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Intranasal administration of a PARG inhibitor profoundly decreases ischemic brain injury

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Abstract
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Keywords
Intranasal, administration, PARG, inhibitor, profoundly, decreases, ischemic, brain, injury

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Keywords: Ischemia; brain injury; poly(ADP-ribose) polymerase; poly(ADP-ribose) glycohydrolase; neuronal death; oxidative damage

Abstract

Objectives: Cumulative evidence has indicated a critical role of poly(ADP-ribose) polymerase-1 activation in ischemic brain damage. Poly(ADP-ribose) glycohydrolase (PARG) is a key enzyme in poly(ADP-ribose) catabolism. Our previous studies showed that PARG inhibitors, gallotannin (GT) and nobotanin B, can profoundly decrease oxidative cell death in vitro. Here, we tested the hypothesis that intranasal delivery of GT can decrease ischemic brain damage by inhibiting PARG. Intranasal delivery of GT within 5 hours after the onset of focal brain ischemia markedly decreased the infarct formation and neurological deficits of rats. The GT administration also increased poly(ADP-ribose) in the ischemic brains, suggesting that GT acts as a PARG inhibitor in vivo. Furthermore, the GT treatment abolished nuclear translocation of apoptosis-inducing factor (AIF) in the ischemic brains, suggesting that prevention of AIF translocation may contribute to the protective effects of GT. In contrast, intravenous injection of GT, at 2 hours after ischemic onset, did not reduce ischemic brain damage. Collectively, our findings suggest that PARG inhibition can significantly decrease ischemic brain injury, possibly by blocking AIF translocation. This study also highlights distinct merits of intranasal drug delivery for treating CNS diseases.

1. INTRODUCTION

Oxidative stress plays a critical role in ischemic brain damage (1). Many in vitro studies have shown that excessive poly(ADP-ribose) polymerase-1 (PARP-1) activation mediates the cell death induced by oxidative stress, the excitotoxin N-methyl-D-aspartate (NMDA), and oxygen-glucose deprivation (2-4). A number of in vivo studies have also reported that PARP-1 inhibition can decrease ischemic brain injury (2-4). Collectively, these in vitro and in vivo findings have indicated an important pathological role of PARP-1 in ischemic brain damage. Cumulative evidence has also suggested that PARP-1 is a significant pathological factor in several other neurological diseases, including Parkinson’s disease and brain trauma (3, 5, 6). The studies by us and other researchers have further indicated that NAD⁺ depletion, mitochondrial permeability transition and AIF translocation play critical roles in PARP-1-induced cell death (5-11).
PARP-1-generated poly(ADP-ribose) (PAR) is rapidly degraded to ADP-ribose by PARG - the pivotal enzyme for PAR catabolism (12). We have hypothesized that PARG inhibition may prevent PARP-1 toxicity by the following mechanisms (13, 14): First, PARG inhibition could slow the rapid PAR turnover thus preventing NAD⁺ depletion; and second, PARP-1 can auto-poly(ADP-ribosyl)ate itself, leading to PARP-1 inhibition (15). Therefore, PARG inhibition could prevent removal of PAR from PARP-1 leading to sustained PARP-1 activation. Moreover, several recent studies have suggested a novel mechanism by which PARG inhibition may decrease oxidative cell death: Intracellular ADP-ribose is capable of activating TRPM2 receptors (16), leading to Ca²⁺ influx and subsequent cell death. Therefore, PARG inhibition may also decrease cell death by blocking PARG-mediated generation of ADP-ribose from PAR. The previous in vitro studies of us and other researchers have suggested that PARG inhibition can decrease the cell death induced by various PARP activators (13, 14, 17-19). Based on these in vitro findings, it appears to be increasingly important to determine the roles of PARG in cell death in vivo. The major goal of our current study is to test our hypothesis that administration of GT - a PARG inhibitor - can decrease ischemic brain damage by blocking AIF translocation in a rat model of brain ischemia.

Due to the presence of the blood-brain barriers (BBB), most drugs can not be effectively delivered into the brain. A number of studies have suggested that intranasal drug delivery can produce neuroprotective effects in several animal models of CNS diseases (6, 20-23). However, there has been no sufficient study that directly compares the efficacy of intranasal drug delivery with that of intravenous (i.v.) drug injection in decreasing brain injury. Based on this information, another goal of our current study is to test our hypothesis that intranasal GT administration can profoundly decrease ischemic brain injury, while i.v. GT injection can not produce protective effects.

2. MATERIALS AND METHODS

2.1. Materials

Reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA) except where noted.

2.2. Focal brain ischemia

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Transient focal brain ischemia of male Sprague Dawley rats (250-280 grams) was produced by intraluminal middle cerebral artery occlusion (MCAo) with a nylon suture, as described previously (6). The rats were anesthetized by 2% isoflurane in 30% oxygen and 70% nitrous oxide. The rectal temperature of the rats was controlled at 37.0 ± 0.5°C during surgery by a feedback-regulated heating pad. The left external carotid artery was exposed by a midline skin incision, followed by electrocoagulation of its branches. A 20-mm 3-0 surgical monofilament nylon suture with a blunted end was introduced into the left internal carotid artery through the external carotid artery stump. Two hours (hrs) after MCAo, the blood flow was restored by withdrawal of the nylon suture.

2.3. Intranasal delivery of gallotannin
While the rat was lying on his back, six µl of GT stock solutions prepared in distilled water was applied to one side of the nose of the rat each time, while the other side of the nose was blocked for 5 seconds. This procedure was repeated every 2 minutes alternatively on each side of the nose, totally for 10 times.

2.4. Determinations of infarct volume

As described previously (6), rats were killed at either 24 or 72 hrs after ischemia. The brains were rapidly removed, which were carefully evaluated for macroscopic hemorrhagic changes before 2,3,5-triphenyltetrazolium chloride (TTC) staining. TTC staining has been widely used for determinations of infarct volume either at 24 hrs or later time points after ischemia (24, 25). The brain tissue was sliced into coronal sections with 2-mm-thickness by a rodent matrix. The brain slices were stained in 2% TTC for 20 min, which were subsequently immersed in 4% paraformaldehyde (PFA). Six coronal sections per rat were scanned for determinations of infarct volume. Striatal and cortical areas of infarction were measured and analyzed by Image J (NIH) version 1.63.

2.5. Determinations of neurological deficits

Neurological deficits were evaluated at either 24 or 72 hrs after ischemia as described previously (6, 26). The following grading system was used to determine motor neurological deficits: Grade 0 (no observable deficit); Grade 1 (forelimb flexion); Grade 2 (forelimb flexion with decreased resistance to lateral push); Grade 3 (forelimb flexion with decreased resistance to lateral push in addition to unilateral circling); and Grade 4 (forelimb flexion and difficulty or inability to ambulate).

2.6. PAR immunostaining

PAR immunostaining was conducted as previously described (14, 27). Rats were sacrificed at various time points after ischemia/reperfusion, which were transcardially perfused with 0.9% saline solution, followed by perfusion with 4% PFA for 10 min. The brains were fixed in 4% PFA for 1 hr, followed by incubation in 30% sucrose for 24 hrs. Coronal sections with 60 µm thickness were prepared and immunostained. The sections were blocked and permeabilized with phosphate buffered saline containing 10% goat serum and 0.1% Triton X-100, which were subsequently incubated with monoclonal anti-PAR antibody (Trevigen, Gaithersburg, MD) at 1:2000 dilution at 4°C overnight. After three washes, the sections were incubated with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, Eugene, OR) at 1:500 dilution at room temperature (RT) for 1 hr. After counterstaining with 0.5 g/ml propidium iodide for 5 min at RT, the slices were placed on coverslips and mounted with Prolong Antifade agent. The sections were photographed under a Leica confocal laser-scanning microscope.

2.7. AIF immunostaining

As reported in our previous studies (27), PFA-fixed brains were cut into slices with thickness of 60 m in a cryostat. The brain slices were permeabilized, and nonspecific protein binding was blocked by 1-hr incubation with a blocking buffer containing 10% goat serum and 0.1% Triton X-100 at RT. Slides were then incubated with rabbit anti-AIF antibody (Sigma, St Louis, MO) at 1:1500 dilution at 4 °C for 24 hrs. After washes, the slices were incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen, Eugene, OR) at 1:250 dilution at RT for 2 hrs. After counterstaining with 0.5 µg/ml propidium iodide for 5 min at RT, the slices were placed on coverslips and mounted.
with Prolong Antifade agent. The sections were photographed under a Leica confocal laser-scanning microscope.

**Figure 1:** Intranasal GT administration significantly decreased ischemic brain damage assessed at 24 hrs after ischemia / reperfusion (I/R). Immediately after 2-hr ischemia, rats received intranasal administration of 25 mg / kg GT. Twenty four hrs after I/R, the neurological deficits of the rats were assessed. Subsequently the rat brains were collected, which were used for determinations of infarct volume by TTC staining. As shown in the photographs of the brain sections (Figure 1A), the TTC-stained, red colored tissues are the uninjured tissues, while the white colored tissues are the infarct tissues. Massive infarct formation was found in the rat brains that received I/R only (the upper panel of Figure 1A). In contrast, decreased infarct formation was found in the brain sections from the rats that received both I/R and the intranasal GT administration (the bottom panel of Figure 1A). Figure 1B shows the quantifications of the infarct volume of the rats. The intranasal GT administration also significantly decreased neurological deficits of the rats (Figure 1C). N = 8 - 10; data are Mean ± SE; *, p < 0.05; ** p < 0.01.

2.8. Statistical analyses

All data are presented as means ± standard error (SE). Data were assessed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. p values less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Effects of intranasal GT administration on ischemic brain damage

We used a rat MCAo model of brain ischemia to determine the effects of GT administration on ischemic brain injury. Our pilot studies suggested that intranasal administration of GT at 50 mg / kg may alter the physiological parameters of the rats that have received MCAo, when GT was administered immediately after reperfusion (i.e., 2 hrs after ischemic onset). Thus, in this study we
determined the effects of intranasal administration of 25 mg / kg GT immediately after reperfusion on various major physiological parameters of rats, including body temperature, blood pressure, CO₂ levels and pH. There was no any statistically significant difference in these parameters between the rats that received ischemia only and the rats that received both ischemia and the GT administration, when the measurements were conducted at 1 hr prior to ischemic onset, 1 hr after ischemic onset and 1 hr after reperfusion (not shown).

In order to obtain comprehensive information regarding the effects of intranasal administration of GT on ischemic brain injury, we determined the effects of the GT administration on ischemic brain injury under the following three experimental protocols: First, intranasal administration of GT was conducted at 2 hrs after ischemia, and the ischemic brain injury of the rats was determined at 24 hrs after ischemia (i.e., 22 hrs after reperfusion); second, intranasal administration of GT was conducted at 2 hrs after ischemia, and the ischemic brain injury of the rats was determined at 72 hrs after ischemia; and third, intranasal administration of GT was conducted at 5 hrs after ischemia, and the ischemic brain injury of the rats was determined at 72 hrs after ischemia.

Our study using the first experimental protocol showed that intranasal administration of GT at 2 hrs after ischemia significantly decreased ischemic brain injury, when assessed at 24 hrs after ischemia: The ischemia/reperfusion (I/R) produced massive infarct formation in both striatum and cortex of the rat brains, which was markedly decreased by intranasal administration of 25 mg / kg GT (Figure 1A). Quantifications of the infarct volume show that the GT administration decreased infarct volume by nearly 70% (Figure 1B). Consistent with its effects on infarct volume, the intranasal GT administration also significantly decreased the I/R-induced neurological deficits (Figure 1C).
Figure 2: Intranasal GT administration significantly decreased ischemic brain damage assessed at 72 hrs after I/R. Immediately following 2-hr ischemia, a subset of rats received intranasal administration of 25 mg / kg GT. Seventy two hrs after ischemia, the neurological deficits of the rats were assessed. Subsequently the rat brains were collected, which were used for determinations of infarct volume by TTC staining. Massive infarct formation was found in the rat brains that received I/R only, which was significantly decreased by the intranasal administration of 25 mg / kg GT (Figure 2A). The GT administration also significantly decreased the neurological deficits of the rats (Figure 2B). N = 8 - 10; data are Mean ± SE; *, p < 0.05; ** p < 0.01.

Our study using the second experimental protocol also showed that intranasal administration of GT at 2 hrs after ischemia can significantly decrease ischemic brain injury, when assessed at 72 hrs after ischemia: Quantifications of the infarct volumes indicate that intranasal administration of 25 mg / kg GT decreased infarct volume by nearly 60% (Figure 2A), and significantly attenuated neurological deficits (Figure 2B). Our study using the third experimental protocol further showed that intranasal administration of GT at 5 hrs after ischemia significantly decreased the extent of infarct formation and neurological deficits, when assessed at 72 hrs after ischemia (Figure 3A and Figure 3B).

3.2. Effects of intravenous GT administration on ischemic brain damage

To obtain information for directly comparing the efficacy of intranasal GT administration with that of i.v. injection of GT on ischemic brain damage, we determined the effects of i.v. injection of GT at 2 hrs after ischemia on ischemic brain injury: Injection of GT at 25 mg / kg led to significantly increased death rate of rats, while i.v. injection of 12.5 mg / kg GT did not reduce ischemic brain injury (Figure 4). These results indicate that i.v. injection of GT can not produce protective effects against ischemic brain damage.

Figure 3: Intranasal GT administration at 5 hrs after ischemic onset significantly decreased the extent of infarct formation and neurological deficits of the rats that received I/R. Five hrs after ischemic onset (i.e., 3
hrs after reperfusion), rats received intranasal GT administration. Seventy two hrs after I/R, the neurological deficits of the rats were assessed. Subsequently the rat brains were collected, which were used for determinations of infarct volume by TTC staining. Intranasal GT administration significantly decreased infarct volume induced by I/R (Figure 3A). The GT administration also significantly decreased neurological deficits (Figure 3B). N = 8 - 10; data are Mean ± SE; *, p < 0.05; ** p < 0.01.

3.3. Effects of intranasal GT administration on PAR levels and AIF translocation in rat brains after ischemia-reperfusion

To determine if the intranasal GT administration can produce PARG inhibition in vivo, we assessed the effects of intranasal GT administration at 2 hrs after ischemia on PAR levels in the rat brains at various time points after reperfusion: We found significantly increased PAR immunostaining (green fluorescence) at 4 hrs after reperfusion in the penumbra area of the rat brains, which persisted for at least 12 additional hrs (Figure 5). The PAR was mainly localized at the cell nuclei that were stained by propidium iodide (red fluorescence). Interestingly, we found that the intranasal GT administration remarkably increased PAR immunostaining in the rat brains, at 4 and 16 hrs after reperfusion (Figure 5). These results provide the first evidence that GT acts as a PARG inhibitor in vivo.

Because mitochondrial alterations, including AIF translocation, could play significant roles in PARP-1 toxicity and ischemic brain damage (10, 11, 28), we determined the effects of intranasal GT administration on nuclear translocation of AIF in the rat brains after I/R. Assessed at 4 and 16 hrs after reperfusion, there was extensive translocation of AIF (green fluorescence) into the cell nuclei (red fluorescence) in the penumbra area of the rat brains that underwent I/R. The intranasal administration of GT at 25 mg / kg nearly abolished the AIF translocation (Figure 6).

Figure 4: Intravenous GT administration could not decrease ischemic brain damage of rats. Immediately following 2-hr ischemia rats received i.v. injection of 12.5 mg / kg GT. Seventy two hrs after ischemia, the neurological deficits of the rats were assessed. Subsequently the rat brains were collected, which were used
for determinations of infarct volume by TTC staining. The GT administration did not significantly reduce the extent of infarct formation and neurological deficits of the rats. N = 5; data are Mean ±SE.

4. DISCUSSION

There are four pieces of major findings from this study: First, intranasal GT administration can significantly decrease ischemic brain damage with extended window of opportunity; second, the intranasal GT administration can completely prevent I/R-induced AIF translocation in rat brains; third, GT acts as a PARG inhibitor in vivo; and fourth, intranasal delivery of GT is markedly more effective than i.v. injection of GT in reducing ischemic brain injury. Collectively, our findings have three major theoretical and therapeutic implications: First, PARG inhibition can decrease ischemic brain damage with extended window of opportunity, possibly by blocking AIF translocation; second, the intranasal delivery of GT may be used in clinical settings to decrease ischemic brain injury; and third, our study provides direct evidence suggesting that intranasal drug delivery is a more effective approach of drug delivery than i.v. drug injection for treating cerebral ischemia.

PARP-1 is a nuclear enzyme that catalyzes poly(ADP-ribosyl)ation of target proteins by consuming NAD⁺ (5). Protein poly(ADP-ribosyl)ation plays critical roles in various biological functions, including DNA repair and regulation of gene expression, cell cycle and genomic stability (15). PARG is the key enzyme that degrades the PAR formed on poly(ADP-ribosyl)ated proteins (12, 15). While there are only insufficient studies regarding PARG, multiple studies have suggested that PARG plays significant roles in regulation of gene expression, cell cycle and cell differentiation (29-31). Our previous studies provided first in vitro evidence that PARG may be a new target for decreasing oxidative cell death (13, 14). A number of studies using various structurally different PARG inhibitors, including GPI 18214 (32), GPI 16552 [N-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide] (33), GT and nobotanin B (13, 14, 17, 18, 34), have supported our hypothesis that PARG inhibition may be a new strategy for preventing oxidative cell death (5, 35); GT and nobotanin B can decrease cell death induced by oxidative stress and other PARP activators in vitro (13, 14, 17, 18, 34); the PARG inhibitor GPI 16552 can reduce spinal cord injury (36); and the PARG inhibitor GPI 18214 is also beneficial for septic shock-like syndrome (32) and inflammatory bowel disease (37). Protective effects of PARG inhibition have also been found in several studies in which PARG activity is decreased by RNA silencing or PARG antisense oligonucleotides: Decreases of PARG levels by RNA silencing (38) or PARG antisense oligonucleotides (19) led to decreases in PARP-1-mediated cell death. Of particular interest, a latest study using a cell model that has increased PARG activity has further suggested that PARG inhibition could be protective: The increased PARG activity was found to accelerate NAD⁺ depletion and increase cell death (39). Our current study has provided first direct evidence that GT acts as a PARG inhibitor in vivo, which can profoundly decrease ischemic brain damage at least partially by blocking AIF translocation. Thus, our findings have provided new information for understanding the roles of PARG in tissue injury in vivo.

It is noteworthy that there have been variable results from the studies using PARG knockout mice: Compared with wild type mice, the mice that have genetic deletion of the 110 kDa PARG isoform showed significantly decreased spinal cord injury (36) and ischemic damage of intestine (40) and kidney (41). However, there are also studies suggesting that genetic deletion of PARG could produce detrimental effects (42, 43). Precaution may be needed in interpreting the results from the studies using PARG knockout mice for the following reasons: PARG can significantly affect gene
expression and other biological properties (29-31); and PARG gene is closely associated with the inner mitochondrial membrane translocase 23 (TIM23) gene (44). Indeed, it has been shown that there are significant changes of the gene expression of heat shock protein 70 and cyclooxygenase 2 -- two important proteins in cell survival --- in PARG knockout mice (42). Therefore, future studies that use conditional PARG knockout mice may be necessary for further elucidating the role of PARG in cell death.

GT has been shown to be a PARG inhibitor in both test tube assays and cell culture studies (14, 17). However, there has been no in vivo study demonstrating that certain drugs can act as PARG inhibitors in vivo. Our current study provides the first evidence that GT can act as a PARG inhibitor in vivo. In our study there was significant PAR immunostaining in the rat brains at 4-16 hrs after reperfusion, which was significantly increased by the GT administration. This observation, together with our findings that GT can significantly decrease ischemic brain damage and abolish AIF translocation, suggests that GT acts as a PARG inhibitor in vivo.

Our study also argues against the possibility that GT decreases ischemic brain damage by acting as a free radical scavenger or a PARP inhibitor. We have found that the intranasal GT administration increased PAR formation in vivo, which is completely different from the expected effects of free radical scavengers or PARP inhibitors on the PAR immunostaining: Both free radical scavengers and PARP inhibitors should decrease the PAR immunostaining. Increased PAR immunostaining has been widely used as a major index for increased PARP activity. However, our current results challenge the validity of this approach for assessing PARP activity: Increased PAR immunostaining can be produced not only by increased PARP activation, but also by PARG inhibition in the absence of increased PARP activation.
**Figure 5**: Intranasal GT administration significantly increased PAR immunostaining in the rat brains after I/R. At 4 and 16 hrs after reperfusion, the rat brains were collected, which were immunostained with anti-PAR antibody and PI. The immunostained brain sections were photographed under a confocal microscope at 40X amplification. There was significant PAR staining (green fluorescence) in the cell nuclei (red fluorescence) in the penumbra area of the rats at 4 (Figure 5A) and 16 hrs (Figure 5B) after reperfusion. The yellow color indicates the overlay of the green fluorescence and the red fluorescence. The images are representatives of the images of the samples prepared from at least two independent experiments.
Figure 6: Intranasal GT administration significantly increased nuclear translocation of AIF in the rat brains after I/R. At 4 and 16 hrs after ischemia, the rat brains were collected, which were immunostained with anti-AIF antibody and PI. The immunostained brain sections were photographed under a confocal microscope at 40X amplification. There was significant AIF staining (green fluorescence) in the cell nuclei (red fluorescence) in the penumbra area of the rats at 4 (Figure 6A) and 16 hrs (Figure 6B) after reperfusion. The yellow color indicates the overlay of the green fluorescence and the red fluorescence. The images are representatives of the images of the samples prepared from at least two independent experiments.
AIF is a FAD-binding protein that localizes at mitochondria under normal conditions (45). Under various cell death-inducing conditions, AIF is translocated into cytosol and cell nucleus, which can lead to large-scale DNA condensation and some other apoptotic changes (45). AIF translocation has been indicated as an important factor mediating PARP-1-induced cell death in cell culture studies (11). Multiple in vivo studies have also shown that ischemic insults induce AIF translocation, which precedes neuronal death (46-48). It has also been reported that TAT-mediated delivery of Bcl-xL protein can decrease neonatal hypoxic-ischemic brain injury via inhibition of AIF and caspases (28). Several studies have further suggested that PARP activation contributes to I/R-induced AIF translocation (49, 50). Collectively, these results have suggested that ischemic insults could induce AIF translocation by activating PARP, which may contribute to ischemic brain damage. Our current study shows that intranasal GT administration can nearly abolish the AIF translocation in the rat brains following I/R, which is the most profound blockage of AIF translocation ever reported in vivo studies. Based on this information, our findings suggest that GT administration could decrease ischemic brain injury at least in part by preventing AIF translocation.

One of the critical challenges for treating CNS diseases is the limitations of drug delivery into the brains by the BBB. Increasing evidence suggests that intranasal drug delivery can deliver drugs into the brain both indirectly through the blood and directly along both olfactory and trigeminal neural pathways which allow drugs to bypass the BBB (20-23). It has been suggested that the cells in olfactory regions, including olfactory receptor cells, may play critical roles in the drug entrance into the brains (20-23). A number of studies have found that the drug delivery by the intranasal approach can decrease brain injury in several models of neurological diseases (6, 20-23). Our latest study has shown that intranasal NAD⁺ delivery at 2 hrs after ischemic onset can decrease ischemic brain damage by nearly 90% (6).

Intranasal drug delivery approach could have multiple merits over traditional drug delivery approaches: It may decrease the amount of drugs that is needed to affect the CNS, which could significantly decrease the cost of treatments. This approach may also be helpful for studying the mechanisms of drug effects on the brain, in light of the increasing evidence suggesting contributions of the alterations of peripheral systems to CNS diseases (51-52): It could decrease the probability that the protective effects of certain drugs on the CNS may result from the drug effects on peripheral systems. One of the key goals of our current study is to use GT as a model drug to directly compare the efficacy of intranasal drug delivery with that of i.v. drug injection in decreasing ischemic brain damage.

Our study demonstrates that intranasal GT administration can produce profound protective effects against ischemic insults, while i.v. injection of GT cannot decrease ischemic brain injury. Therefore, our study strongly supports the proposition that intranasal drug delivery may be used for treating cerebral ischemia and other CNS diseases under clinical settings.

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