Pregnenolone Attenuates the Ischemia-Induced Neurological Deficit in the Transient Middle Cerebral Artery Occlusion Model of Rats
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ABSTRACT: Neurosteroids are apparent to be connected in the cerebral ischemic injury for their potential neuroprotective effects. We previously demonstrated that progesterone induces neuroprotection via the mitochondrial cascade in the cerebral ischemic stroke of rodents. Here, we sought to investigate whether or not pregnenolone, a different neurosteroid, can protect the ischemic injury in the transient middle cerebral artery occlusion (tMCAO) rodent model. Male Wistar rats were chosen for surgery for inducing stroke using the tMCAO method. Pregnenolone (2 mg/kg b.w.) at 1 h postsurgery was administered. The neurobehavioral tests and (TTC staining) 2, 3, 5-triphenyl tetrazolium chloride staining were performed after 24 h of the surgery. The mitochondrial membrane potential and reactive oxygen species (ROS) were measured using flow cytometry. Oxygraph was used to examine mitochondrial bioenergetics. The spectrum of neurobehavioral tests and 2, 3, 5-triphenyltetrazolium chloride staining showed that pregnenolone enhanced neurological recovery. Pregnenolone therapy after a stroke lowered mitochondrial ROS following ischemia. Our data demonstrated that pregnenolone was not able to inhibit mitochondrial permeability transition pores. There was no effect on mitochondrial bioenergetics such as oxygen consumption and respiratory coupling. Overall, the findings demonstrated that pregnenolone reduced the neurological impairments via reducing mitochondria ROS but not through the inhibition of the mitochondria permeability transition pore (mtPTP).

INTRODUCTION
Stroke is still one of the leading causes of mortality and impairment around the globe, affecting millions of people. Ischemic stroke occurs when blood supply to the brain is suddenly interrupted, resulting in cell death and damage, as well as neurological deficits. Despite the plethora of research and a significant number of encouraging outcomes in many laboratories using rodent models of stroke, no medicine or preventive agent has been proven to be useful in the treatment of ischemic stroke. Despite the plethora of research and a significant number of encouraging outcomes in many laboratories using rodent models of stroke, no medicine or preventive agent has been proven to be useful in the treatment of ischemic stroke. The current therapeutics is limited to thrombolytics, which is only applicable to certain cases of stroke patients. Only one medicine is currently approved for usage in clinical settings, and novel therapies that provide ischemia neuroprotection are badly needed. Because of the scarcity of treatment alternatives, only around 10% of stroke patients can benefit from them. Thus, the development and discovery of new therapeutics is a challenge for researchers in the field of ischemic stroke. In addition, there is an urgent need for finding new therapeutics that can have higher translational efficacy for ischemic stroke-related deficits.

There are plenty of compounds that have been explored in ischemic stroke; still there is no effective treatment that is currently available. Neurosteroids have drawn strong attention because of their positive results at both preclinical and clinical levels. A number of neurosteroids have produced some promising results that have made them attractive for further investigations. Nerve cells manufacture neurosteroids by a process known as neurosteroidogenesis, which is regulated by numerous steroidalogenetic enzymes found in different parts of the brain. Out of these neurosteroids, (progesterone) P4, (pregnenolone) P5, and (allopregnanolone) ALLO are thought to have immense neuroprotective potential. Previously, we have demonstrated the role of P4 in ischemic stroke and its mechanism via the mitochondria.7 Our results have revealed that P4 exerts neuroprotection by ameliorating the mitochondrial functions such as the electron transport chain (ETC), mitochondrial membrane potential, and mitochondrial bioenergetics and also inhibits the mtPTP.7 These results on P4 prompted us to investigate the possible...
neuroprotective role of P5 in ischemic stroke. P5 is one of the pivotal inactive precursors of all neurosteroids, and its potential functional benefits have not been well explored. It is a steroid through which other essential neurosteroids are synthesized in the cells. Baulieu and his colleagues were the first who ascertained the neuronal synthesis of neurosteroids.8 P5 is synthesized by the cleavage reaction of cholesterol in various brain cells. P5 is one of the crucial neurosteroids, where from other neurosteroids are synthesized such as P4, ALLO, estrone, and so forth. P5 was originally thought to be produced only in the adrenal glands and gonads; later, in mouse hippocampus cells, it was discovered to have neuroprotective properties against glutamate and amyloid beta protein neurotoxicity.9 The release of steroid hormones and their functions in myelin synthesis and repair in both the central and peripheral nerve systems have long been known. Schwann cells were found to be a key producer of steroid hormones in the peripheral nervous system, and P5 synthesis by P450scc was a crucial regulatory step during myelination.10 Furthermore, P5 reduced subsequent histological changes in vivo after spinal cord damage, protected neural tissue from secondary lesions, and improved motor function recovery. P5 may affect the neuronal cytoskeleton dynamics via binding to microtubule-associated protein 2. This neuroprotective effect could be due to P5's direct action on the spinal cord neurons (MAP2).11 However, the significance of P5 in ischemic stroke has not been dogged yet. P5’s mitochondria-mediated neuroprotective effects in rat ischemic stroke were investigated in the current research. In rats given the transient middle cerebral artery occlusion (tMCAO) model, a model of focal ischemia, we looked at the impact of P5 on the size of the infarction, the neurobehavioral outcome, and the mitochondrial function.

RESULTS

Effect of P5 on Neurological Deficit. There was a significant ($P < 0.001$) increase in neurological deficit performance in the tMCAO group as matched to the sham group (Figure 1A). The administration of the P5 attenuated the neurological deficit significantly ($P < 0.05$) in the treated group as compared to only the tMCAO group.

Effect of P5 on Grip Strength. We investigated at grip strength to see whether P5 could help with neurological impairments, following tMCAO surgery. The grip strength of the sham, tMCAO, and P5-administered groups was scored in the first test. Grip strength declined significantly ($P < 0.001$) in rodents subjected to tMCAO as compared to the sham group. The repeated administration of P5 improved the grip strength ($P < 0.05$) as compared to the tMCAO alone group (Figure 1B).

Effect of P5 on Motor Coordination. Following that, we measured the duration spent on the rota rod apparatus (in seconds) and took the average of three trials for each rat. There was a substantial ($P < 0.001$) decrease in the rota rod performance in the tMCAO group as compared to the sham group (Figure 1C). The administration of P5 improved the rota rod performance significantly ($P < 0.05$) in the treated group as compared to only the tMCAO group (Figure 1C).

Effect of P5 on Adhesive Tape Removal. Animals took longer to remove the adhesive tape from the damaged forelimb, reaching a peak of 24 h after the stroke (Second) (Figure 1D). There was a substantial difference [$F (2, 28) = 13.41, P = 0.001$] among groups (Figure 1D). Post stroke treatment with P5 rats take less time to remove tape with a dose of 2 mg/kg ($P < 0.05$) (Figure 1D).
Effect of P5 on the Infarct Volume after tMCAO Injury. TTC staining is a rapid and reliable approach for determining infarction volume in ischemic stroke models. In comparison to just tMCAO rats, brain slices from the P5-treated tMCAO group after 24 h stained with TTC exhibited a substantial (P < 0.05) reduction in the infarction volume (Figure 2A–C).

Effect of P5 on Mitochondrial Complex Enzymes. The enzyme mitochondrial complex I (NADH dehydrogenase) is required for ETC and is unregulated in ischemia-induced mitochondrial change. P5 therapy massively improved the NADH dehydrogenase activity (P < 0.05) when compared to tMCAO surgery rats, who showed a significant (P < 0.001) reduction in the NADH dehydrogenase activity as compared to the sham group (Figure 3A). Complex II (succinate dehydrogenase), another ETC enzyme, displayed a similar pattern and was depleted because of ischemia damage. The tMCAO group had significantly reduced the enzyme activity of complex II than the sham group (P < 0.001). P5 therapy significantly (P < 0.05) boosted complex II activity in the treated group as compared to the tMCAO group (Figure 3B). To test the influence on mitochondrial ETC, we used complex III (MTT decrease frequency). Cell viability was greatly enhanced (P < 0.05) in the P5 group, but dramatically decreased (P < 0.001) in the tMCAO group (Figure 3C). Complex V (ATPase synthase) activity is also suppressed in mitochondrial change caused by ischemia injury. The P5 group’s ATPase synthase levels were greatly (P < 0.05) restored, but the tMCAO surgery rats’ levels were dramatically (P < 0.001) reduced (Figure 3D).

Effect of P5 on Mitochondrial ROS. DCF fluorescence was used to detect mitochondrial reactive oxygen species (ROS). Changes in the dichlorofluorescin (DCF) fluorescence intensity were used to measure ROS generation in the sham, tMCAO, and tMCAO + P5 groups (Figure 4A–C). The tMCAO group had a significantly higher amount of mitochondrial ROS than the sham group (P < 0.001). In contrast to the sole tMCAO group, P5 therapy considerably (P < 0.05) reduced mitochondrial ROS levels, as measured by DCF fluorescence intensity (Figure 4D).

Effect of P5 on MMP. The MMP is expressed as a TMRE fluorescence measurement. Alteration in tetramethylrhodamine, ethyl ester (TMRE) fluorescence reflected changes in MMP in sham, tMCAO, and tMCAO + P5 groups (Figure 5A–C). In the tMCAO group, there was a significant (P < 0.001) reduction in MMP, as evidenced by low fluorescence intensity, when compared to the sham group. In comparison to the tMCAO alone group, P5 was unable to regulate MMP, as evidenced by TMRE fluorescence intensity (Figure 5D).

Effect of P5 on Mitochondrial Swelling and Mitochondrial Bioenergetics. After 24 h of tMCAO, the effect of P5 on mitochondrial oxygen consumption (state 3 respiration) and the respiratory control ratio (RCR) was studied. When tMCAO animals were compared to sham animals, oxygen consumption was shown to be lower (P < 0.05) (Figure 6A). Oxygen consumption was not appreciably reduced by P5. In addition, when tMCAO animals were compared to the sham group, the RCR was lower (P < 0.001). P5 was also unable to significantly restore the RCR in tMCAO animals (Figure 6B). Before adding Ca2+, there was no discernible difference in light transmission between any of the groups. After adding Ca2+ at a concentration of 400 m, the baseline value was measured for 5 min. With the addition of Ca2+, there was a substantial (P < 0.001) decrease in light transmission in the tMCAO group compared to the sham group. P5 treatment had no effect on mitochondrial swelling (P < 0.05) when compared to the tMCAO group alone (Figure 6C).

DISCUSSION

We studied the putative mitochondrial mechanism behind P5-enabled neuroprotection in cerebral ischemia in the current work. P5, as a parent neurosteroid, has the potential to influence the release of numerous neurotransmitters.5,9 It may affect the neuronal activity by inhibiting the gamma-amino butyric acid (GABA) receptors. It has been demonstrated that P5 attenuates glutamate neurotoxicity in adult rats.10 P5 affects myelin formation and repair in both the central and peripheral neurological systems, according to research.11,12 Moreover, P5 alleviates the histopathological changes in the spinal cord injury that elicits the neurological recovery.13 P5 is implicated in neuroprotection, strain, anxiety, depression, psychosis-related disorders, and addiction processes with a significant impact on cannabinoid-related dysfunctions, according to convergent evidence from animal studies and human clinical research.8 Here, we investigated the neuroprotective role of P5 in the ischemic stroke of rats. We used different neurobehavioral tests to validate our results. To underpin the possible role of P5 via the mitochondria, we used flow cytometry and oxygraph techniques in our study.

P5 promotes neurological recovery in ischemic stroke rats, according to our findings. To support the existence of neurological abnormalities associated with cerebral ischemia and the action of P5, we conducted a number of behavioral experiments in rats. P5’s effect on neuronal processes that are crucial for neurological functioning may explain why ischemia-induced neurological deficits were reduced. P5 has been shown to be a cognitive enhancer, capable of directly stimulating neuronal activation in brain regions important for cognitive function.14–16 In humans, muscle or motor dysfunction is a
common complaint following a stroke. Our findings showed that ischemia causes severe motor coordination impairment, which was alleviated by the P5 therapy. P5 sulfate has previously been shown to normalize extracellular GABA and the glutamate-NO-cGMP pathway activity in the cerebellum of hyperammonemic rats, resulting in improved motor coordination.17 P5 has been shown to enhance motor coordination and a variety of other neurological impairments, including spinal cord damage, according to prior findings from other study groups.11 The effect of P5 injection after occlusion on grip strength in tMCAO rats was also examined. P5 was able to reduce the muscle damage caused by ischemia. With P5, sensory function was also consolidated, as treated rats were able to remove the sticky tape in shorter time. These findings are consistent with prior findings that P5 improves neurological disability.8 Intranasal injection of P5 enhances memory recovery in mice, according to previous research.18,19 P5 has been effective in enhancing the release of acetylcholine and increases the spatial memory in rats.20,21 To confirm that infarct volume is a measure of how serious the ischemia damage is, TTC staining was utilized, which accurately identifies the infarct volume. Because of ischemia, the rats given tMCAO had a considerable amount of lesioned regions.22 In the frontotemporal areas of the cerebral cortex, ischemia caused neuronal death. The administration of P5 was able to reduce the infarct volume in the peripheries of the frontotemporal areas. Other studies have revealed that P5 inhibited the neuronal death, but the mechanism has not been thoroughly elucidated.13 The fact that these behavioral and TTC findings coupled in with flow cytometry data, resulting in a reduction in mitochondrial ROS in the frontal cortex part of the rat brain, could be the explanation. Ischemia-induced brain increased mitochondrial ROS and weakened ETC complexes, affecting mitochondrial constituents and ROS accumulation.23 Mitochondrial ROS, which impacts mitochondrial function and is one of the hallmarks of reperfusion injury, was found in the frontal cortex region of the mitochondria.24,25 P5 treatment reduced mitochondrial ROS, boosting the activity of the ETC complexes in stroke rats. P5’s antioxidant properties appear to have scavenged ROS in the mitochondria and refilled the ETC complex enzymes. We used TMRE and Ca2+ induced swelling to investigate the influence on mtPTP in frontal cortex-isolated mitochondria. P5 treatment after stroke had no effect on mitochondrial edema and had no effect on the mitochondrial membrane potential. The effects could be explained by the participation of GABAergic mechanisms.17,26 In our previous studies, we have determined that P4 inhibits the mtPTP in ischemic stroke, as its parent compound P5 could not inhibit the mtPTP. P5 metabolites such as P4 and ALLO block the mtPTP, preventing the discharge of cytochrome c from mitochondrial cascade, according to prior research. P4 and ALLO show binding affinity for the mtPTP, according to cumulative evidence from patch clamp and flow cytometry.7,27 We believe that P5 does not have a high affinity for mtPTP, but more research is needed to fully understand the mechanism. Cerebral oxidative metabolism produces ATP, carbon dioxide, and water mostly from oxygen and glucose.28 The uncoupling of oxidative phosphorylation and ETC causes dysregulation of mitochondrial bioenergetics, which is the first stage in ischemia. Changes in mitochondrial activities, such as reduced electron transport chain activity, adenosine diphosphate (ADP)-stimulated mitochondrial respiration, and oxygen

Figure 3. Effect of P5 on mitochondrial complexes. Values are expressed as mean ± SEM (n = 6). Effect of P5 on (A) complex I (NADH dehydrogenase), (B) complex II (succinate dehydrogenase), (C) complex III (MTT reduction rate), (D) complex V (ATPase synthase). ***P < 0.001 versus sham, significant (‡P < 0.05) differences were seen between tMCAO and the P5 treated group.
Figure 4. Effect of P5 on the mitochondrial ROS. Values are expressed as mean ± SEM (n = 6). (A–C) Production of mitochondrial ROS in sham, tMCAO, and tMCAO + P5, as shown by changes in DCF fluorescence. (D) Relative changes in DCF fluorescence intensity. ***P < 0.001 versus sham, and significant (#P < 0.05) differences were found between tMCAO and the P5 treated group.

Figure 5. Effect of P5 on MMP. Values are expressed as mean ± SEM (n = 6). (A–C) Changes in mitochondrial membrane potential in sham, tMCAO, and tMCAO + P5, as reflected by changes in TMRE fluorescence. (D) Relative changes in the TMRE fluorescence intensity are shown. ***P < 0.001 versus sham, ns versus tMCAO.
utilization, have been seen in various hypoxia–ischemia models. Our studies have shown that ischemia promotes the alterations in the mitochondrial respiration, as demonstrated by less oxygen consumption. P5 was not able to improve the mitochondrial oxygen consumption and respiratory coupling. These findings demonstrate that P5 does not have any effect on mitochondrial bioenergetics. Indeed, there was alleviation of mitochondrial ROS by P5, but it seems that P5 has no effect on ETC components. The possible mechanism may be the failure of P5 to stabilize the ETC components due to excessive ROS in the mitochondria. P5 enhances neurological recovery after ischemic stroke, as evidenced by improvements in neurological functions such as muscular strength and motor coordination, according to our findings. P5 does not inhibit the mtPTP like ALLO and P4 did; hence, further studies are warranted for elucidating the possible mechanisms. ALLO effect can be pronounced at very lower concentration, whereas P4 cannot have an inhibitory effect at lower concentrations. Keeping in view our previous studies and current studies on neurosteroids, we can conclude that both P4 and ALLO are more effective than P5. Thus, additional further studies need to be undertaken with various dose regimens and with longer therapeutic windows to elucidate the possible mechanism.

■ CONCLUSIONS

The current study has demonstrated that P5 has been able to promote the neurological recovery in ischemic stroke of rats by reducing mitochondrial ROS. This mechanism needs to be further examined to unveil the neuroprotective mechanism of P5. Further experimental findings are required to explain the connection between mitochondrial ROS-mediated neurobehavioral alterations to identify the specific mechanism of P5 neuroprotection and the potential relevance of P5 in the treatment of cerebral ischemia.

■ EXPERIMENTAL PROCEDURE

Animals. Animal Ethics Committee, Jamia Hamdard, approved all experiments and animals. The Central Animal House Facility of Jamia Hamdard, New Delhi, India, provided male Wistar rats weighing 250–300 g (16–18 weeks old). Animals were housed in groups of three to four in a cage with constant light (12 h light/dark cycle), temperature, humidity, and free access to food and water.

tMCAO Model. We employed transient cerebral ischemia induced by the blockage of the right MCA, as reported earlier with minor modifications. Animals were anesthetized with chloral hydrate (400 mg/kg b.w.) prior to tMCAO operation. The animals were placed on the ventral side of the operating table and kept warm using thermal heaters. The right common
carotid artery was exposed by making a midline incision on the ventral aspect of the neck. The external carotid artery (ECA) was ligated, and the internal carotid artery (ICA) near the bifurcation was isolated. An intraluminal monofilament with a silicon rubber-covered tip and a filament size of 4.0, length 30 mm, and diameter 0.19 mm was inserted into the ECA and advanced through the ICA to the origin of the middle cerebral artery (MCA). After 2 h of MCA blockage, the suture was gently released, and the rats were returned to their cages for 22 h of reperfusion. ECA was surgically prepped in the sham group, but the filament was not placed. The animals were returned to their normal surroundings in an air-conditioned room with 12 h light/dark cycles and free access to the pellet meal and purified drinking water at an ambient temperature of 25 °C and a relative humidity of 45–50%.

**Drug Administration.** The dose and route of administration of the medications were chosen in accordance with the literature. Pregnlenone (147664) injections were given intraperitoneally (i.p.) at a dose of 2 mg/kg b.w.t. 1 h after occlusion, with subcutaneous (s.c) follow-up at 6, 12, and 18 h after occlusion.

**Experimental Design.** All Wistar rats were taught for six days in a row to establish a baseline score for a variety of neurobehavioral tests. Animals were examined for a variety of behavioral tests after 24 h postsurgery, and subsequently euthanized for TTC staining and mitochondrial parameters. (1) Sham-operated group, (2) tMCAO group, (3) tMCAO + pregnlenone (P5) (2 mg/kg b. w.).

A randomized block pattern was used to divide the animals, and the experimenter was blinded to the grouping of animals. All measurements were obtained in the frontal cortex of the brain, with n = 6 for each set of parameters in each group.

**Behavioral Training of Animals.** Prior to the start of the experiments, all animals were trained for various behavioral metrics for five days to acclimate them.

**Assessment of Neurobehavioral Activity.** Rats’ neurobehavioral activity were recorded in an animal cage for 5 min using the approach described earlier. The following criteria were used to rate neurological deficits on a scale of 0–4 (0, no neurological deficit; 4, severe neurological deficiency): 0 = normal; the rat actively explored the cage area and walked around; 1 = the rat could timidly relocate in the cage but did not approach all sides; 2 = the rat had postural and movement irregularities and had difficulty resembling all walls of the cage; 3 = the rat with postural abnormalities tried to move in the cage but did not acknowledge one wall of the cage; 4 = the rat was unable to move in the cage and remained in the center.

**Motor Impairment.** The rats were tested in the rota rod task before being sacrificed to assess the influence on motor impairment, as described before. The rota rod unit (Omni Rotor, Omnitech Electronics, Inc., Columbus, OH, USA) was used to evaluate motor function in this investigation. It comprises a rotating rod with a diameter of 75 mm that is separated into four compartments to test four animals at a time after 23 or 22 h postocclusion. Each animal’s time lingering at the rotating rod was recorded for three trials at a 5 min interval, with a maximum trial length of 180 s each trial. The apparatus automatically recorded the time in 0.1 s till the rat falls on the floor. The speed was set at 10 rotations per min, and the cut off time was 180 s. The score was presented as mean off three trials to which rat remains on the rotating rod.

**Assessment of Sensory Impairment.** To assess somatosensory function, an adhesive removal test was performed, as described earlier on the ventral side of the contralateral forepaw, adhesive (0.5 in round) labels were applied. The investigator timed how long each rat took to remove the adhesive label with its tongue, up to a maximum of 2 min. The effect of neuroprotectants on sensory impairment was tested one day before surgery and 24 h after surgery.

**Grip Strength.** A 50 cm long string is pulled strongly between two vertical supports and elevated 40 cm from the level surface to create the apparatus. A method described earlier was used to conduct the test. The rats were placed in the middle of the string and scored using the following scoring scale: 0 = falls off, 1 = clings to string with two forepaws, 2 = clings to string with two forepaws while simultaneously attempting to climb it, 3 = clings to string with two forepaws and one or both hind paws, 4 = clings to string with all forepaws and tail wrapped around the string, and 5 = escape.

**Assessment of Infarction Volume.** Animals were euthanized 24 h after surgery, and the brains were coronally sectioned into 1.5-mm thick sections in a rat brain matrix, dyed in a 2% 3, 3',5-triphenyltetrazolium chloride solution, and fixed in 10% formalin overnight. The procedure was carried out in the manner outlined earlier. A scanner was used to image the infarction area, which was then analyzed using ImageJ. (Wayne Rasband National Institute of Health, USA). The volume of the infarction was determined by adding the infarct volumes of the parts. The following formula was used to calculate the infarct size and represent it as a percentage:

\[
\text{Contralateral volume} - \text{ipsilateral undamaged volume} \times 100 / \text{contralateral volume},
\]

to eliminate the effects of edema, as described previously.

**Mitochondrial Preparations.** According to the previous method, differential centrifugation was used to isolate mitochondria from the frontal cortex of the brain. Animals were decapitated, and the frontal cortex was dissected and homogenized in ice cold buffer A using a mechanically powered Teflon-fitted Potter–Elvehjem homogenizer. Mitochondria were separated from three different buffers (A, B, and C). Sucrose (250 mM), 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N' ′-tetraacetic acid (EGTA), and 0.1% fat-free bovine serum albumin (BSA) in Buffer A, adjusted to pH 7.4 with Tris, and centrifuged at 1000 g for 8 min at 4 °C. The supernatant was collected and centrifuged for 10 min at 4 °C at 10,000 g. The pellet was then resuspended and washed twice with washing media (B) comprising 250 mM sucrose, 10 mM HEPES, and 0.1 mM EGTA adjusted to pH 7.4 with Tris, then centrifuged at 12,300 g for 10 min. Finally, the pellet was resuspended in 250 mM sucrose, 10 mM HEPES, and 0.1% fat-free BSA in an isolation medium (C) containing 250 mM sucrose, 10 mM HEPES, and 0.1% fat-free BSA adjusted to pH 7.4 by Tris and centrifuged at 12,300 g for 10 min. The protein content of the mitochondrial pellet was measured using the Bradford assay after it was resuspended in buffer C. All of the isolation techniques were performed in ice cold temperatures, and the mitochondria were utilized within 2 h of the animal being decapitated. Mitochondrial purity was determined by flow cytometry after staining the final mitochondrial preparations with NAO (100 nmol/L, excitation 488 nm, and emission 525 nm).

**Biochemical Analysis of Mitochondrial Complexes.** The mitochondrial complexes I, II, III, and V (NADH dehydrogenase, succinate dehydrogenase, cytochrome c
reductase, and ATPase synthase) were assayed spectrophotometrically, as described previously.7

Flow Cytometric Analysis of Mitochondrial ROS and Membrane Potential. Flow cytometry has significant benefits over IHC, including the ability to distinguish different cell populations based on their size and granularity, the potential to rule out dead cells, better sensitivity, and the ability to measure several antigens using multicolor assessment. A FACs Calibur with a 488 nm argon laser and a 635 nm red diode laser was used for flow cytometry analysis.7 The Cell Quest software was used to examine the data from the studies (BD Bioscience). To exclude garbage, samples were gated using light scattering properties in the side scattering (SSC) and forward scattering (FSC) modes, with a total of 20,000 events per sample recorded within the R1 gate. At pH 7.4, the mitochondrial fractions were suspended in an analytical buffer comprising 250 mmol/L sucrose, 20 mmol/L MOPS, 10 mmol/L Tris-base, 100 mol/L Pi(K), 0.5 mmol/L Mg2+, and 5 mmol/L succinate. The mitochondria were then stained with tetramethylrhodamine, ethyl ester (TMRE) (100 nmol/L, excitation at 488 nm and emission at 590 nm) and 2′,7′-dichlorofluorescein diacetate (H2DCFDA) (10 mmol/L, excitation at 488 nm and emission at 525 nm), which were used to measure the mitochondrial membrane potential and the production of mitochondrial ROS, respectively.

Mitochondria Respiration by Oxygen Graph. According to the previous method, mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instrument) at 37 °C, pH 7.4, in a KCl medium containing 0.1 mM EDTA, MgCl2, sucrose, and KH2PO4 in a KCl medium containing 0.1 mM EDTA, MgCl2, sucrose, and KH2PO4 in a KCl medium containing 0.1 mM tMCAO after 24 h, animals were euthanized, and the frontal cortex was separated for mitochondrial preparations.37 By introducing mitochondrial preparations to a measurement chamber supplemented with 10 mM succinate in a total volume of 1.5 mL, oxygen consumption in the respiratory medium was determined. State 3 was induced in the measuring chamber by adding 2 mM ADP, while state 4 was taken without the ADP. The RCR was calculated as the ratio of ADP-induced state 3 respirations to state 4 respirations without ADP to assess mitochondrial respiratory energy coupling. Nanomoles of oxygen (O2)/min/mg of protein were used to calculate the rate of mitochondrial oxygen consumption. The RCR was determined as a ratio of state 3 to state 4 on the basis of state 3. Each reaction was run for 10−20 min.

Ca2+−Induced Mitochondrial Swelling. A previously published approach was used to examine mitochondria.37,38 Light transmission increases as a result of mitochondrial enlargement produced by the entry of solutes through open PT pores (i.e., a reduced turbidity). By measuring absorbance in mitochondrial suspensions, this turbidity change provides a straightforward and widely used MPT test. Mitochondrial permeability was measured using a spectrophotometer after Ca2+−induced mitochondrial swelling. After the last round of washing, the mitochondrial pellet was resuspended in ice cold BSA- and EDTA-free sucrose buffer (300 mmol sucrose and 10 mmol/L Tris-Base, pH 7.4). After 5 min, an aliquot of 100 g of mitochondria was added to 1 mL of BSA- and EDTA-free buffer, 400 μM Ca2+ was added, and a reading was taken at 540 nm for 5 min.

Determination of Protein. The protein content of various fractions of the brain frontal cortex, such as the mitochondria, cell lysate, cytosolic fractions, and mitochondria fractions, was determined using the Bradford method with BSA as a standard.

Statistical Analysis. The standard error of the mean was used to examine the data (SEM). GraphPad Prism 5 was used to analyze all of the data (GraphPad Software Inc., San Diego, CA, USA). The one-way analysis of variance (ANOVA) was used to assess behavioral data, followed by a post-hoc Tukey’s test. All other data was examined using a two-way ANOVA followed by a Tukey post hoc test. The Cell Quest programme (BD Bioscience) was used to examine the flow cytometry data. P<0.05 values are computed significant.

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Notes
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