Xiao-mei Chen, M.D.,* Li-na Zhang, M.D.,* Lize Xiong, M.D., Ph.D.‖

ABSTRACT

Background: Recent studies suggest that the novel neuropeptide orexin-A may play an essential role during neuronal damage. However, the function of orexin-A during brain ischemia remains unclear. Recently, hypoxia-inducible factor-1α (HIF-1α) was shown to be activated by orexin-A. The aim of the current study is to test the hypothesis that administration of exogenous orexin-A can attenuate ischemia-reperfusion injury through the facilitation of HIF-1α expression.

Methods: Sprague-Dawley rats were subjected to transient middle cerebral artery occlusion for 120 min. Rats were treated with different doses of orexin-A or vehicle before the ischemia and at the onset of reperfusion. To investigate the action of HIF-1α in the neuroprotective effects of orexin-A, the HIF-1α inhibitor YC-1 was used alone or combined with orexin-A. Neurologic deficit scores and infarct volume were assessed. Brains were harvested for immunohistochemical staining and western blot analysis.

Results: Orexin-A significantly ameliorated neurologic deficit scores and reduced infarct volume after cerebral ischemia reperfusion. Administration of 30 μg/kg orexin-A showed optimal neuroprotective effects. This effect was still present 7 days after reperfusion. Furthermore, orexin-A decreased the number of apoptotic cells and significantly enhanced HIF-1α expression after cerebral ischemia reperfusion. Moreover, the facilitation of HIF-1α expression was accompanied with inhibition of von Hippel-Lindau expression. Administration of HIF-1α inhibitor suppressed the increase of HIF-1α and reversed the neuroprotective effects of orexin-A.

Conclusions: Orexin-A has a neuroprotective effect against cerebral ischemia–reperfusion injury. These effects may be mediated through the HIF-1α pathway.

What We Already Know about This Topic

- Reductions in the endogenous neuropeptide orexin-A have been implicated in the pathophysiology of stroke.

What This Article Tells Us That Is New

- Intravenous orexin-A was neuroprotective in a rat model of transient cerebral ischemia by a mechanism involving hypoxia-inducible factor-1α, representing a novel potential therapy for stroke and neuroprotection.

OREXINS are neuropeptides that were initially characterized as potent stimulants of food intake,[1] but they also have various physiologic effects, including energy homeostasis, neuroendocrine and autonomic nervous system functions, pain perception, and sleep-wake cycle control.[2,3] For example, Peyron *et al.*[4] discovered that reduced production of orexin peptides occurred in patients with narcolepsy. In addition, deletion of the orexin gene in mice produces a condition similar to human narcolepsy.[5] Recently, we demonstrated that orexinergic signals are involved in regulation of the anesthesia-awake cycle.[6,7]

It is also well known that various physiologies, including sleep patterns,[8,9] energy homeostasis,[10] autonomic functions, and neuroendocrine functions[11,12] are often abnormal in patients with stroke. These phenomena inspired us to speculate that there is a correlation between the orexin system and cerebral ischemic injury.

Recently, a series of clinical and empirical studies have provided solid evidence supporting our speculation. A new published clinical study observed a significant transient decline in orexin-A concentrations at the onset of delayed ischemic neuronal deficits resulting from symptomatic vasospasm in patients with subarachnoid hemorrhage.[13] Nishino *et al.*[8] also found that patients with cerebral infarction showed a persistent decrease of cerebrospinal orexin-A concentrations. In addition, a number of animal studies have shown that global or focal cere-

* Postgraduate student, † Associate Professor and Chief, ‖ Professor, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China. ‡ Research Assistant, Department of Emergency and General Dentistry, Stomatological College of Fourth Military Medical University, Xi'an, Shaanxi, China. § Associate Professor and Chief, Department of Anesthesiology, General Hospital of People's Liberation Army Chengdu Military Region, Chengdu, Sichuan, China.

Received from the Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China. Submitted for publication April 29, 2010. Accepted for publication October 14, 2010. Supported by the National Science Foundation for Distinguished Young Scholars (Beijing, China) Grant no. 30725039 (to Dr. Xiong), the State Key Program of the National Natural Science Foundation of China (Beijing) Grant no. 30930091 (to Dr. Xiong), and the National Natural Science Foundation of China (Beijing) Grant nos. 30772059 and 30972853 (to Dr. Dong). Drs. Yuan and Dong contributed equally to this work.

Address correspondence to Dr. Xiong: Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi, China. lxiong@fmmu.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

 Anesthesiology 2011; 114: 340–54

Table 1. Physiological Parameters, Mean ± SD (N = 6)

Time Point	Temperature, °C	PaO_2, mmHg	$PaCO_2$, mmHg	pH	Glucose, mmol/L
Baseline	36.9 ± 0.1	90.9 ± 7.31	38.2 ± 2.61	7.36 ± 0.02	4.32 ± 0.26
Ischemia, 60 min	37.1 ± 0.2	89.9 ± 6.56	39.3 ± 4.18	7.39 ± 0.03	4.27 ± 0.32
Reperfusion, 30 min	37.0 ± 0.2	88.7 ± 5.41	39.2 ± 3.81	7.38 ± 0.02	4.25 ± 0.28

bral ischemia induces increased expression of orexin 1 receptors (OX1R) in the brain, which correlated with decreases of orexin-A in cerebrospinal fluid.[14–16] The accumulated evidence indicates that orexins, especially orexin-A, may play a pivotal role in neuronal cell death in cerebrovascular diseases and ischemia-reperfusion injury. Nevertheless, the direct effect of orexin-A on brain ischemic injury is poorly understood.

Hypoxia-inducible factor-1α (HIF-1α) is an important transcription factor involved in ischemic and hypoxic conditions.[17] Recently, an *in vitro* experiment revealed that orexin-A can activate expression of HIF-1α by inhibiting von Hippel-Lindau (vHL) expression.[18] Thus, the current study was designed to test the hypothesis that administration of exogenous orexin-A can induce neuroprotective effects—as mediated by facilitation of HIF-1α expression—against cerebral ischemia–reperfusion injury in rats.

Materials and Methods

Animals

All procedures were carried out according to protocols approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (Xi'an, Shaanxi, China) and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Adult male Sprague-Dawley rats (280–320g) obtained from the laboratory animal center of the Fourth Military Medical University were used in this study.

Experimental Protocol

Part I: Neuroprotective Effects of Orexin-A. To investigate the neuroprotective effects of orexin-A (Phoenix Biotech, Mississauga, Ontario, Canada), 48 rats were randomly assigned to one of six groups (n = 8): sham, control, OXA-3, OXA-10, OXA-30, and OXA-100 groups. At the onset of reperfusion, rats were randomized to receive either vehicle (phosphate buffered saline [PBS]) or orexin-A (3, 10, 30, or 100 μg/kg, diluted in PBS) by intravenous injection (0.5 ml). At 24 and 72 h after reperfusion, neurologic scores were evaluated in a masked fashion. At 72 h after reperfusion, rats were sacrificed for infarct volume measurement.

Part II: Sustained Neuroprotective Effects of Orexin-A. To investigate the sustained neuroprotective effects of orexin-A as well as differences between preischemic and postischemic administration, 32 rats were randomly assigned to one of four groups (n = 8): sham, control, OXA-pre, and OXA-post groups. For the OXA-pre group, orexin-A (30 μg/kg, diluted in PBS) was administered by intravenous injection (0.5 ml) 10 min before ischemia. For the OXA-post group, orexin-A (30 μg/kg, diluted in PBS) was administered by intravenous injection (0.5 ml) at the onset of reperfusion in rats. For sham and control groups, PBS (0.5 ml) was administered intravenously 10 min before ischemia and at the onset of reperfusion in rats. At 3 and 7 days after reperfusion, neurologic scores were evaluated in a masked fashion and rats were sacrificed for infarct volume assessment.

Part III: HIF-1α and vHL Regulation after Orexin-A. To explore whether the neuroprotective effects of orexin-A are mediated by the HIF-1α pathway, the expression of HIF-1α and vHL were observed after cerebral ischemia reperfusion with or without orexin-A treatment. According to the results from the first part of this experiment, 30 μg/kg orexin-A was used in the treatment group. Rats were randomly assigned to one of three groups (n = 36): sham, control, and orexin-A treatment groups. At the onset of reperfusion, rats were randomized to receive either vehicle (PBS) or orexin-A (30 μg/kg diluted in PBS) by intravenous injection (0.5 ml). Immunohistochemistry and western blot analysis were performed 2, 24, and 72 h after reperfusion (n = 12 at each time point).

Part IV: Effects of HIF-1α Inhibitor YC-1 on Neuroprotective Properties of Orexin-A and HIF-1α Protein Expression. To demonstrate the involvement of the HIF-1α pathway in the neuroprotective effects of orexin-A, 40 rats were ran-

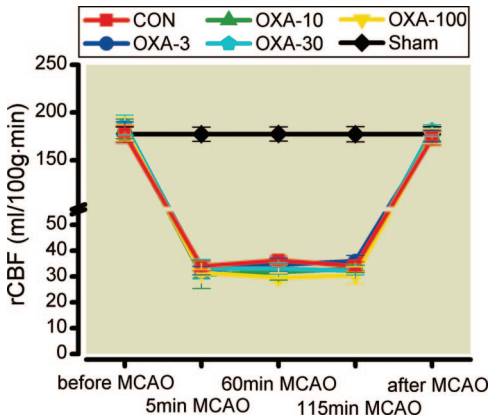

Fig. 1. Regional cerebral blood flow (rCBF) was monitored 5 min before transient middle cerebral artery occlusion (MCAO), at 5, 60, and 115 min during MCAO, and 5 min after MCAO. Regional cerebral blood flow monitoring demonstrated if the MCAO model was successful. CON = control; OXA-3 = 3 μg/kg orexin-A; OXA-10 = 10 μg/kg orexin-A; OXA-30 = 30 μg/kg orexin-A; OXA-100 = 100 μg/kg orexin-A.

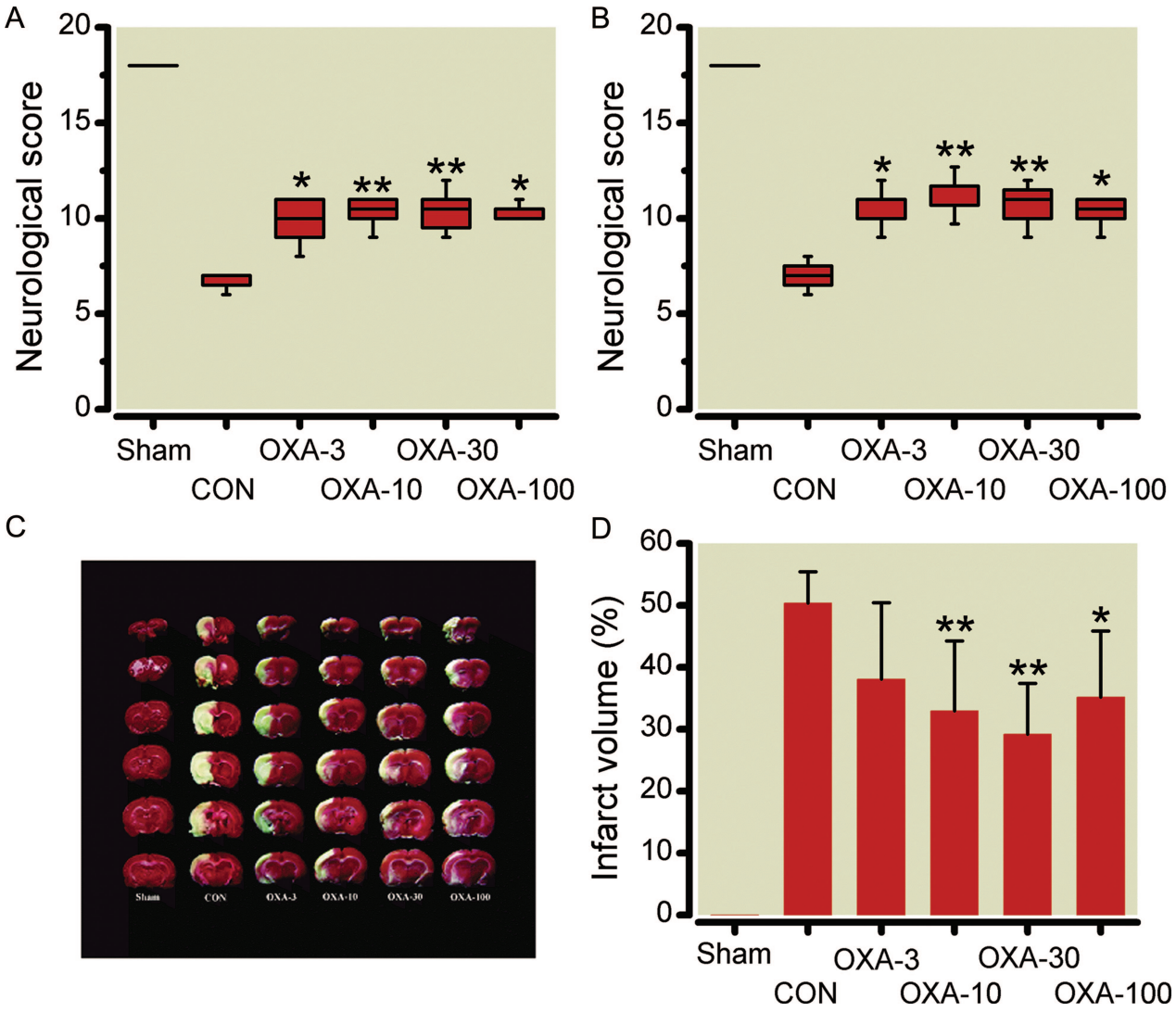

Fig. 2. Neurologic scores and infarct volume after 120 min of transient middle cerebral artery occlusion in sham, control (CON), and orexin-A treatment groups. Orexin-A was administered at the onset of reperfusion by intravenous injection. Neurologic scores are presented as median (range); data for infarct volume are expressed as mean ± SD. Neurologic scores were evaluated at 24 h (*A*) and at 72 h (*B*) after reperfusion using the Garcia scoring system. (*C*) Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in the rat brain at 72 h after reperfusion. (*D*) Statistical analysis of the percentage of infarct volume was determined for each study group (n = 8). OXA-3 = 3 μg/kg orexin-A; OXA-10 = 10 μg/kg orexin-A; OXA-30 = 30 μg/kg orexin-A; OXA-100 = 100 μg/kg orexin-A. *$P < 0.05$ *versus* control. **$P < 0.01$ *versus* control.

domly assigned to one of five groups (n = 8): sham; control; YC-1(3-(50-hydroxymethyl-20-furyl)-1-benzylindazole, the inhibitor of HIF-1α; orexin-A; and orexin-A + YC-1. At the onset of reperfusion, rats were randomized to receive either vehicle (1% dimethyl sulfoxide), YC-1 (1 mg/kg diluted in 1% dimethyl sulfoxide PBS), orexin-A (30 μg/kg diluted in PBS), or orexin-A combined with YC-1 by intravenous injection (0.5 ml). At 24 and 72 h after reperfusion, neurologic scores were evaluated in a masked fashion. At 72 h after reperfusion, rats were sacrificed for infarct volume measurement.

Western blot analysis was used to check the effect of YC-1 on HIF-1α protein expression. Sixty rats were randomly assigned to one of five groups: sham, control, YC-1, orexin-A, and orexin-A + YC-1 group. At 24 and 72 h after reperfusion, rats (n = 6 at each time point) were sacrificed and the hippocampus and penumbral cortex of the right hemisphere was dissected for western blot analysis.

Transient Middle Cerebral Artery Occlusion Model

The transient middle cerebral artery occlusion (MCAO) model was performed as previously described.[19] After an overnight fast, animals were anesthetized by intraperitoneal injection of 40–50 mg/kg sodium pentobarbital, 2% (v/v), for all surgical procedures. Transient focal cerebral ischemia was produced by intraluminal suture occlusion of the right middle cerebral artery using a 3-0 monofilament nylon suture (Ethicon, Inc., Osaka, Japan). After 2 h of MCAO, the suture was carefully removed from the internal carotid artery.

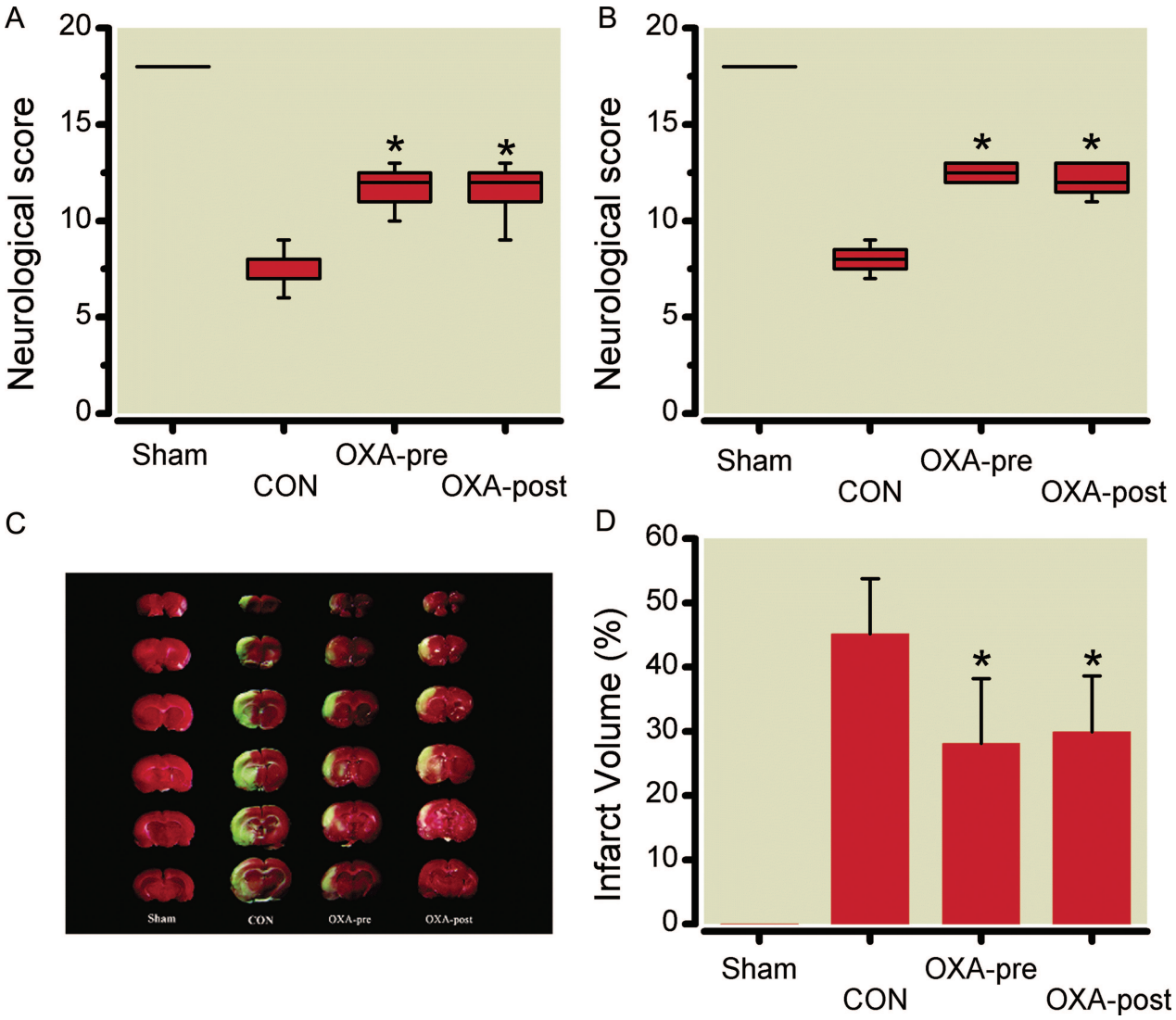

Fig. 3. Neurologic scores and infarct volume after 120 min of transient middle cerebral artery occlusion in sham, control (CON), and orexin-A treatment groups. Orexin-A was administered preischemia (OXA-pre) and postischemia (OXA-post). Neurologic scores are presented as median (range); data for infarct volume are expressed as mean ± SD. Neurologic scores were evaluated at 3 days (*A*) and at 7 days (*B*) after reperfusion using the Garcia scoring system. (*C*) Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in the rat brain at 7 days after reperfusion. (*D*) Statistical analysis of the percentage of infarct volume was determined for each study group (n = 8). *$P < 0.05$ *versus* control.

The neck incision was closed and rats were allowed to recover. Sham-operated rats underwent the same surgical procedure except that the suture was not inserted into the internal carotid artery.

Regional cerebral blood flow was monitored using a flexible optical fiber probe attached to the skull over the ipsilateral parietal cortex at one point (1 mm posterior and 5 mm lateral to the bregma) by laser Doppler flowmetry (PeriFlux system 5000; Perimed AB, Stockholm, Sweden). Rats in which ipsilateral blood flow was not reduced to less than 20% of the baseline after placement of the intraluminal filament and whose cerebral blood flow signal was not rapidly restored during reperfusion were excluded from subsequent experiments. Cranial temperature was maintained at 36.8–37.5°C with a heating pad. In a separate experiment, physiologic parameters (cranial temperature, arterial pH, $PaCO_2$, PaO_2, glucose) were monitored and analyzed in six additional rats. Arterial blood samples were taken 3 min before ischemia (baseline), 60 min after ischemia, and 30 min after reperfusion for gases and plasma glucose measurements.

Neurologic Scores

Neurologic scores were evaluated 24 and 72 h after reperfusion by using a scoring system reported by Garcia *et al.*[20] in a masked fashion. This system consisted of the following six tests: (1) spontaneous activity, (2) symmetry in the movement of four limbs, (3) forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. The score given to each rat at the completion of the evalua-

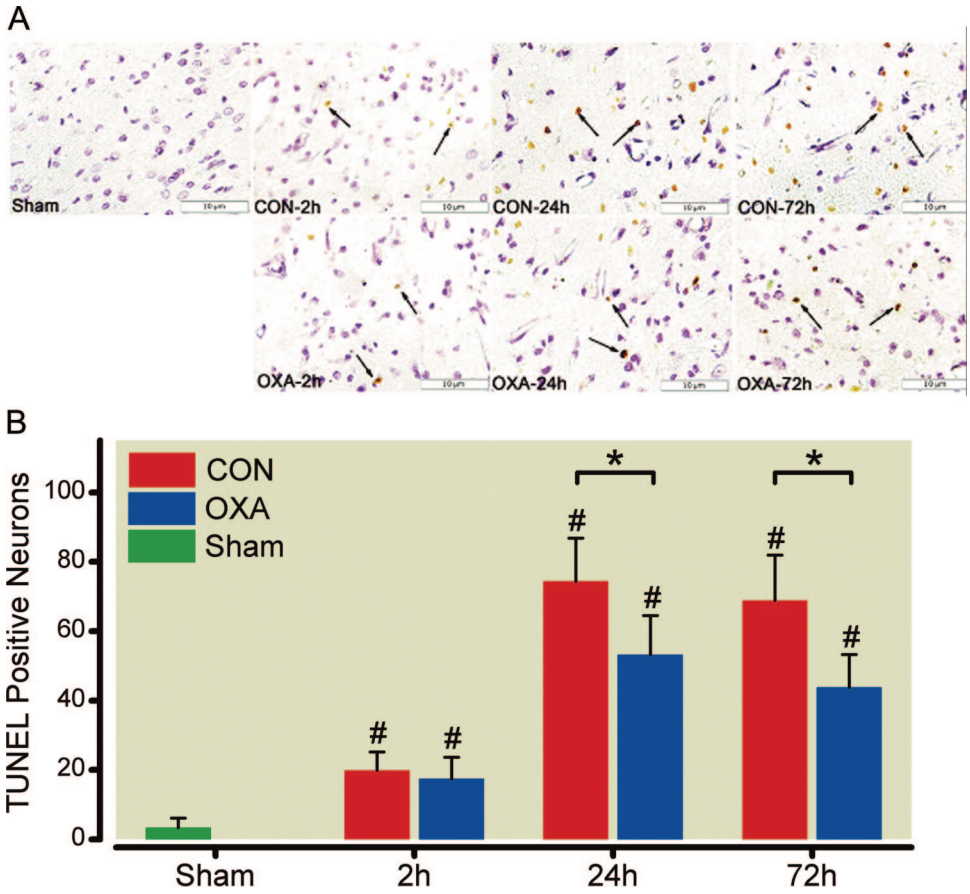

Fig. 4. (*A*) Representative sections of nuclear DNA fragmentation assays performed by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining in the penumbral cortex in sham, control (CON) and 30 μg/kg orexin-A (OXA) treatment groups (n = 6). (*B*) Quantitative analysis of the number of TUNEL-positive neurons in the penumbral cortex. Data are presented as mean ± SD. Scale bar, 10 μm. #$P < 0.05$ *versus* sham. *$P < 0.05$ *versus* control.

tion was the summation of all six individual test scores. Minimum neurologic score was 3; maximum was 18.

Infarct Volume Measurement

To compare infarct sizes in the ischemia-treated groups, infarct volume was measured (at 72 h after reperfusion; n = 8 per group) using 2,3,5-triphenyltetrazolium chloride staining (Sigma-Aldrich, St. Louis, MO) as described in our previous report.[19] Infarct and total hemispheric areas of sections were traced from slices taken at 2-mm intervals and measured using an image analysis system (Adobe Photoshop 8.0, Adobe Systems Incorporated, San Jose, CA). To compensate for the effect of brain edema, corrected infarct volumes were calculated as previously described using the following equation: corrected infarct area = measured infarct area × {1 − [(ipsilateral hemisphere area − contralateral hemisphere area)/contralateral hemisphere]}.[21] Infarct volumes were expressed as percentages of contralateral hemispheric volumes.

Specimen Preparation

For immunohistochemical analysis, animals (n = 6 at each time point) were anesthetized with an overdose of 2% (v/v) sodium pentobarbital and perfusion-fixed *via* the left ventricle as previously described at 2, 24, and 72 h after MCAO.[22,23] Animals were first perfused with 200 ml 0.1 M PBS and then with 400 ml paraformaldehyde, 4% (w/v), in 0.1 M PBS (pH 7.4). Brains were removed and postfixed in 4% (w/v) paraformaldehyde in 0.1 M PBS. Brain blocks were embedded in paraffin and cut into 3-μm coronal sections. Sections were used for immunohistochemical staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end label (TUNEL) staining.

For western blot analysis, rats (n = 6 at each time point) were sacrificed at designated time intervals. Brains were removed and the hippocampus and penumbral cortex of the right hemisphere was dissected according to well-established protocols in rodent models of MCAO.[24] Samples were frozen immediately on dry ice and stored at −80°C until ready for use.

Immunohistochemical Staining

Sections were immunostained for rabbit anti–HIF-1α antibody (ab51608, 1:200; Abcam, Cambridge, MA) and goat anti-vHL protein antibody (ab77262, 1:300; Abcam). A microwave oven was used to dewax paraffin sections for 10 min

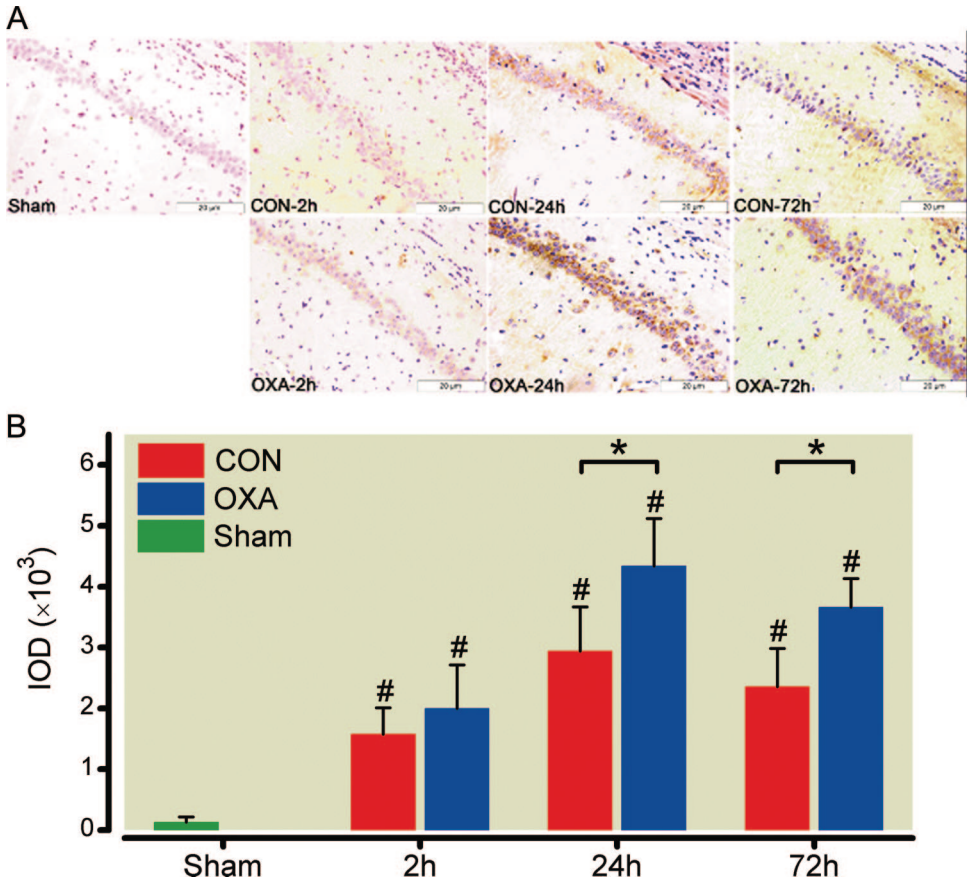

Fig. 5. (*A*) Representative sections of immunostaining for hypoxia-inducible factor-1α in the hippocampus CA1 region in sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups. (*B*) Quantitative analysis of the integrated optical density (IOD). Data are presented as mean + SD. Scale bar, 20 μm. #$P < 0.05$ *versus* sham. *$P < 0.05$ *versus* control.

in 0.01 M citrate buffer solution (pH 6.0). Sections were incubated in 3% (v/v) H_2O_2 for 10 min, to prevent reaction with endogenous peroxidases, and 30-min incubation with 10% (v/v) normal goat serum or rabbit serum at room temperature. Sections were then incubated with the primary antibodies overnight at 4°C.

For HIF-1α staining, after washing in PBS, sections were incubated with peroxidase anti-rabbit IgG secondary antibody solution (1:500; Vector Laboratories, Burlingame, CA) in a humidified chamber at room temperature for 1 h. For vHL staining, sections were washed with PBS and incubated with biotinylated anti-goat IgG secondary antibody solution (1:800; Vector Laboratories) in a humidified chamber at room temperature for 1 h. Sections were then incubated with streptavidin-biotin complex in a humidified chamber for 30 min at room temperature. All sections were developed with diaminobenzidine for 5 min at room temperature. Sections were counterstained with hematoxylin, dehydrated with a series of ethanol solutions, coverslipped, and observed under a microscope (BX51; Olympus Corporation, Tokyo, Japan). Application of control serum instead of the primary antibody on other sections of the same brain provided a negative control. No immunostaining was observed in these sections.

Integrated optical density, a parameter representing expression of HIF-1α and vHL proteins in the hippocampus CA1 region and penumbral cortex, was measured quantitatively *via* digital image analysis (Image-Pro Plus 5.0; Media Cybernetics, Inc., Bethesda, MD) in four fields per slide. The average value of the integrated optical density in six animals in each group was calculated.

TUNEL Staining and Quantification of Apoptosis

Apoptotic cells were detected by TUNEL. Fragmented DNA in cell nuclei was labeled using an *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to manufacturer instructions. Sections were stained by TUNEL and then were counterstained with hematoxylin. A neuron with a nucleus stained dark brown was considered a positive cell. By counterstaining with hematoxylin (a blue dye), viable neurons were also observed. TUNEL-positive cell numbers were counted in a masked fashion in four fields in the penumbral cortex of the right hemisphere at high-power microscopic magnification (×400) and expressed as the number of positive cells per high-power field. Data were analyzed from six animals per group at each time point.

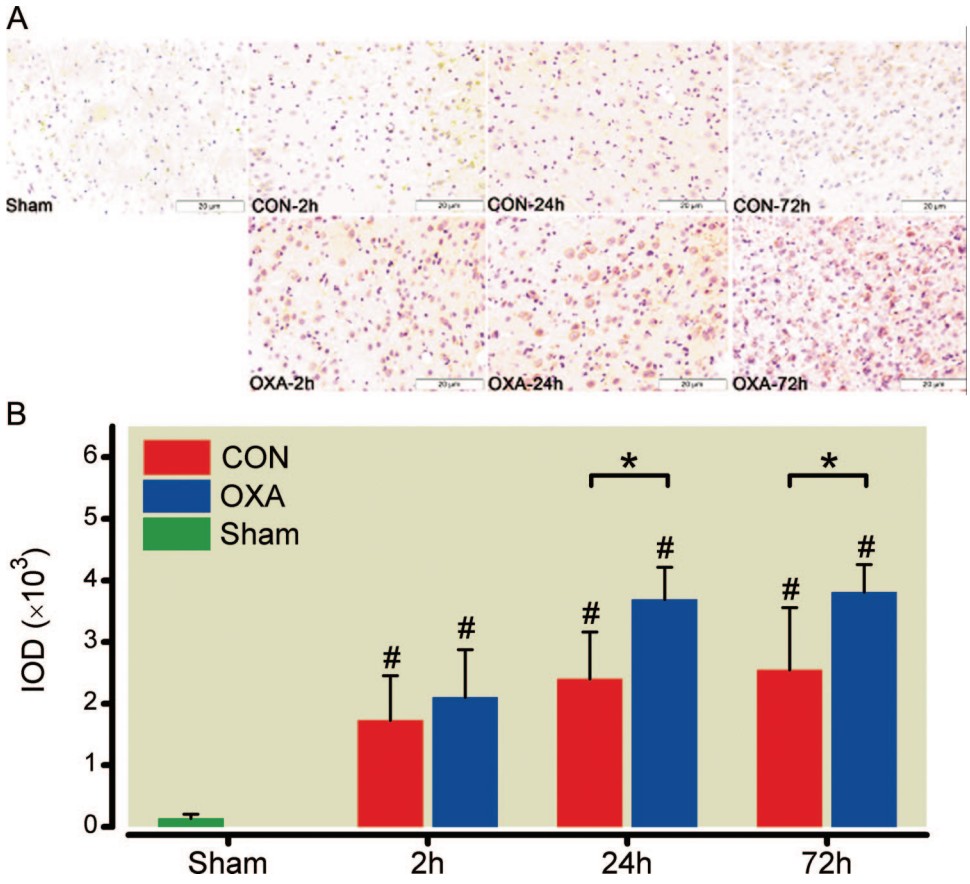

Fig. 6. (*A*) Representative sections of immunostaining for hypoxia-inducible factor-1α in the penumbral cortex in sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups. (*B*) Quantitative analysis of the integrated optical density (IOD). Data are presented as mean ± SD. Scale bar, 20 μm. #$P < 0.05$ *versus* sham. *$P < 0.05$ *versus* control.

Western Blot Analysis

Methods for western blot analysis have been described previously.[25] Tissue samples were homogenized using a whole protein extract kit (Millipore Corporation, Billerica, MA) with a glass tissue grinder. Protein content was determined using the BCA protein assay kit (Millipore Corporation). Equal amounts of protein per lane (50 μg) were loaded onto an 8% (v/v) polyacrylamide gel and separated by electrophoresis at 80 V for 30 min and 120 V for 70 min. Proteins were then transferred to nitrocellulose (Bio-Rad Laboratories, Inc., Hercules, CA) at 20 V for 50 min and the membrane was blocked with 10% (w/v) nonfat dry milk and 0.5% (v/v) Tween-20 in Tris-buffered saline. Nitrocellulose was incubated with two different antibodies overnight at 4°C, namely rabbit anti–HIF-1α antibody (ab51608, 1:1,000; Abcam) and goat anti-vHL protein antibody (ab77262, 1:1,000; Abcam). The membrane was treated with horseradish peroxidase–conjugated secondary antibody (anti-rabbit IgG or anti-goat IgG, 1:2000, Vector Laboratories) for 45 min at 37°C. The specific protein bands were visualized using the standard chemical luminescence method (ECL Kit; Amersham Pharmacia Biotech, Piscataway, NJ). Image analysis was accomplished with the assistance of computerized analysis software (Bio-Rad Laboratories, Inc.). Protein concentration was analyzed densitometrically and corrected with values determined on anti–β-actin blots. The density of proteins was expressed as relative values.

Statistical Analysis

All data, except for neurologic scores, are expressed as mean ± SD. Physiologic parameters were analyzed by repeated-measures ANOVA. Infarct volumes among different groups were compared by one-way ANOVA. When ANOVA showed significant differences, the *post hoc* multiple-comparison Tukey test was applied. For the analysis of neuron apoptosis and interest protein expression quantification, two-way ANOVA was applied to determine if there was interaction between the two main effects (treatment and time). In case of a significant interaction, the *post hoc* multiple comparison was performed to compare the difference among treatment groups at each time point. Otherwise, global conclusions were drawn based on the main effect. Neurologic scores were expressed as median (range) and were compared by Kruskal-Wallis test and Mann–Whitney U test with Bonferroni correction. The nature of hypothesis testing was two-tailed test, with $P <$ 0.05 accepted as statistically significant. All statistical

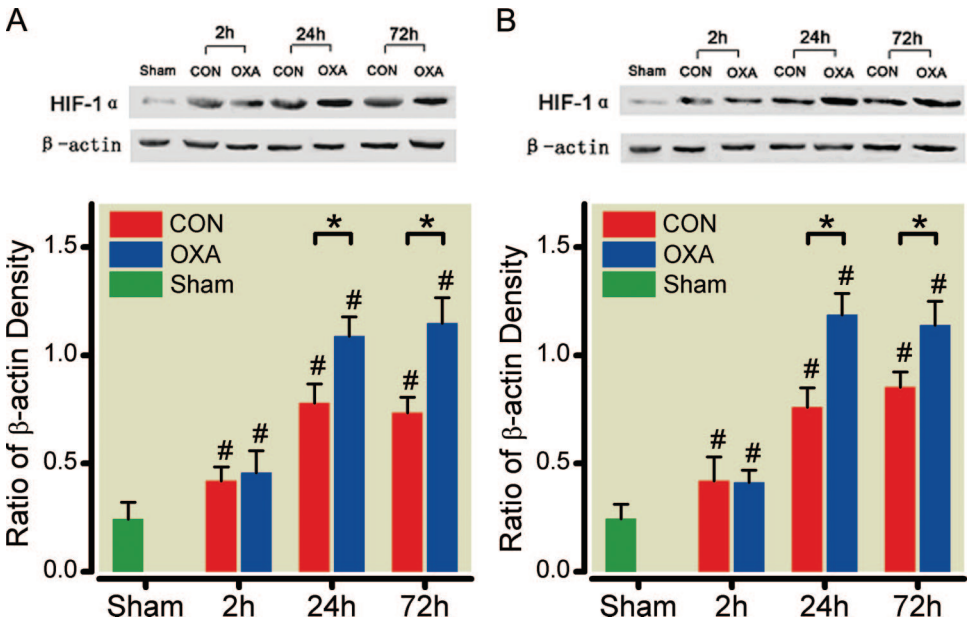

Fig. 7. Protein expression of hypoxia-inducible factor-1α (HIF-1α) in the hippocampus (*A*) and penumbral cortex (*B*) in sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups were evaluated by Western blot analysis. Representative bands and corresponding β-actin bands (*top*) and analysis of HIF-1α expression (*bottom*) are shown for each study group. Data are presented as mean ± SD. #$P < 0.05$ *versus* sham. *$P < 0.05$ *versus* control.

analyses were performed with SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL).

Results

Physiologic Parameters and Regional Cerebral Blood Flow

No statistical significance was noted among different time points for any of the physiologic parameters including cranial temperature, blood gas, and glucose concentrations (table 1). Physiologic parameters remained in the normal range during the experimental period. Monitoring of regional cerebral blood flow demonstrated successful MCAO (fig. 1).

Effects of Orexin-A–induced Neuroprotection on Neurologic Scores and Infarct Volume

Neurologic scores evaluated at 24 and 72 h after reperfusion in the sham, control, and the orexin-A–treated groups are shown in figure 2. Orexin-A significantly attenuated early neurologic deficits after MCAO ($P < 0.05$).

Representative coronal brain sections stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride from the six groups are shown in figure 2C. Mean infarction volume percentage in the control group was 50.4%, whereas treatment with orexin-A reduced brain infarct volume in a dose-dependent manner from 10 to 30 μg/kg. However, when the dose of orexin-A was increased to 100 μg/kg, a slight increase in infarct volume was observed when compared with the 30 μg/kg dose. These results demonstrate that orexin-A exerts greater neuroprotection at a therapeutic dose of 30 μg/kg in rats (fig. 2D; $P = 0.001$).

Sustainable Neuroprotective Effects of Orexin-A

Neurologic scores evaluated at 3 and 7 days after reperfusion in the sham group, control group, OXA-pre, and OXA-post groups are shown in figure 3. Both administration schedules significantly attenuated neurologic deficits at 3 and 7 days after MCAO ($P < 0.05$).

Representative coronal brain sections stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride from the four groups are shown in figure 3C. The mean infarction volume percentage in the control group was 44.7%, whereas treatment with orexin-A preischemia and postischemia reduced brain infarct volume (fig. 3D; OXA-pre, $P = 0.003$; OXA-post, $P = 0.008$). For neurologic scores and infarction volume, there was no significant difference between orexin-A groups regardless of administrative timing. These results demonstrate that orexin-A has sustainable neuroprotective effects regardless of administration timing (preischemia *vs.* postischemia).

TUNEL Staining

Ischemia-induced brain injury and the neuroprotective effects of orexin-A treatment were also assessed by observation of cell apoptosis in the brain with TUNEL staining. Figure 4A shows TUNEL staining in the penumbral cortex of the right hemisphere. The bar graph (fig. 4B) shows quantitative analysis of the number of TUNEL-positive neurons. Apoptotic neurons appeared 2 h after reperfusion and increased at 24 and 72 h after reperfusion in the control group. The number of apoptotic neurons in the 30-μg/kg orexin-A treatment group was significantly lower than that in the control group at 24 h ($P = 0.008$) and 72 h ($P = 0.001$) after

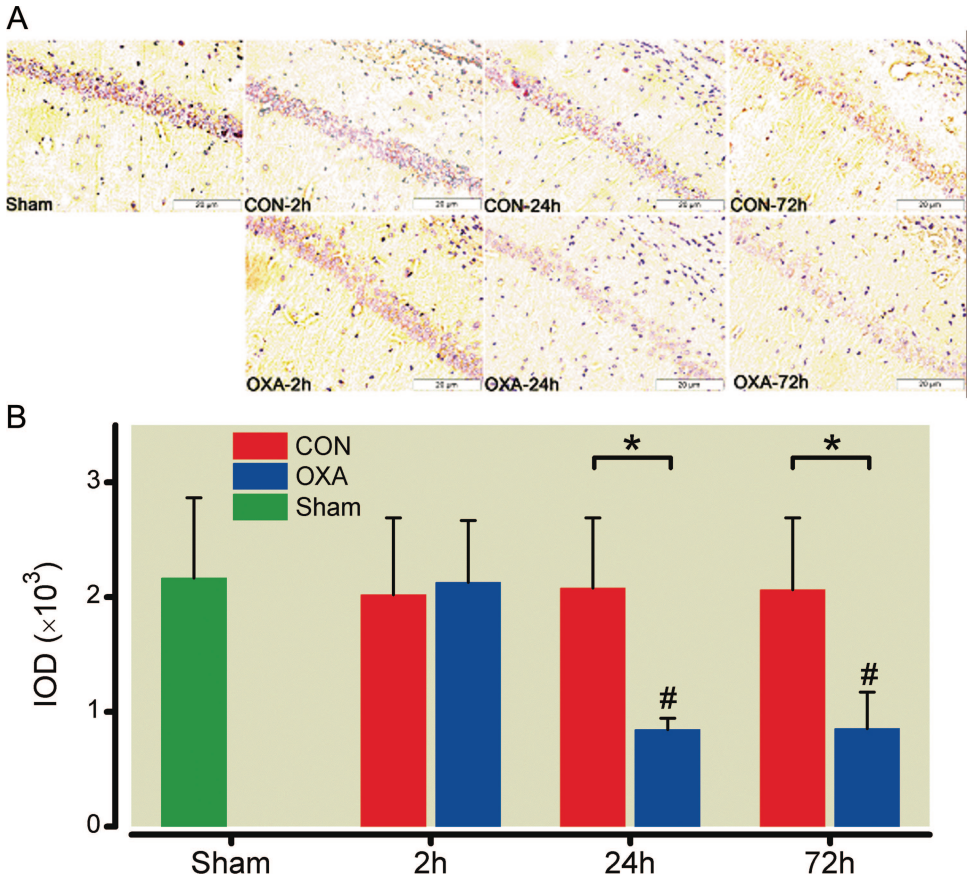

Fig. 8. (*A*) Representative sections of von Hippel-Lindau immunohistochemical staining in the hippocampus CA1 region in sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups. (*B*) Quantitative analysis of the integrated optical density (IOD). Data are presented as mean ± SD. Scale bar, 20 μm. #$P < 0.05$ *versus* sham. *$P < 0.05$ *versus* control.

reperfusion. At 2 h after reperfusion, there was no significant difference in the number of apoptotic neurons between the control group and orexin-A treatment group.

Expression of HIF-1α

Immunohistochemistry. Immunohistochemical staining of HIF-1α in the hippocampus CA1 region and penumbral cortex are shown in figure 5 and figure 6. At 2 h after reperfusion, a small number of HIF-1α–positive neurons were observed in the control group. Increased expression of HIF-1α was observed in sections from the control group 24 and 72 h after reperfusion. Orexin-A treatment (30 μg/kg) enhanced the staining of HIF-1α in the hippocampus CA1 region (24 h, $P = 0.026$; 72 h, $P = 0.007$) and penumbral cortex (24 and 72 h, $P = 0.008$) after reperfusion. However, there was no conspicuous distinction of HIF-1α staining between the control group and orexin-A group 2 h after reperfusion. There was no significant immunohistochemical staining for HIF-1α observed in the hippocampus CA1 region and the penumbral cortex in the sham group.

Western Blot Analysis. Protein expression of HIF-1α in the hippocampus in the control group increased significantly at 2 h ($P = 0.035$) and peaked at 24–72 h ($P < 0.001$) after reperfusion when compared with the sham group. Orexin-A treatment (30 μg/kg) significantly increased the expression of HIF-1α at 24 h ($P = 0.002$) and 72 h ($P < 0.001$) after reperfusion when compared with the control group (fig. 7A). Similar HIF-1α protein expression was observed in the penumbral cortex. Orexin-A treatment increased HIF-1α expression at 24 h ($P < 0.001$) and 72 h ($P = 0.003$) after reperfusion (fig. 7B).

vHL Expression

Immunohistochemistry. Immunohistochemical staining of vHL in the hippocampus CA1 region and penumbral cortex are shown in figure 8 and figure 9. vHL expression in the control group did not show any significant changes when compared with the sham group. Orexin-A treatment (30 μg/kg) inhibited the increasing expression of vHL at 24 and 72 h after reperfusion in the hippocampus CA1 ($P = 0.016$) region and penumbral cortex ($P = 0.002$). However, there was no significant difference between the control group and orexin-A group at 2 h after reperfusion.

Western Blot Analysis. Immunohistochemical findings were confirmed by western blot analysis. The expression of vHL in the hippocampus in the control group did not show

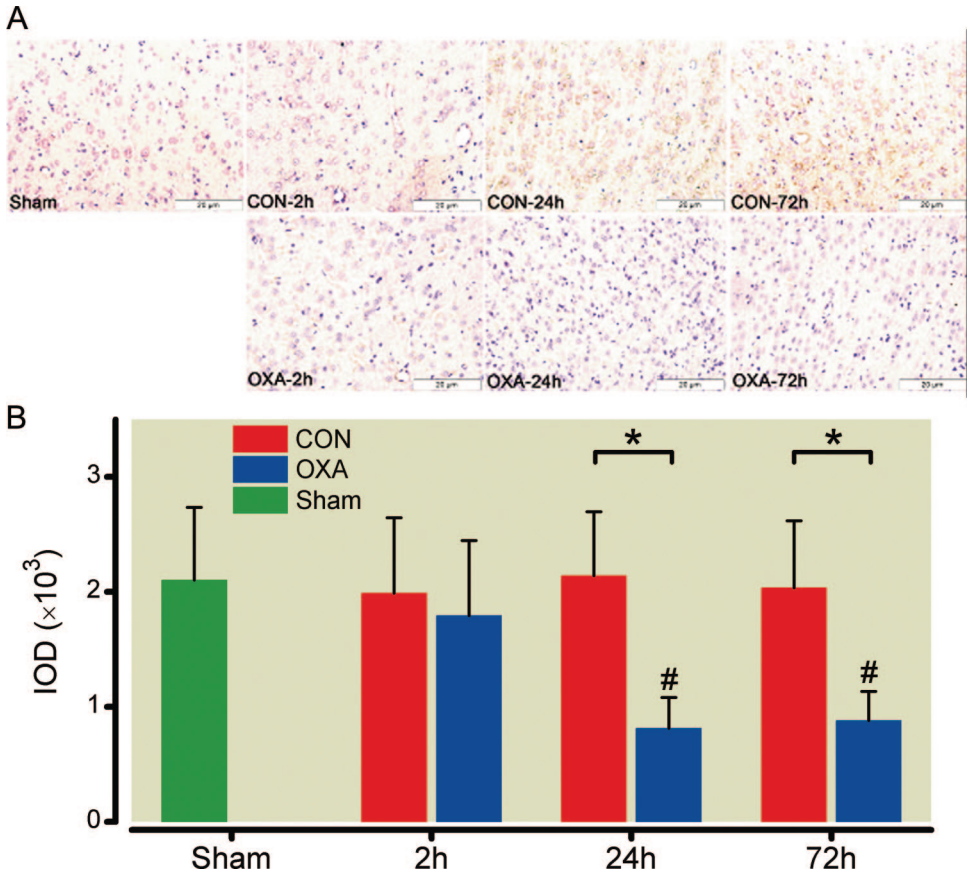

Fig. 9. (*A*) Representative sections of von Hippel-Lindau immunohistochemical staining in the penumbral cortex in sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups. (*B*) Quantitative analysis of the integrated optical density (IOD). Data are presented as mean ± SD. Scale bar, 20 μm. #$P < 0.05$ *versus* sham, *$P < 0.05$ *versus* control.

significant changes after ischemia reperfusion when compared with the sham group. vHL expression was significantly inhibited by 30 μg/kg orexin-A treatment at 24 h ($P = 0.039$) and at 72 h ($P = 0.037$) after reperfusion as compared with the control group (fig. 10A). Results from western blot analysis revealed that the penumbral cortex region and hippocampus had similar vHL expression patterns (fig. 10B; 24 h, $P = 0.028$; 72 h, $P = 0.031$).

Effect of HIF-1α Inhibitor YC-1 on Neuroprotective Effects of Orexin-A

Neurologic scores evaluated at 24 and 72 h after reperfusion are shown in figure 11. In figures 11A and B, the neurologic score of orexin-A combined with YC-1 group was reduced as compared with orexin-A group. This result indicates that administration of orexin-A combined with YC-1 deteriorated rather than reduced the neurologic deficits when compared to OXA group.

Representative coronal brain sections stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride from the five groups are shown in figure 11C. The mean infarction volume percentage in the orexin-A + YC-1 group is 43.2%, which was significant higher than orexin-A group ($P = 0.013$). There was no significant difference between control group and the orexin-A + YC-1 group in neurologic scores or infarction volume (fig. 11D).

Effect of YC-1 on HIF-1α Protein Expression

Orexin-A treatment (30 μg/kg) significantly increased the expression of HIF-1α in the hippocampus at 24 and 72 h after reperfusion when compared with the control group ($P < 0.001$). YC-1 treatment significantly suppressed the elevation of HIF-1α expression in the orexin-A + YC-1 group ($P < 0.001$ *vs.* orexin-A) as well as in YC-1 group (fig. 12A; $P < 0.001$ *vs.* control).

Similar HIF-1α protein expression tendency was observed in the penumbral cortex (fig. 12B).

Discussion

In the current study, we demonstrate that orexin-A treatment can induce prominent neuroprotective effects on transient cerebral ischemia in rats by improving neurologic function and decreasing infarct size. Ischemia reperfusion–induced neuronal apoptosis was also markedly attenuated by administration of orexin-A. The results of the current study also indicate that the neuroprotective effects of exogenous orexin-A treatment are mediated by HIF-1α activation and vHL inhibition.

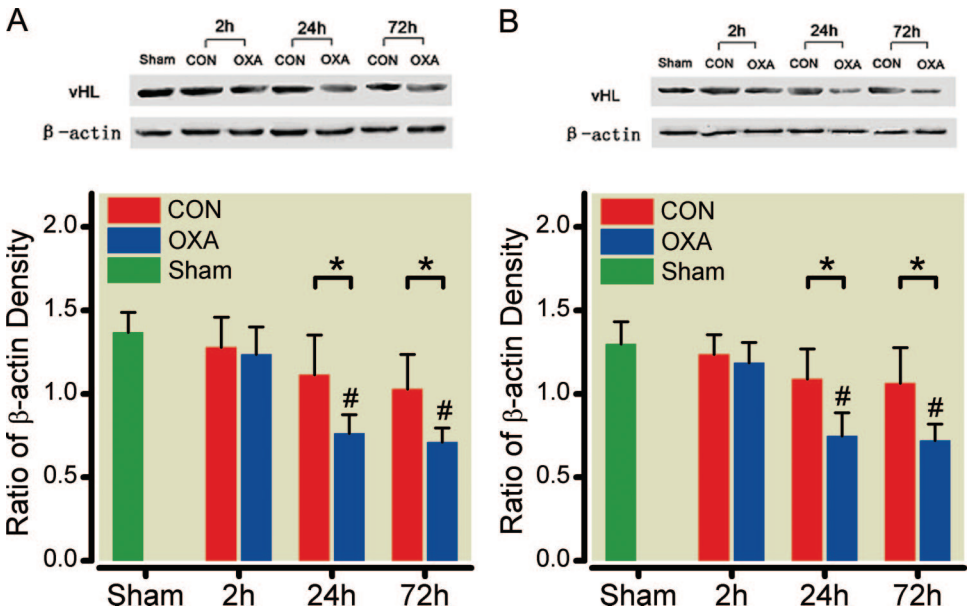

Fig. 10. Western blot analysis of von Hippel-Lindau expression in the hippocampus (*A*) and in the penumbral cortex (*B*) in the sham, control (CON), and 30 μg/kg orexin-A (OXA) treatment groups. Representative von Hippel-Lindau bands, corresponding β-actin bands (*top*), and statistical results (*bottom*) are shown for each study group (n = 6). Data are presented as mean ± SD. #$P < 0.05$ *versus* sham, *$P < 0.05$ *versus* control.

Orexins (orexin-A and orexin B) are novel neuropeptides. Neuronal fibers of the orexin-containing neurons are widely distributed throughout the brain.[1,26] Previous reports have revealed that orexins are involved in the regulation of many neurologic functions such as wakefulness, appetite, and metabolism.[3] The physiologic function of orexins is mediated through two receptor types, the OX1R and the orexin 2 receptor.[27–29] OX1R and the orexin 2 receptor are G protein–coupled receptors, which transmit information into cells by activating heterotrimeric G proteins. Activation of the signaling pathways associated with distinct G proteins may contribute to the diverse physiologic roles of orexin in particular neurons. Ramanjaneya *et al.*[30] found both extracellular receptor kinase 1/2 and p38 activation were predominantly Gq-mediated and, to a lesser extent, Gi-mediated; these two signaling pathway were phosphorylated rapidly in response to orexin-A and orexin B. The multiple roles for orexin mediated mitogen-activated protein kinase activation may help to explain the diverse biologic actions of orexins. Orexin-A increases cell surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid receptors and potentiates α-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid receptors' mediated synaptic transmission in the striatum, providing a potential link between the activation of orexin signaling in the striatum in response to addictive substances and neural adaptations in the reward circuitry.[31] A recent study[32] also revealed that orexin-A, but not orexin B, induces a state-dependent long-term potentiation of synaptic transmission in hippocampal slices from adult mice. Long-term potentiation of synaptic transmission is blocked by pharmacologic inhibition of OX1R and plasticity-related kinases, including serine/threonine (calmodulin kinase II, protein kinase C, protein kinase A, mitogen-activated protein kinase), lipid, phosphatidylinositol 3-kinase, and receptor tyrosine kinases. Peltonen *et al.*[33] also found orexin-A acting at OX1R-triggered oscillatory Ca^{2+} responses through transient receptor potential channel 3, which provides a novel mechanism for sustained activation of cellular processes.

Orexins derive from a single precursor prepro-orexin. The prepro-orexin gene is located on chromosome 17, which contains genes relevant to neurodegenerative diseases.[1] Therefore, it is speculated that the orexin system may be involved in maintaining neuronal survival and brain integrity. A number of clinical studies have shown that sleep patterns are altered in stroke patients. For example, many patients with stroke were reported to have incidents of insomnia,[34] although strokes are also associated with hypersomnia.[8] Because the orexin system plays an important role in sleep regulation, a change in the sleep-arousal cycle after a stroke indicate that orexin signal activities may be affected by cerebral ischemia–reperfusion injury. Recently, it was reported that cerebral ischemia induces increased cortical expression of the OX1R, suggesting involvement of the orexin system in response to an ischemic insult.[16] Irving *et al.*[14] reported that gene and protein expression of OX1R, but not the orexin 2 receptor, was increased in the ischemic cortex after focal ischemia in the rat. Furthermore, a decrease in orexin-A concentrations was observed in several neurodegenerative diseases and trauma in humans.[35] As orexin-A has a higher affinity than orexin B to bind with OX1R[1] and it can cross the blood-brain barrier rapidly by simple diffusion,[36] we have only focused on whether exogenous orexin-A is a promising neuroprotective agent against cerebral ischemia–reperfusion injury.

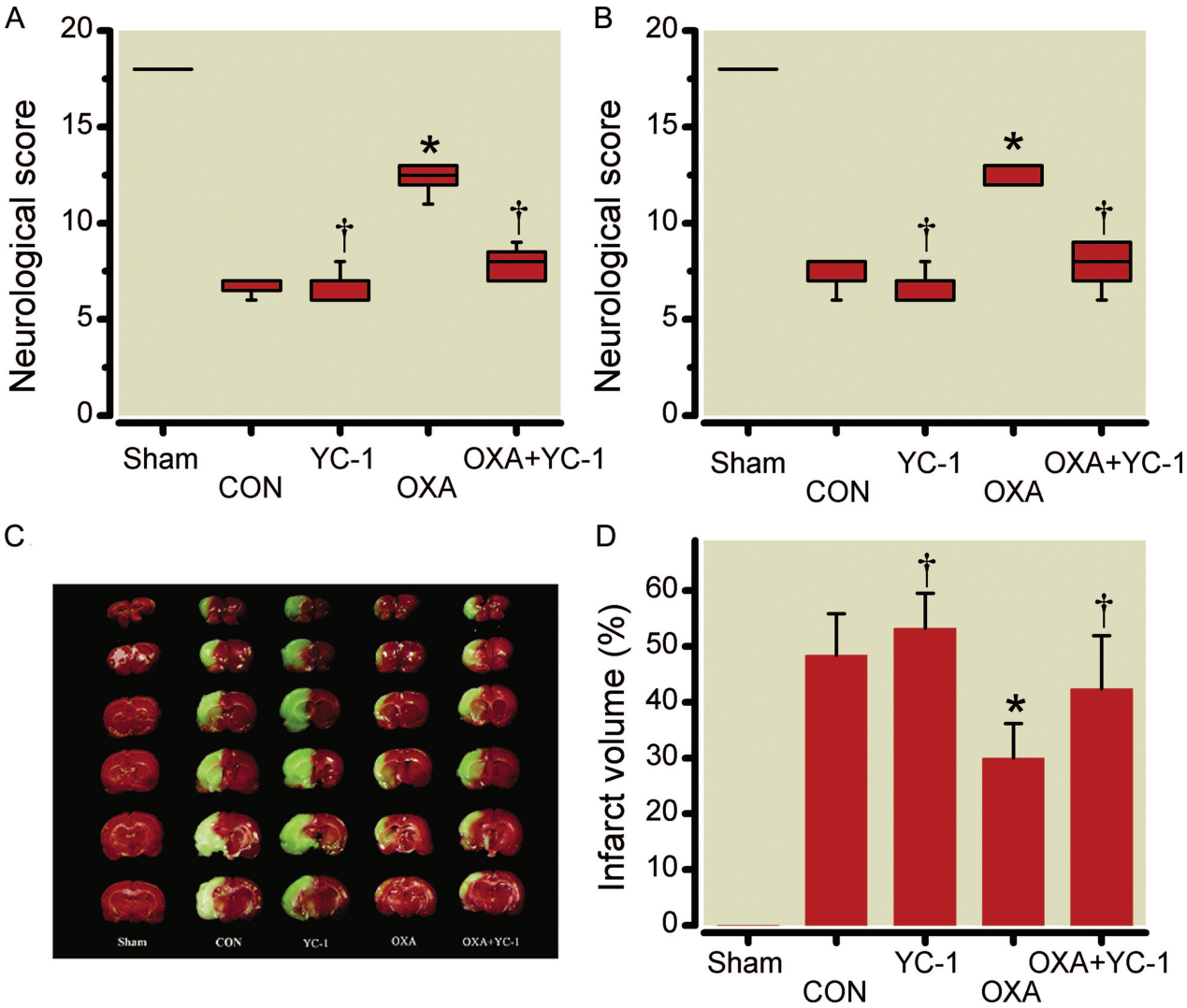

Fig. 11. Neurologic scores and infarct volume after 120 min of transient middle cerebral artery occlusion in sham, control (CON), YC-1(3-(50-hydroxymethyl-20-furyl)-1-benzylindazole, orexin-A (OXA), and orexin-A + YC-1 groups. Neurologic scores are presented as median (range); data for infarct volume are expressed as mean ± SD. Neurologic scores were evaluated at 24 h (*A*) and at 72 h (*B*) after reperfusion using the Garcia scoring system. (*C*) Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in the rat brain at 72 h after reperfusion. (*D*) Statistical analysis of the percentage of infarct volume was determined. *$P < 0.05$ *versus* control. †$P < 0.05$ *versus* orexin-A.

In this study, we have demonstrated that orexin-A treatment at the onset of reperfusion can induce neuroprotection by attenuating infarct volume and improving neurologic function in a transient rat MCAO model. These effects occurred in a dose-dependent manner at 10–30 μg/kg orexin-A. However, administration of 100 μg/kg orexin-A did not further improve the neuroprotective effects of orexin-A. This result indicates that the neuroprotective effects of orexin-A have a ceiling effect. The results of the neurologic function assessment also correlated with the reduction in infarct volume. Therefore, from the neurologic and histologic outcomes, we conclude that 30 μg/kg orexin-A treatment provides optimal neuroprotective effects. We also demonstrated these effects could exist as long as 7 days after MCAO regardless of administrative timing.

To evaluate the antiapoptotic effect of orexin-A treatment, TUNEL staining was performed in the penumbral cortex in the current study. Apoptotic cells appeared in the penumbral cortex at 2 h after ischemia reperfusion in the rat. At 24 and 72 h after reperfusion, an increased number of apoptotic neurons was observed in the control group as a result of ischemia-reperfusion injury. Administration of orexin-A at the onset of reperfusion significantly reduced the number of TUNEL-positive cells after reperfusion. These results indicate that orexin-A has an antiapoptotic function that may contribute to its neuroprotective effects.

HIF-1α is a transcription factor that has proven important for cell survival under hypoxic ischemia. HIF-1α increases the expression of HIF-1α target genes, such as vascular endothelial growth factor[37] and erythropoietin,[38] which

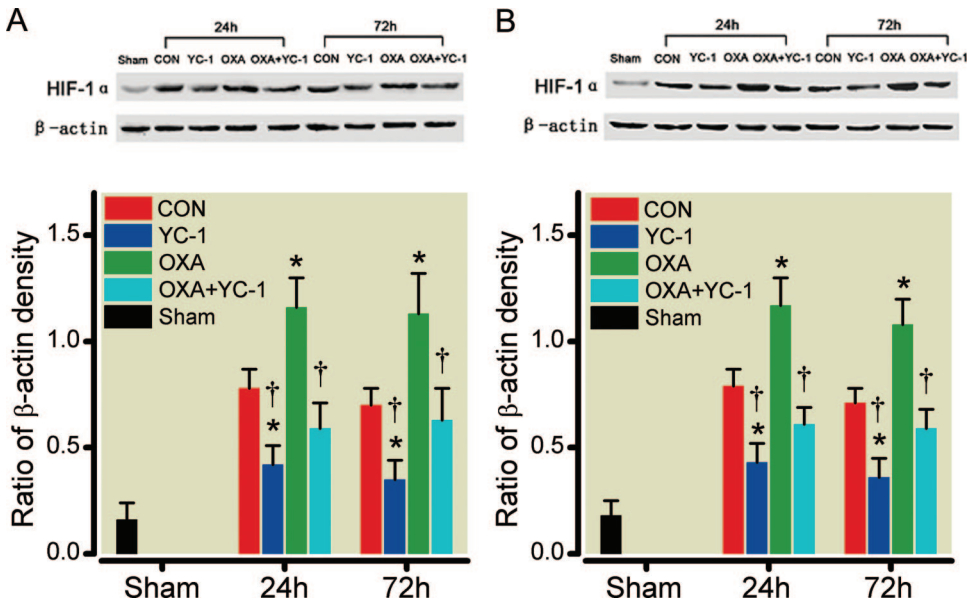

Fig. 12. Protein expression of hypoxia-inducible factor-1α (HIF-1α) in the hippocampus (*A*) and penumbral cortex (*B*) in sham, control (CON), YC-1(3-(50-hydroxymethyl-20-furyl)-1-benzylindazole, orexin-A (OXA), and orexin-A + YC-1 groups were evaluated by Western blot analysis. Representative bands and corresponding β-actin bands (*top*) and analysis of HIF-1α expression (*bottom*) are shown for each study group. Data are presented as mean ± SD. **P* < 0.05 *versus* control. †*P* < 0.05 *versus* orexin-A.

have been shown to mediate neuroprotective effects. HIF-1α–induced angiogenesis and glycolytic metabolism result in the increased delivery of oxygen and nutrition that is critical for neuron survival under hypoxic and ischemic conditions.[39] This protein has been linked to homeostatic regulation of metabolism,[40] a function common to orexins. In addition, an *in vitro* experiment[15] revealed that orexins can activate HIF-1α under normoxic conditions, and that this activation is partly the result of a down-regulation of vHL, an E3 ubiquitin ligase that mediates turnover of HIF-1α *via* the ubiquitin-proteasome pathway. Therefore, we wanted to know whether the neuroprotective effects induced by orexin-A are relevant to HIF-1α regulation.

As shown in the results of western blot analysis and immunohistochemistry, the expression of HIF-1α increased significantly in the penumbral cortex and hippocampus CA1 region at 2, 24, and 72 h after reperfusion in the control group when compared with the sham group. These findings are consistent with previous reports. Li *et al.*[41] have demonstrated that HIF-1α expression increased in the hippocampus and cortex at 6 h after ischemia, peaked at 24 h, and persisted for 96 h after reperfusion in a rat model of global ischemia. Another study[42] reported a marked increase in HIF-1α proteins by 20 h after focal cerebral ischemia reperfusion. It is noteworthy that we found that treatment with orexin-A at the onset of reperfusion resulted in a further enhancement of HIF-1α expression, which increased at 24 and 72 h after reperfusion as compared with the control group.

To further demonstrate the involvement of the HIF-1α pathway in the neuroprotective effects of orexin-A, HIF-1α inhibitor YC-1 was used. Our results showed that YC-1 significantly suppressed increased HIF-1α expression induced by orexin-A and reversed the neuroprotective effects of orexin-A. These findings support our hypothesis that the neuroprotective effects of orexin-A may be mediated by the up-regulation of HIF-1α.

Under normoxic conditions, HIF-1α protein concentrations are low as a result of proteasomal degradation by vHL. However, HIF-1α escapes from this degradation during hypoxia, a process that is recognized as the hypoxic pathway.[43,44] Recently, it was revealed that HIF-1α can also be induced *via* a nonhypoxic pathway by angiotensin II, tumor necrosis factor-α, and nitric oxide.[45–48] Kakinuma *et al.*[49] reported that in acute myocardial ischemia, vagal nerve stimulation increased HIF-1α expression and reduced infarct size. Therefore, it is speculated that neurons possess a similar system for regulating HIF-1α through orexin-A, independent of oxygen concentration. Our current results indicate that the neuroprotective effects of orexin-A against ischemic stress can be attributed to additional HIF-1α induction.

It has been demonstrated that orexin-A can induce HIF-1α expression by regulating vHL under normoxic conditions.[18] To explore whether orexin-A–induced increase of HIF-1α expression is accompanied with vHL regulation, we observed protein expression of vHL at 2, 24, and 72 h after reperfusion. Western blot analysis showed that vHL protein concentrations in the penumbral cortex and hippocampus in the control group did not change significantly after ischemia reperfusion when compared with the sham group. Nevertheless, vHL protein concentrations decreased at 24 and 72 h after reperfusion in rats treated with orexin-A. These results

revealed that orexin-A may reduce vHL protein expression after administration *in vivo*.

Our current study confirmed that increased HIF-1α concentrations, as triggered by orexin-A, have neuroprotective effects in acute transient cerebral ischemia. It is conceivable that HIF-1α induction is a powerful cellular response against hypoxic ischemia, and that additional induction of HIF-1α during ischemia *via* a nonhypoxic pathway could provide further protection. We also revealed that the increase of HIF-1α by orexin-A administration was related to a reduction in the expression of vHL.

There are some limitations to the current study. First, the underlying mechanism of how orexin-A modulates vHL expression remains unclear. Further studies are needed to demonstrate whether other factors, such as HIF-1α gene transcription and prolyl-hydroxylase activity, also contribute to HIF-1α accumulation. Second, although the intraluminal suture MCAO model is the most frequently used model among experimental ischemic stroke models, it has some shortcomings. Hyperthermia occurs in most animals subjected to suture occlusion of MCAO lasting 2 h or more. This phenomenon appears to be associated with hypothalamic injury. The stroke condition induced by this model is different from the clinical stroke situation in some aspects. Withdrawal of the intraluminal thread induces instantaneous reperfusion whereas spontaneous or thrombolysis-induced recanalisation results in slowly progressing recirculation. As postischemic recovery is greatly influenced by the dynamics of reperfusion, the outcome and pharmacologic responsiveness of transient filament occlusion is distinct from most clinical situations of reversible ischemia.

In summary, we have demonstrated for the first time that exogenous orexin-A can induce neuroprotective effects against cerebral ischemia–reperfusion injury. Furthermore, we have shown that the neuroprotection of orexin-A may be mediated through the HIF-1α pathway. Therefore, it is imperative to determine the mechanism of neuroprotection of orexin-A, which may be a novel neuroprotectant for future use. Further experiments using orexin-deficient animals will be extremely useful.

References

1. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M: Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998; 92:573–85
2. Nishino S: The hypocretin/orexin system in health and disease. Biol Psychiatry 2003; 54:87–95
3. Willie JT, Chemelli RM, Sinton CM, Yanagisawa M: To eat or to sleep? Orexin in the regulation of feeding and wakefulness. Annu Rev Neurosci 2001; 24:429–58
4. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E: A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med 2000; 6:991–7
5. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M: Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. Cell 1999; 98:437–51
6. Dong HL, Fukuda S, Murata E, Zhu Z, Higuchi T: Orexins increase cortical acetylcholine release and electroencephalographic activation through orexin-1 receptor in the rat basal forebrain during isoflurane anesthesia. ANESTHESIOLOGY 2006; 104:1023–32
7. Dong H, Niu J, Su B, Zhu Z, Lv Y, Li Y, Xiong L: Activation of orexin signal in basal forebrain facilitates the emergence from sevoflurane anesthesia in rat. Neuropeptides 2009; 43: 179–85
8. Nishino S, Kanbayashi T: Symptomatic narcolepsy, cataplexy and hypersomnia, and their implications in the hypothalamic hypocretin/orexin system. Sleep Med Rev 2005; 9:269–310
9. Culebras A: Cerebrovascular disease and sleep. Curr Neurol Neurosci Rep 2004; 4:164–9
10. Rousseaux M, Muller P, Gahide I, Mottin Y, Romon M: Disorders of smell, taste, and food intake in a patient with a dorsomedial thalamic infarct. Stroke 1996; 27:2328–30
11. Espiner EA, Leikis R, Ferch RD, MacFarlane MR, Bonkowski JA, Frampton CM, Richards AM: The neuro-cardio-endocrine response to acute subarachnoid haemorrhage. Clin Endocrinol 2002; 56:629–35
12. Payne RS, Tseng MT, Schurr A: The glucose paradox of cerebral ischemia: Evidence for corticosterone involvement. Brain Res 2003; 971:9–17
13. Dohi K, Ripley B, Fujiki N, Ohtaki H, Shioda S, Aruga T, Nishino S: CSF hypocretin-1/orexin-A concentrations in patients with subarachnoid hemorrhage (SAH). Peptides 2005; 26:2339–43
14. Irving EA, Harrison DC, Babbs AJ, Mayes AC, Campbell CA, Hunter AJ, Upton N, Parsons AA: Increased cortical expression of the orexin-1 receptor following permanent middle cerebral artery occlusion in the rat. Neurosci Lett 2002; 324:53–6
15. Dohi K, Nishino S, Nakamachi T, Ohtaki H, Morikawa K, Takeda T, Shioda S, Aruga T: CSF orexin A concentrations and expressions of the orexin-1 receptor in rat hippocampus after cardiac arrest. Neuropeptides 2006; 40:245–50
16. Nakamachi T, Endo S, Ohtaki H, Yin L, Kenji D, Kudo Y, Funahashi H, Matsuda K, Shioda S: Orexin-1 receptor expression after global ischemia in mice. Regul Pept 2005; 126: 49–54
17. Semenza GL: HIF-1, O(2), and the 3 PHDs: How animal cells signal hypoxia to the nucleus. Cell 2001; 107:1–3
18. Sikder D, Kodadek T: The neurohormone orexin stimulates hypoxia-inducible factor-1 activity. Genes Dev 2007; 21:2995–3005
19. 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
20. Garcia JH, Wagner S, Liu KF, Hu XJ: Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke 1995; 26:627–34
21. Belayev L, Khoutorova L, Deisher TA, Belayev A, Busto R, Zhang Y, Zhao W, Ginsberg MD: Neuroprotective effect of SolCD39, a novel platelet aggregation inhibitor, on transient middle cerebral artery occlusion in rats. Stroke 2003; 34: 758–63
22. Badr AE, Yin W, Mychaskiw G, Zhang JH: Effect of hyper-

baric oxygen on striatal metabolites: A microdialysis study in awake freely moving rats after MCA occlusion. Brain Res 2001; 916:85–90

23. Meguro T, Clower BR, Carpenter R, Parent AD, Zhang JH: Improved rat model for cerebral vasospasm studies. Neurol Res 2001; 23:761–6
24. Ashwal S, Tone B, Tian HR, Cole DJ, Pearce WJ: Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. Stroke 1998; 29:1037–46
25. Calvert JW, Yin W, Patel M, Badr A, Mychaskiw G, Parent AD, Zhang JH: Hyperbaric oxygenation prevented brain injury induced by hypoxia-ischemia in a neonatal rat model. Brain Res 2002; 951:1–8
26. Chen CT, Dun SL, Kwok EH, Dun NJ, Chang JK: Orexin A-like immunoreactivity in the rat brain. Neurosci Lett 1999; 260: 161–4
27. Suzuki R, Shimojima H, Funahashi H, Nakajo S, Yamada S, Guan JL, Tsurugano S, Uehara K, Takeyama Y, Kikuyama S, Shioda S: Orexin-1 receptor immunoreactivity in chemically identified target neurons in the rat hypothalamus. Neurosci Lett 2002; 324:5–8
28. Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK: Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol 2001; 435:6–25
29. Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA: Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 2001; 103:777–97
30. Ramanjaneya M, Conner AC, Chen J, Kumar P, Brown JE, Jöhren O, Lehnert H, Stanfield PR, Randeva HS: Orexin-stimulated MAP kinase cascades are activated through multiple G-protein signalling pathways in human H295R adrenocortical cells: Diverse roles for orexins A and B. J Endocrinol 2009; 202:249–61
31. Shin HS, Cho HS, Sung KW, Yoon BJ: Orexin-A increases cell surface expression of AMPA receptors in the striatum. Biochem Biophys Res Commun 2009; 378:409–13
32. Selbach O, Bohla C, Barbara A, Doreulee N, Eriksson KS, Sergeeva OA, Haas HL: Orexins/hypocretins control bistability of hippocampal long-term synaptic plasticity through co-activation of multiple kinases. Acta Physiol 2010; 198: 277–85
33. Peltonen HM, Magga JM, Bart G, Turunen PM, Antikainen MS, Kukkonen JP, Akerman KE: Involvement of TRPC3 channels in calcium oscillations mediated by OX(1) orexin receptors. Biochem Biophys Res Commun 2009; 385:408–12
34. Bassetti CL, Aldrich MS: Sleep electroencephalogram changes in acute hemispheric stroke. Sleep Med 2001; 2:185–94
35. Ripley B, Overeem S, Fujiki N, Nevsimalova S, Uchino M, Yesavage J, Di Monte D, Dohi K, Melberg A, Lammers GJ, Nishida Y, Roelandse FW, Hungs M, Mignot E, Nishino S: CSF hypocretin/orexin levels in narcolepsy and other neurological conditions. Neurology 2001; 57:2253–8
36. Kastin AJ, Akerstrom V: Orexin A but not orexin B rapidly enters brain from blood by simple diffusion. J Pharmacol Exp Ther 1999; 289:219–23
37. Jin KL, Mao XO, Greenberg DA: Vascular endothelial growth factor: Direct neuroprotective effect in *in vitro* ischemia. Proc Natl Acad Sci U S A 2000; 97:10242–7
38. Zaman K, Ryu H, Hall D, O'Donovan K, Lin KI, Miller MP, Marquis JC, Baraban JM, Semenza GL, Ratan RR: Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. J Neurosci 1999; 19:9821–30
39. Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR: Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol 2000; 48:285–96
40. Simon MC: Coming up for air: HIF-1 and mitochondrial oxygen consumption. Cell Metab 2006; 3:150–1
41. Li Y, Zhou C, Calvert JW, Colohan AR, Zhang JH: Multiple effects of hyperbaric oxygen on the expression of HIF-1 alpha and apoptotic genes in a global ischemia-hypotension rat model. Exp Neurol 2005; 191:198–210
42. Bergeron M, Yu AY, Solway KE, Semenza GL, Sharp FR: Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. Eur J Neurosci 1999; 11:4159–70
43. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ: The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399:271–5
44. Min JH, Yang H, Ivan M, Gertler F, Kaelin WG Jr, Pavletich NP: Structure of an HIF-1alpha -pVHL complex: Hydroxyproline recognition in signaling. Science 2002; 296:1886–9
45. Pagé EL, Robitaille GA, Pouysségur J, Richard DE: Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. J Biol Chem 2002; 277:48403–9
46. Richard DE, Berra E, Pouyssegur J: Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem 2000; 275:26765–71
47. Zhou J, Schmid T, Brüne B: Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. Mol Biol Cell 2003; 14:2216–25
48. Sandau KB, Fandrey J, Brüne B: Accumulation of HIF-1alpha under the influence of nitric oxide. Blood 2001; 97:1009–15
49. Kakinuma Y, Ando M, Kuwabara M, Katare RG, Okudela K, Kobayashi M, Sato T: Acetylcholine from vagal stimulation protects cardiomyocytes against ischemia and hypoxia involving additive non-hypoxic induction of HIF-1alpha. FEBS Lett 2005; 579:2111–8