Progesterone Changes VEGF and BDNF Expression and Promotes Neurogenesis After Ischemic Stroke

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Abstract

Studies have shown that progesterone enhances functional recovery after ischemic stroke, but the underlying mechanisms are not completely understood. Therefore, we investigated the effect of progesterone on vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and neurogenesis in a rodent stroke model. Rats underwent permanent middle cerebral artery occlusion (pMCAO) and then received intraperitoneal injections of progesterone (15 mg/kg) or vehicle at 1 h followed by subcutaneous injections at 6, 24, and 48 h. We examined VEGF and BDNF expression by Western blotting and/or immunostaining and microvessel density by lectin immunostaining. Neurogenesis in the subventricular zone was determined by immunostaining of Ki67 and doublecortin, and double BrdU/Nestin immunostaining. We calculated brain water content with the wet-dry weight method on day 3 and assessed neurologic deficits with the modified neurological severity score on days 1, 3, 7, and 14. Progesterone-treated rats showed a significant decrease in VEGF expression, but an increase in BDNF expression, compared with that of vehicle-treated pMCAO rats on day 3 post-occlusion. Progesterone did not alter the microvessel density, but it reduced brain water content compared with that in vehicle-treated rats on day 3 post-occlusion. Progesterone treatment increased the numbers of newly generated neurons in the subventricular zone and doublecortin-positive cells in the peri-infarct region on day 7 post-occlusion. In addition, progesterone improved neurologic function on days 7 and 14 post-occlusion. Our data suggest that the enhancement of endogenous BDNF and subsequent neurogenesis could partially underlie the neuroprotective effects of progesterone.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Keywords

Brain-derived neurotrophic factor; Cerebral ischemia; Neurogenesis; Progesterone; Vascular endothelial growth factor

Introduction

Ischemic stroke is the third leading cause of death and disability in humans, but treatments to reduce the degree of brain damage or restore brain function are restricted to thrombolytic and rehabilitation therapies. Repair of brain damage is essential to restoring neurologic function. However, self-repair mechanisms are insufficient and functional recovery is limited [1].

Progesterone, although primarily a female sex hormone, is pleiotropically neuroprotective and could be a useful therapeutic and neuroprotective agent for central nervous system (CNS) injury [2]. The neuroprotective effects of progesterone have been reported in various CNS disease models, including traumatic brain injury (TBI) [3, 4], ischemic stroke injury [5–7], and spinal cord injury [8]. We and others have shown that progesterone may exert its neuroprotective effects by reducing inflammation [9–11] and blood-brain barrier permeability [10], and by promoting myelin repair [12].

In the adult brain, neurogenesis from neural stem cells and progenitor cells persists primarily in two regions: the subventricular zone (SVZ), which lines the lateral ventricles, and the subgranular zone of the dentate gyrus [13]. Neural progenitor cells also reside in the adult mouse striatum [14]. Post-ischemic neurogenesis occurs in animal models and in humans [15, 16] and may represent an endogenous repair mechanism and therapeutic target for ischemic stroke [16]. Recent evidence has revealed an important role for vascular endothelial growth factor (VEGF) as a neurotrophic factor and neuroprotectant [17]. VEGF is also a potent endothelial cell mitogen and key regulator of angiogenesis [18]. The angiogenesis promoted by VEGF is essential for the survival of resident or newly generated neurons [19].

Brain-derived neurotrophic factor (BDNF) is traditionally thought to be a key neurotrophic factor for neurogenesis after brain injury. It is secreted by various neural cells and plays an important role in adult neurogenesis [20, 21]. Previous studies have reported that progesterone promotes a neurotrophic response to TBI [22] and induces significant increases in the expression of BDNF at both the mRNA and protein level after TBI and spinal cord injury [23, 24]. Progesterone also was shown to increase BDNF expression and protect brain tissue after exposure to glutamate toxicity [25]. However, the effect of progesterone on BDNF expression and neurogenesis after ischemic stroke remains unexplored.

In this study, we investigated the relationship between the neuroprotective effects of progesterone, VEGF, and BDNF. Using a rat model of ischemic stroke, we examined whether progesterone can increase VEGF expression and alter microvessel density and tested the hypothesis that progesterone increases BDNF expression and neurogenesis, thereby improving functional outcomes.

Materials and Methods

Animals

All studies were performed in accordance with National Institutes of Health and institutional guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee, Zhengzhou University, Zhengzhou, China. Adult, male Sprague-Dawley rats (260–300 g, 3 months old) were purchased from the Animal Experimental Center of Zhengzhou University and were given free access to food and water throughout the study.

Occlusion of the Middle Cerebral Artery (MCA)

After cerebral infarction, only 2–5 % of patients achieve blood flow restoration in the ischemic area [26]. Therefore, to mimic clinical conditions [27], we used a permanent MCA occlusion (pMCAO) model in this study. Procedures were carried out aseptically. Rats were anesthetized with 10 % chloral hydrate (400 mg/kg, intraperitoneally [i.p.]) and then underwent pMCAO with the filament model as described previously [28–30]. Only the left MCA was occluded. Laser Doppler flowmetry was used to monitor cerebral blood flow in the MCA territory for 5 min after insertion of the filament. Rats with less than 50 % drop in blood flow were excluded from this study. Sham-operated rats were subjected to the same surgical procedure, except that the filament was not advanced to occlude the MCA. After surgery, the wound was sutured and rats were returned to their cage, which was maintained at 29 °C.

Experimental Groups

One hundred and forty-four male rats were used in the study. Five rats died during surgery, and seven were excluded because of inadequate occlusion. The remaining 132 rats were randomly divided into four groups of 33 rats each: sham + vehicle, sham + progesterone, pMCAO + vehicle, and pMCAO + progesterone. Progesterone (15 mg/kg, Sigma, St. Louis, MO) was dissolved in 22.5 % 2-hydroxypropyl- β -cyclodextrin (Sigma) and administered i. p. at 1 h after MCAO and then by subcutaneous injection at 6, 24, and 48 h after MCAO. Vehicle-treated rats received only 2-hydroxypropyl- β -cyclodextrin. The dosing and duration regimens were based on previous work in rats [10, 31, 32]. Animals in each group were assigned to subgroups for immunostaining ($n = 5$ per group), Western blot analysis ($n = 5$ per group), neurogenesis detection ($n = 5$ per group), brain water content measurement ($n = 6$ per group), and neurologic function assessment ($n = 12$ per group). We did not monitor the effects of progesterone on physiologic parameters because others have already reported that various doses of progesterone have no effect on temperature, pH, PCO₂, PO₂, hematocrit, blood glucose, heart rate, or mean arterial pressure in rats [32–34].

Protein Isolation and Immunoblotting

Rats were anesthetized with an overdose of chloral hydrate and underwent transcardial perfusion with phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) in 0.1 M PBS on day 3 post-occlusion ($n = 5$ per group). The brains were carefully removed and placed in chilled saline for measurement of VEGF and BDNF with Western blot

analysis as in our previous studies [35, 36]. Total protein was isolated directly from the cortex by lysing the tissue in Laemmli buffer containing 2 % sodium dodecyl sulfate (SDS), 10 % glycerol, 2 % 2-mercaptoethanol, and 0.002 % bromophenol blue in 75 mM Tris-HCl. The samples were heated to 95 °C for 10 min before being separated on 10 % Tris/glycine/SDS acrylamide gels (Bio-Rad, Hercules, CA). The proteins were subsequently transblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked for 2 h at room temperature in 5 % milk. The immunoblots were incubated for 2 h at 37 °C with rabbit anti-VEGF and anti-BDNF antibodies (1:100; Santa Cruz Biotechnology, Dallas, TX). After three washes with Tris-buffered saline/0.05 % Tween-20, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:500; Santa Cruz Biotechnology) for 1 h at 37 °C. Protein signal was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected with an imaging system. β -Actin protein served as a loading control. A UVTEC scanner (UVTEC, Beijing) was used to capture images of blots, and Scion software (Scion Corp., Frederick, MD) was used for densitometric analysis of bands. Background values were subtracted, and multiple blots were combined for statistical analysis.

Bromodeoxyuridine (BrdU) Labeling

BrdU (Sigma) was used to label mitotically active cells. Rats were administered i.p. injections of BrdU (100 mg/kg in saline with 0.007 N NaOH) once daily for 7 days beginning on day 1 after pMCAO. The rats were killed 2 h after the last BrdU injection for analysis of BrdU immunostaining. This timing was selected based on previous research showing that SVZ proliferation peaks approximately 1 week after ischemic stroke [37].

Immunofluorescence

We immunostained for lectin and BDNF on day 3 post-occlusion ($n = 5$), as described previously [38]. To detect neurogenesis, we stained for Ki67 and doublecortin (DCX) and double stained for BrdU and nestin on day 7 after occlusion ($n = 5$). Briefly, after being blocked for 2 h with 1 % bovine serum albumin in PBS/Tween-20 (PBS-T), brain sections were incubated overnight at 4 °C with primary antibodies of fluorescein lycopersicon esculentum (tomato) lectin (1:1000; Vector laboratories, Burlingame, CA); rabbit anti-BDNF, anti-Ki67, or anti-DCX primary antibody (1:100; Santa Cruz Biotechnology); rat anti-BrdU IgG (1:50; Oxford Biotechnology, Kidlington, UK); or mouse anti-Nestin IgG (marker for neural stem cells, 1:500; MAB344, Chemicon International, Temecula, CA) in PBS-T containing 1 % bovine serum albumin. The sections were rinsed three times in PBS-T (10 min each) and then incubated for 2 h at room temperature with biotinylated chicken anti-rat IgG (1:200; Vector Laboratories), rinsed in PBS-T three times (10 min each), and/or incubated for 2 h at 4 °C with AlexaFluor 488-conjugated goat anti-rat or anti-rabbit IgG (1:1500; Molecular Probes, Eugene, OR) and AlexaFluor 594-conjugated goat anti-rabbit or anti-mouse IgG (1:1500; Molecular Probes, Eugene, OR). The sections were then rinsed three times (10 min each) at 4 °C in PBS, and coverslips were mounted with VECTASHIELD (Vector Laboratories). We viewed lectin and DCX in the peri-infarct area, and Ki67, DCX, and double-labeled BrdU⁺/Nestin⁺ cells in the SVZ in four equidistant anterior striatal sections/animal under a fluorescence microscope (ZEISS, Germany). Two areas in each section were chosen at random and photographed by an investigator blinded to

the experimental groups. BDNF-positive cells were not calculated. Vessel branch points and other cell counts were averaged and expressed as positive branch points or cells per field.

Assessment of Brain Water Content

The rats ($n = 6$) were anesthetized and decapitated on day 3 after pMCAO as previously described [10] for determination of water content. Briefly, cerebral tissue was divided with a blade into two hemispheres from the anatomic midline. The left hemisphere was immediately weighed with an electronic analytical balance to obtain the wet weight. Then brain samples were dried at 100 °C in an electric blast drying oven for 24 h and the dry weight measured. Brain water content was calculated as: (wet weight – dry weight)/wet weight $\times 100\%$ [39].

Neurologic Assessment

An investigator blinded to treatment group used the modified neurological severity score (mNSS) system to test the neurologic function of rats on days 1, 3, 7, and 14 after pMCAO or sham surgery ($n = 12$) [28]. The mNSS comprises motor, sensory, balance, and reflex tests. Neurologic function was graded on a scale of 0–22 (no deficit = 0, maximal deficit = 22).

Statistical Analysis

Results are expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by LSD test was used to compare changes in VEGF and BDNF expression, microvessel density, neurogenesis in the SVZ, and brain water content after pMCAO. Student's t test was used to analyze DCX-positive cells around the infarct area. Repeated measures ANOVA was used to analyze changes in neurologic deficit score after pMCAO. Differences were considered statistically significant at $p < 0.05$.

Results

Progesterone Treatment Decreases VEGF Expression but Does Not Influence Microvessel Density

Immunostaining revealed that the quantity of lectin-positive microvessels was significantly reduced on day 3 after pMCAO when compared with that in the sham group ($F = 9.07$, $p = 0.01$). However, the microvessel density did not differ significantly between the progesterone-treated and vehicle-treated pMCAO rats ($p = 0.537$, $n = 5$ rats/group; Fig. 1a, c). Immunoblotting results showed that the expression of VEGF was significantly greater in the pMCAO + vehicle group than in the other three groups on day 3 ($F = 160.46$, $p < 0.001$). Additionally, progesterone decreased the expression of VEGF compared with that in the vehicle-treated pMCAO group on day 3 post-occlusion ($p = 0.001$, $n = 5$ rats/group; Fig. 1b, d).

Progesterone Treatment Increases BDNF Expression After pMCAO

On day 3 after brain ischemia, the number of BDNF-positive cortical neurons in the ischemic penumbra was greater in the vehicle-treated pMCAO rats than in the sham-

operated rats. Progesterone treatment increased the number of BDNF-positive neurons in the ischemic penumbra (Fig. 2a). Western blot results further confirmed that protein expression of BDNF was very low in sham-operated rats and that it was higher in progesterone-treated pMCAO rats than in vehicle-treated pMCAO rats on day 3 ($F = 55.10$, $p < 0.001$, $n = 5$ /group; Fig. 2b, c).

Progesterone Treatment Increases Neurogenesis in the SVZ After pMCAO

Progesterone treatment significantly increased the numbers of Ki67-positive ($F = 106.79$, $p < 0.001$; Fig. 3a, c) and DCX-positive ($F = 21.65$, $p < 0.001$; Figs. 4 and 5b) cells in the SVZ area on day 7 post-occlusion. The number of DCX-positive cells also was significantly greater around the infarct area of progesterone-treated rats than of vehicle-treated rats ($p = 0.003$; Fig. 5a, b). We observed few BrdU/Nestin-positive cells in the dorsal and ventral SVZ regions of sham-operated rats across the 7-day assessment period, indicating that the surgical procedure itself did not affect neurogenesis ($p > 0.05$, data not shown). Although we observed BrdU-positive cells in the SVZ region of both vehicle-treated and progesterone-treated pMCAO rats, the number of BrdU/Nestin-positive cells was significantly higher in progesterone-treated pMCAO rats than in vehicle-treated pMCAO rats on day 7, indicating enhanced neurogenesis ($p = 0.001$, $n = 5$ rats/group; Fig. 3b, d).

Progesterone Treatment Reduces Brain Water Content After pMCAO

Brain water content in the ipsilateral hemisphere was significantly higher in pMCAO rats than in sham-operated rats on day 3 post-occlusion ($F = 11.95$, $p < 0.001$). However, progesterone treatment significantly reduced the brain water content in the ipsilateral hemisphere when compared with that of vehicle-treated rats on day 3 post-occlusion ($p = 0.01$, $n = 6$ rats/group; Fig. 6a).

Progesterone Treatment Improves Neurologic Function After pMCAO

Neurologic function was assessed on days 1, 3, 7, and 14 after pMCAO. Prior to ischemia, rats exhibited no neurologic deficits. All rats showed substantial neurologic deficit on day 1 after pMCAO, but neurologic function gradually improved on days 3, 7, and 14. Rats treated with progesterone had neurologic deficit scores that were significantly lower than those of vehicle-treated pMCAO rats ($F = 22.51$, $p < 0.001$, $n = 12$ rats/group; Fig. 6b).

Discussion

Clinical studies of cell-based restorative therapies for cerebral infarction have revealed that the safety and feasibility of neuronal transplantation warrants further study [40]. Until transplantation of stem cells becomes a viable option, we must look for ways to enhance endogenous neurogenesis. In this study, we showed that progesterone treatment beginning 1 h after pMCAO decreases VEGF expression and reduces brain edema without influencing microvessel density. In addition, we show that progesterone enhances BDNF expression, increases neurogenesis, and improves neurologic outcome after ischemic stroke. These results suggest that progesterone treatment might promote stroke recovery by reducing brain edema and enhancing neurogenesis.

Drug-induced neurogenesis has been shown to contribute to post-stroke recovery in animal models [41, 42]. We and others have shown previously that progesterone treatment after focal cerebral ischemia reduces lesion volume and brain edema [7, 43] and improves early functional outcome [5, 7, 31, 44]. Consistent with these reports, our data show that progesterone-induced neurogenesis and reductions in brain edema [7] may contribute to long-term functional recovery after stroke. Combined with data from other groups [41, 42, 45], our findings raise the possibility that augmentation of self-repair mechanisms could be of therapeutic value for patients with ischemic stroke.

Progesterone, a neurosteroid, can be synthesized in the brain, where it regulates neuron growth, myelination, and synaptogenesis [46]. VEGF, a key regulator of angiogenesis, stimulates neurogenesis through Flk-1 signaling [47]. To explore the neuroprotective effects of progesterone, we studied the influence of progesterone on VEGF expression in ischemic brain. Progesterone significantly reduced VEGF expression on day 3 after ischemic stroke in rats, consistent with a previous study in which progesterone reduced hemorrhagic transformation after delayed tPA treatment in an experimental model of stroke [48]. Nevertheless, we found that progesterone treatment did not affect microvessel density on day 3 after stroke. Taken together, these findings suggest that changes in VEGF expression in the acute phase may not contribute to the neurotrophic effect of progesterone.

Previous studies have shown that progesterone induces the expression of BDNF after various CNS injuries [23–25]. However, reports have been conflicting in regard to the effect of progesterone on BDNF expression in the MCAO model [32, 49]. As a traditional neurotrophin, BDNF has a major role in protecting brain tissue from injury and in promoting neuronal plasticity and neurogenesis [20, 21, 50, 51]. Intravenous administration of BDNF has been shown to stimulate neurogenesis and enhance post-stroke functional recovery [52]. Intraventricular infusion of BDNF or a viral vector encoding BDNF also induces neurogenesis in the adult mouse brain [53, 54]. Therefore, BDNF is a prime candidate for modulation by progesterone. Our results further confirm that progesterone can increase BDNF expression after MCAO and are consistent with those of Ishrat et al. [32]. The increase in BDNF might contribute to enhanced neurogenesis and the improvement in neurologic function on days 7 and 14 after stroke. Additional studies are needed to focus on progesterone's long-term effects, including its effects on brain atrophy and functional recovery.

A previous report revealed that progesterone can attenuate blood–brain barrier breakdown and hemorrhagic transformation by reducing VEGF and matrix metalloproteinase-9 expression after delayed tPA treatment in an experimental model of stroke [48]. Others have demonstrated that increases in VEGF expression and newly generated microvessels are associated with the occurrence and aggravation of brain edema or hemorrhagic transformation [55]. We have previously shown that progesterone has the ability to reduce matrix metalloproteinase-9 expression and brain water content after ischemic stroke [10, 56]. Although the current study indicates that the reduction in VEGF expression in the acute phase of ischemic stroke may not contribute to the neurotrophic effect of progesterone, our results suggest that it may relate to the effect of progesterone on post-stroke brain water content.

Our study is limited because we assessed only the short-term effects of progesterone on VEGF and BDNF expression and assessed neurogenesis only on day 7 in the SVZ after pMCAO. The long-term effects of progesterone on neurogenesis and the fate of the newly formed neurons are still not known, and we did not analyze whether the newly generated neurons are integrated into the brain circuitry. It would be interesting to determine whether BDNF receptor antagonist can block the effects of progesterone on neurogenesis and functional recovery after pMCAO. Additional research is needed to validate the long-term neurotrophic effect of progesterone in the ischemic brain. The knowledge gained will enhance our understanding of the mechanisms by which progesterone may offer neuroprotection after ischemic stroke.

In conclusion, the conventional view that progesterone is only a female gonadal steroid and involved only in reproductive functions has changed completely over the past few years. Many experimental studies, most performed in rodents, have shown that progesterone has multiple action in the CNS and that it has considerable influence on the functioning of neurons and glial cells [57, 58]. Our findings support the premise that progesterone contributes to post-stroke functional recovery by decreasing VEGF and increasing BDNF and neurogenesis. These neurotrophic and pleiotropic neuroprotective effects suggest that progesterone may hold therapeutic promise for patients with ischemic stroke.

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Highlights

- Rats underwent a permanent middle cerebral artery occlusion model of stroke.
- Progesterone increased BDNF but decreased VEGF on day 3 post-occlusion.
- Progesterone reduced brain edema.
- Progesterone increased neurogenesis and improved functional outcome.
- Progesterone treatment provides a therapeutic effect after ischemic stroke.

Fig. 1.

Progesterone (PROG) decreases VEGF expression but does not affect microvessel density in the cortex on day 3 after pMCAO. **a** Representative photomicrographs of brain slices showing microvessel density in each group (*scale bar* = 100 μ m). **b** Representative Western blot showing relative protein expression of VEGF in the four groups. β -Actin was used as a loading control ($n = 5$ /per group). **c** The quantitative results for microvessel density from each group ($\#p < 0.05$ vs. sham + vehicle or sham + PROG). **d** Bar graph shows the quantitative analysis for VEGF expression in each group ($n = 5$ /per group; $*p < 0.05$ vs. pMCAO + vehicle). All data are shown as mean $\pm$ SEM

Fig. 2.

Progesterone (PROG) increases protein expression of brain-derived neurotrophic factor (BDNF) on day 3 after pMCAO. **a** Representative photomicrographs of brain slices showing BDNF immunoreactivity in vehicle-treated and progesterone-treated pMCAO rats (*scale bar* = 100 μ m). **b** Representative Western blot showing relative protein expression of BDNF in brain lysates from sham-operated and pMCAO rats post-treated with vehicle or progesterone. β -Actin was used as a loading control ($n = 5$ /per group). **c** Bar graph shows the quantitative analysis of BDNF expression from each group ($n = 5$ per group). All data are shown as mean $\pm$ SEM. * $p < 0.05$ vs. pMCAO + vehicle

Fig. 3. Progesterone (PROG) increases neurogenesis on day 7 after pMCAO. **a** Representative photomicrographs of Ki67 staining in the subventricular zone near the ventrolateral ventricle in vehicle- and progesterone-treated pMCAO rats. **b** Representative photomicrographs of double BrdU/Nestin staining in the subventricular zone near the ventrolateral ventricle in vehicle- and progesterone-treated pMCAO rats. **c, d** Bar graphs show quantitative analysis for Ki67-stained cells (**c**) and BrdU/Nestin-stained cells from each group ($n = 5$ per group). All data are shown as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle-treated pMCAO rats. Scale bar = 100 μ m

Fig. 4.

Progesterone (PROG) increases DCX-positive cells in the subventricular zone (SVZ) on day 7 after pMCAO. Only a few DCX-positive cells were visible in the SVZ of sham-operated rats, but the quantity increased after 7 days of pMCAO. Significantly more DCX-positive cells were visible in the SVZ of progesterone-treated pMCAO rats than in that of vehicle-treated pMCAO rats on day 7 after stroke. $n = 5$ per group. *Scale bar* = 100 μm

Fig. 5.

Progesterone (PROG) increases doublecortin (DCX)-positive cells in the peri-infarct area on day 7 after pMCAO. **a** Representative photomicrographs of brain slices showing DCX immunoreactivity in vehicle-treated and progesterone-treated pMCAO rats. **b** Bar graph shows quantitative analysis for DCX-stained cells in the subventricular zone (SVZ) and peri-infarct area from each group ($n = 5$ per group). All data are shown as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle-treated pMCAO rats. Scale bar = 100 μ m

Fig. 6.

Progesterone (PROG) reduces brain water content and improves neurologic recovery after pMCAO. **a** The brain water content was significantly increased on day 3 after pMCAO. Progesterone treatment significantly reduced brain water content compared with that in the vehicle-treated pMCAO group ($n = 6$). **b** The modified neurological severity score was used to assess the neurologic deficits of rats after pMCAO. Neurologic deficit scores were significantly different among the four groups on days 1, 3, 7, and 14 ($n = 12$ per group). Post-treatment with progesterone significantly reduced the neurologic deficit scores as compared with those in the vehicle-treated pMCAO rats on days 7 and 14. All data are shown as mean $\pm$ SEM. $^{**}p < 0.01$ vs. vehicle-treated pMCAO rats