Effects of Brain-Derived Neurotrophic Factor on Local Inflammation in Experimental Stroke of Rat

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1. Introduction

Stroke is a major cause of death and long-term disability worldwide [1, 2]. Brain-derived neurotrophic factor (BDNF) can decrease infarct volume and improve neurological outcome either by exogenously supplied or overexpression in vivo using genetic methods in experimental stroke. Inhibition of BDNF exaggerates damage of ischemia. BDNF exerts neuron protection against ischemic injury through binding to two membrane receptors, p75 neurotrophin receptor and tyrosine kinase receptor B (trkB) [3]. 7,8-dihydroxyflavone as a bioactive high-affinity TrkB agonist also protects neurons from apoptosis and decreases infarct volumes in animal model of stroke [4].

Inflammation plays an essential role in the pathogenesis of ischemic stroke [5, 6]. Rapid activation of resident inflammatory cells (mostly microglia), productions of inflammatory cytokines such as interleukin10 (IL-10) and tumor necrosis factor α (TNF-α) and translocation of intercellular transcription factors such as nuclear factor-kappa B (NF-kB) are characters of local inflammatory responses to ischemia in brain [7–11]. Different responses may have different functions in the pathogenesis of stroke. Activated microglia could exert neurotrophic effects such as BDNF to alleviate ischemic injury and exhibit phagocytic activity disposing of degenerating elements [7, 11]. There are several cytokines involved in inflammatory process. TNF-α, as an important proinflammatory cytokine, appears to exacerbate cerebral injury of ischemia [8] while IL-10, an anti-inflammatory cytokine, ameliorates ischemic insult of brain [9]. Activation of NF-kB, an important transcription factor, could mediate translation of many downstream genes and promote survival of neurons [10].

BDNF promotes cell proliferation, increases phagocytic activity and inhibits apoptosis of microglia in brain [12]. BDNF downregulates the expression of TNF-α and upregulates the expression of IL10 in the model of multiple sclerosis [13]. NF-kB activated by BDNF protects cells from damages, such as the serum starvation and glutamate toxicity [14]. However, whether BDNF modulates inflammatory processes in ischemic stroke is unclear. In present study, we evaluated the effect of BDNF on local cerebral inflammatory process on
rats in antibody group were given 5 μg BDNF antibody (PeproTech, USA) diluted in 10 μL PBS (pH 7.4); rats in BDNF group were given 10 μg BDNF (PeproTech, USA) diluted in 10 μL PBS (pH 7.4). At the end of injection, the needle was left in place for 5 minutes before being slowly withdrawn.

2.4. Transient Middle Cerebral Artery Occlusion (MCAO) and Reperfusion. The MCAO procedure has been described before [15]. In brief, rats were anesthetized with pentobarbital sodium (40 mg/kg i.p.). Carotid artery was exposed and a 4-0 silicone-coated nylon filament was gently advanced from external carotid artery into the lumen of internal carotid artery until the rounded tip blocked the origin of the middle cerebral artery. A laser Doppler flow meter (LDF; Perimed PF5000, Stockholm, Sweden) was used to confirm the decrease of the middle cerebral artery blood flow immediately after the occlusion to about 20% of the basic cerebral blood flow. After 2 hours, rats were briefly reanesthetized and the filament withdrawn. Rectal temperature was maintained at 37°C using a rectal probe and heating pad during the surgery. Physiological parameters were monitored pre- and during ischemia (Table 2).

2.5. Tissue Processing. Rats were sacrificed at 6 h and 24 h of reperfusion. Rats were deeply anesthetized. For real-time quantitative PCR, enzyme-linked immunosorbent assay and electrophoretic mobility shift assay, rats were perfused transcardially with 0.1 M PBS (pH 7.4) only, and brains were removed rapidly and stored in the liquid nitrogen until used; for immunofluorescence, rats were perfused transcardially with 0.1 M PBS (pH 7.4) followed by a fixative solution containing 4% paraformaldehyde in PBS (pH 7.4). Brains were removed and fixed in the same fixative solution for an additional 6–12 h in 4°C. Prior to cytosectioning,
tissues were cryoprotected using 20% sucrose in PB for 24 h followed by 30% sucrose in PB for 48 h.

2.6. 2,3,5-Triphenyltetrazolium Chloride (TTC) and Terminal Deoxynucleotidyl Transferase-Mediated dUTP End-Labeling (TUNEL) Assay. For TTC staining, five coronal sections were made from the bregma to the cerebellum and stained with 2% TTC (Sigma-Aldrich, USA) at 37°C for 30 minutes. The infarct area of each section was measured in a blinded manner using Image J (NIH, USA). The infarct volume was then calculated by Swanson’s method [16]. To determine apoptosis-like cell death, TUNEL staining (In Situ Cell Death Detection Kit, POD; Roche, USA) was performed 24 h after reperfusion (n = 6). The brain slices were mounted onto slides, fixed 20 minutes with 4% paraformaldehyde in PBS (pH 7.4), and pretreated with 3% H2O2 in methanol and 0.1% Triton X-100. The TdT enzyme and nucleotide mix were then added at proportions specified by the kit for 60 minutes at 37°C. The slides were washed 3 times with PBS (pH 7.4), 50 μL horse-radish peroxidase (POD) was added and slides were incubated in a humidified chamber for 30 minutes at 37°C. After washed 3 times with PBS (pH 7.4), 75 μL diaminobenzidine (DAB) was added. Slides were incubated for 10 minutes at room temperature. The slides were mounted under glass coverslip and analyzed under light microscope.

2.7. Behavioral Testing. Following recovery from anesthesia, behavioral neurologic deficits were assessed 24 h after MCAO. The battery consisted of four tests to systematically evaluate motor, sensory, and vestibulomotor deficits. The following behavioral tests were performed. Postural reflex and hemiparesis test as described before [17]: (0) no observable neurologic deficits, (1) left forepaw flexion, (2) decreased resistance to lateral push and forepaw flexion without circling, (3) decreased resistance to lateral push and forepaw flexion with circling, and (4) cannot walk spontaneously.

Forepaw placing test was done as described previously [18]. For each test, limb placing scores were as follows: (0) immediate and complete placing, (1) delayed and/or incomplete (>2 s), and (2) no placing.

Modified beam balance test was done as described [19]: The scale was as follows: (1) steady posture with paws on top of the beam, (2) paws on side of the beam or wavering, (3) one or two limbs slip off, (4) three limbs slip off, (5) attempts with paws on the beam, but falls, and (6) drapes over the beam, then falls or falls with no attempt.

Adhesive tape test was done as before [20]: The scale was evaluated as: (1) <10 s; (2) 10–19 s; (3) 20–29 s; (4) 30–39 s; (5) 40–49 s; (6) 50–59 s; (7) ≥60 s.

2.8. Immunofluorescence. All sections (7 μm) were simultaneously run to ensure identical staining conditions. After rinsing in PBS (pH 7.4), sections were incubated at 37°C for 2 h with the primary antibodies in PBS (OX-42 in pH 7.4, ED1 in pH 7.2) used at the following dilutions: OX-42 (1/200), ED-1 (1/200) (AbD Serotec, UK). After 4 washes, antibody visualization was achieved by the incubation at 37°C for 30 minutes with Alexa Fluor 488-conjugated donkey antiamoouse (1/200) (Invitrogen, USA). Negative controls were prepared by omitting the primary antibodies. Sections were then coverslipped with a fluorescent mounting medium (Sigma-Aldrich, USA). Sections were stored at 4°C until viewing. Sections were viewed under a Leica SP5 confocal microscope (Leica, France).

2.9. Enzyme-Linked Immunosorbert Assay (ELISA). At 6 h and 24 h of reperfusion, 6 rats of each group were sacrificed and brain homogenates were obtained from the ischemic hemisphere. The concentrations of BDNF, IL-10 and TNF-α in brain homogenates were measured using specific ELISA kits according to the manufacturer’s instructions (R&D system, USA).

2.10. Real-Time Quantitative PCR. Total RNA was isolated from frozen brain tissues using the TRIzol reagent (Invitrogen, USA) according to the manufacturer’s recommendation, and subjected to DNase (Promega, USA) treatment. Reverse transcription (RT) reaction was carried out using the First-strand cDNA synthesis kit (Takara, Japan) according to the manufacturer’s instructions. Obtained cDNA were amplified using the following primers: for TNF-α, 5’-GATGTTATCGGAGATGTGGAA-3’ and 5’-AGACCGGCTCGGAGTTCTCTG-3’; for IL-10, 5’-CTTTACTGCAGCATTTTAAGGT-GTTA-3’ and 5’-CTGGGCCCATGTGTTCTCT-3’; and for β-actin, 5’-GACAGGTGAACAAGGAGATTACT-3’ and 5’-TGATCCACATCTGTGAGGAGT-3’. The amplification and data acquisition were run on a real time PCR system (Bio-Rad, USA) using SYBR green PCR Master Mix (Takara, Japan). The conditions were predenaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 55°C for 1 minute. All samples were analysed in triplicates in three independent experiments. Reactions without cDNA were used as no template control and no RT controls were also set up to rule out genomic DNA contamination. Gene expression levels were calculated using the 2-ΔΔct method [21].

2.11. Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared by hypotonic lysis followed by high salt extraction. EMSA was performed using a kit (Gel Shift Assay System, Promega, USA) to assay NF-κB DNA-binding activities. The NF-κB oligonucleotide probe, 5’-AGTTGAGGAGCTTTTCCAGGC-3’, was end-labeled with [γ-32P]TP. Protein-DNA binding assays were performed with 50 μg of nuclear protein. The binding medium contained 4% glycerol, 1% NP40, 1 mM MgCl2, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 10 mM Tris/HCl (pH 7.5). In each reaction, 20000cpm of a radiolabeled probe was included. Samples were incubated at room temperature for 15 minutes, and nuclear protein with 32P-labeled oligonucleotide complex was separated from free 32P-labeled oligonucleotide by electrophoresis through a 4% native polyacrylamide gel in 0.5 × TBE. After separation was achieved,
2.13. Statistical Analysis. The data were presented as mean ± SD. Differences between groups were compared using analysis of variance (ANOVA) followed by post hoc t-test with SPSS13.0 software (USA). \( P < .05 \) was considered to be statistically significant.

3. Results

3.1. BDNF Level was Increased in Brain. To assess the concentration of BDNF in brain tissues, the brain homogenates were obtained 6 h and 24 h after reperfusion and the concentration of BDNF was measured using ELISA kits. BDNF level was significantly increased by 19.7 fold in BDNF group while administration of BDNF antibody did not influence BDNF level in brain (1.00 ± 0.05 versus 0.98 ± 0.13 ng/g, \( n = 6, P > .05 \), Figure 1). The concentration of BDNF was decreased at 24 h of reperfusion than that at 6 h of reperfusion; however, it was significantly increased compared with control group (11.36 ± 0.91 versus 1.42 ± 0.08 ng/g, \( n = 6, P < .05 \), Figure 1). There was no significant difference of BDNF level between antibody group and control group (1.38 ± 0.14 versus 1.42 ± 0.08 ng/g, \( n = 6, P > .05 \), Figure 1).

3.1.1. BDNF Protected Brain against Ischemic Insult and Reduced Neurologic Deficits. We investigated cellular injury using TUNEL assay and measured the infarct volume using the TTC staining while neurologic deficits were tested by four behavioral tests 24 h after reperfusion. The number of TUNEL-positive cells in BDNF group was significantly decreased than that in control group (\( n = 6, 23.05 ± 1.86 \) versus 54.26 ± 3.05%, \( P < .05 \), Figures 2(d), 2(e), 2(h)) while BDNF antibody did not increase the TUNEL positive cells (\( n = 6, 55.06 ± 2.57 \) versus 54.26 ± 3.05%, \( P > .05 \), Figures 2(d), 2(f), 2(h)). However, the infarct volume measurements were found to be similar in the three groups with no statistical differences. Exogenous BDNF significantly improved motor-sensory function (1.5 ± 0.55 versus 2.8 ± 0.41, \( P < .05 \), Table 3), somatosensory function (3.8 ± 0.98 versus 5.2 ± 0.75, \( P < .05 \), Table 3), vestibulomotor function (2.2 ± 0.98 versus 4.2 ± 0.75, \( P < .05 \), Table 3) compared with control group while BDNF antibody did not change the functions above. There was no significant difference of somatosensory function in the three groups (Table 3).

3.2. BDNF Increased the Number of Activated Microglia in Brain. Activated microglia could be marked by OX-42 and detected by immunofluorescence. BDNF increased the number of activated microglia compared to control group (394.7 ± 31.5 versus 272.0 ± 22.6/mm², \( n = 6, P < .05 \), Figure 2) as early as 6 h after reperfusion. On the other side, when endogenous BDNF’s activity was suppressed by BDNF antibody, the number of activated microglia was significantly decreased (90.7 ± 19.4 versus 272.0 ± 22.6/mm², \( n = 6, \)
were activated in BDNF group than in control group (408 ± 59 μTUNEL-positive cells 24 h after ischemia. Scar bar: 100 μm). (f) TUNEL assay in antibody group. (g) No significant difference with triphenyltetrazolium chloride (TTC) and TUNEL assay 24 h after reperfusion. (a) Infarct volume without any treatment. (b) Infarct volume with exogenous BDNF. (c) Infarct volume with exogenous BDNF antibody. (d) TUNEL assay in control group. (e) TUNEL assay in BDNF group. (f) TUNEL assay in antibody group. (g) No significant difference of infarct volume in the three groups. (h) Summary of TUNEL-positive cells 24 h after ischemia. Scar bar: 100 μm. Bars represent mean ± SD (n = 6); *P < .05 versus control group.

Figure 2: BDNF alleviated cellular injury of ischemic insult brain tissue sections obtained from injured cerebral hemispheres were stained with triphenyltetrazolium chloride (TTC) and TUNEL assay 24 h after reperfusion. At 24 h of reperfusion, more microglia were activated in BDNF group than in control group (408.0 ± 22.1 versus 277.3 ± 21.9/mm², n = 6, P < .05, Figure 2). However, there was no significant difference of the number of activated microglia between antibody group and control group (266.7 ± 24.1 versus 277.3 ± 21.9/mm², n = 6, P > .05, Figure 2).

3.3. BDNF Increased the Number of Phagocytic Microglia in Brain. ED1 is a marker of phagocytic microglia which could engulf damaged cells and other inflammatory cells. The number of phagocytic microglia in brain was significantly increased in BDNF group than in control group 6 h after reperfusion (378.7 ± 13.1 versus 237.3 ± 18.7/mm², n = 6, P < .05, Figure 3). BDNF antibody, which could blocked the effect of BDNF, decreased the number of ED1 positive microglia (82.7 ± 12.0 versus 237.3 ± 18.7/mm², n = 6, P < .05, Figure 3) 6 h after reperfusion. At 24 h of reperfusion, exogenous BDNF increased the number of phagocytic microglia (325.3 ± 19.4 versus 138.7 ± 16.5/mm², n = 6, P < .05, Figure 3). However, the number of ED1 positive microglia showed no significant difference in antibody group compared with control group (154.2 ± 26.5 versus 138.7 ± 16.5/mm², n = 6, P > .05, Figure 3).

3.4. BDNF Promoted Anti-Inflammatory Cytokine Expression. IL10 is a well-known anti-inflammatory cytokine. IL-10 and its mRNA expression were tested by ELISA kits and real-time qPCR, respectively. When BDNF antibody blocked activity of endogenous BDNF in brain, IL10 was markedly decreased compared to control group (10.85 ± 0.48 versus 14.28 ± 0.82 ng/g, n = 6, P < .05, Figure 4(a)). Application of BDNF antibody also prevented upregulation of IL10 mRNA 6 h after reperfusion (0.50 ± 0.02 versus 1.01 ± 0.09, n = 6, P < .05, Figure 5(a)). However, there was no significant difference of IL10 and its mRNA expression between BDNF group and control group 6 h after reperfusion. Exogenous BDNF markedly increased local IL10 level in brain tissues 24 h after reperfusion (19.80 ± 0.83 versus 13.31 ± 0.36 ng/g, n = 6, P < .05, Figure 4(a)) and increased mRNA expression by 1.93 fold compared with control group. However, BDNF antibody did not influence the level of IL10 and its mRNA 24 h after reperfusion.

3.5. BDNF Inhibited Proinflammatory Cytokine Expression. TNF-α is a proinflammatory cytokine. To determine whether BDNF inhibited local TNF-α in ischemic brain tissues, we used ELISA kits and real-time qPCR to measure TNF-α and its mRNA expression, respectively. Giving exogenous BDNF inhibited TNF-α (11.23 ± 0.46 versus 14.33 ± 0.63 ng/g, n = 6, P < .05, Figure 4(b)) while suppression of endogenous BDNF by BDNF antibody upregulated TNF-α (19.76 ± 0.81 versus 14.33 ± 0.63 ng/g, n = 6, P < .05, Figure 4(b)) 6 h after reperfusion. Exogenous BDNF also significantly decreased TNF-α (9.59 ± 0.44 versus 12.85 ± 0.67 ng/g, n = 6, P < .05, Figure 4(b)) at 24 h of reperfusion. On mRNA level, BDNF inhibited mRNA expression of brain TNF-α after stroke at both 6 h (0.39 ± 0.01 versus 1.00 ± 0.06, n = 6, P < .05,
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**Figure 3:** BDNF increasing the number of activated microglia after stroke. Activated microglia in brain was marked by OX-42 and detected by confocal microscope following immunofluorescence. (a–c) 6 h after reperfusion and (e–g) 24 h after reperfusion; (a, e) control group, (b, f) BDNF group and (c, g) antibody group. (h) The number of phagocytic microglia in BDNF group was significantly increased compared to control group 6 h and 24 h after reperfusion. BDNF antibody significantly decreased the number of activated microglia 6 h after reperfusion. Bars represent mean ± SD (n = 6). *P < .05 versus control group, #P < .05 versus BDNF group. Scale bars: 25 μm.

**Figure 4:** BDNF upregulating the number of phagocytotic microglia after stroke. ED1 is well known as a marker of phagocytotic microglia. (a–c) 6 h after reperfusion and (d–f) 24 h after reperfusion; (a, d) control group, (b, e) BDNF group and (c, f) antibody group. BDNF significantly increased the number of phagocytotic microglia in brain 6 h and 24 h after reperfusion. The number of activated microglia was decreased in BDNF antibody group at 6 h of reperfusion. Bars represent mean ± SD (n = 6). *P < .05 versus control group, #P < .05 versus BDNF group. Scale bars: 25 μm.

Figure 5(b)) and 24 h (0.77 ± 0.04 versus 0.91 ± 0.06, n = 6, P < .05, Figure 5(b)) of reperfusion. The mRNA of TNF-α was increased by 2.28 fold in antibody group 6 h after reperfusion. However, BDNF antibody did not influence the TNF-α and its mRNA 24 h after reperfusion.

3.6. BDNF Increased DNA-Binding Activity of NF-κB after Stroke. The DNA-binding activity of NF-κB was activated by many stresses such as ischemia, the activity was measured using EMSA and expressed as arbitrary densitometric units (AU). Exogenous BDNF increased DNA binding activity of NF-κB by 10.7% compared with control group 6 h after reperfusion, and when endogenous BDNF was suppressed by BDNF antibody, the activity was significantly decreased (35.92 ± 0.99 versus 39.97 ± 0.70, n = 6, P < .05, Figure 6). At 24 h of reperfusion, exogenous BDNF could significantly increase DNA bind activity compared with control group (45.38 ± 0.86 versus 42.46 ± 0.27, n = 6, P < .05, Figure 6).
**Figure 5:** BDNF modulating local cytokine in brain after stroke. Interleukin 10 (IL-10) and tumor necrosis factor α (TNF-α) in rat brain after stroke were measured using ELISA kits. (a) Exogenous BDNF upregulated IL-10 24 h after reperfusion while BDNF antibody decreased IL-10 6 h after reperfusion. (b) Exogenous BDNF decreased TNF-α at 6 h and 24 h of reperfusion than control group and BDNF antibody overwhelmed the effect. Bars represent mean ± SD (n = 6). *P < .05 versus control group, #P < .05 versus BDNF group.

**Figure 6:** Effect of BDNF on mRNA expression of cytokine. Brain homogenates were obtained from ischemic cortices 6 h and 24 h after reperfusion. The expressions of mRNA of interleukin 10 (IL10) and tumor necrosis factor α (TNF-α) in rat brain after stroke were measured by real-time quantitative polymerase chain reaction (PCR). (a) Levels of IL10 mRNA. Application of BDNF antibody prevented upregulation of IL10 mRNA 6 h after reperfusion. At 24 h of reperfusion, BDNF increased level of mRNA of IL10 by 1.93 fold. (b) Levels if TNF-α mRNA. Exogenous BDNF significantly decreased mRNA of TNF-α 6 h and 24 h after reperfusion. Inhibition of endogenous BDNF significantly increased the expression of TNF-α 6 h after reperfusion. Bars represent mean ± SD (n = 6). *P < .05 versus control group, #P < .05 versus BDNF group.
However, there is no significant difference between antibody group and control group (41.43 ± 1.18 versus 42.46 ± 0.27, \( n = 6, P > .05 \), Figure 6).

4. Discussion

Our data suggested that BDNF could alleviate cellular injury of ischemic insult, reduce the neurologic deficits and modulate local inflammation on cellular, cytokine and nuclear factor levels in brain after stroke. Exogenous BDNF could increase the number of activated and phagocytic microglia, upregulate IL-10, downregulate TNF-\( \alpha \) and increase the DNA-binding activity of NF-\( \kappa \)B while BDNF antibody blocked these effects of BDNF in ischemic brain tissues.

Firstly, we showed that introducing BDNF directly to brain increased the concentration of BDNF. We also found that BDNF antibody may not change the expression of BDNF in brain tissues after stroke. Our data confirm previous reports that injection of BDNF directly to brain could raise concentration of BDNF.

Secondly, exogenous BDNF could protect brain from ischemic injury and reduce the neurologic deficits. We found exogenous BDNF significantly decreased the number of TUNEL-positive cells. We also found that BDNF improved the motor-sensory function, sensorimotor function, and vestibulomotor function. Our results were consistent with previous reports [22, 23]. However, BDNF may not reduce vestibulomotor function. Our results were consistent with other studies [13]. Previous publications showed that exogenous pre/postischemic administration of IL10 can provide neuroprotection following MCAO [9, 29]. Over-expression of IL10 in vivo markedly protected cortical tissue against cerebral ischemia using the IL10 transgene mice [30]. Our data suggested that BDNF might protect brain from ischemia through upregulating local IL10 in brain.

On the transcription level of local inflammation in brain in ischemic stroke of rats, we found that exogenous BDNF increased the mRNA expression of IL10 at 24 h of reperfusion. Once activity of endogenous BDNF was blocked by BDNF antibody, local IL-10 and its mRNA in brain were increased. These results were consistent with other studies [13]. Previous publications showed that exogenous pre/postischemic administration of IL10 can provide neuroprotection following MCAO [9, 29]. Over-expression of IL10 in vivo markedly protected cortical tissue against cerebral ischemia using the IL10 transgene mice [30]. Our data suggested that BDNF might protect brain from ischemia through upregulating local IL10 in brain.

In summary, our data suggested that BDNF may alleviate cellular injury of ischemic insult, reduce the neurologic

5. Conclusions

In our study, effect of BDNF on local inflammation in brain showed no significant difference between antibody group and control group 24 h after reperfusion. This may be that BDNF antibody only blocked the activity of BDNF and may not suppressed the expression of local BDNF in brain after stroke. Our data showed that BDNF antibody did not change BDNF level (Figure 1). 24 h after reperfusion (25 h after BDNF antibody was given), the expression of new BDNF may replace the antibody-conjuncted BDNF, so the effect of BDNF antibody might be removed.

In summary, our data suggested that BDNF may alleviate cellular injury of ischemic insult, reduce the neurologic
deficits and modulate local inflammation on cellular level, cytokine level, and transcription factor level in ischemic stroke.

Conflict of Interests

The authors have no conflict of interests to disclosure.

Authors’ Contributions

Y. Jiang, N. Wei, and X. Liu participated in concept and design of the study, acquisition of raw data, analysis and interpretation of data, drafting manuscript, critical revision of the manuscript for scientific validity, statistical analysis. J. Zhu, T. Lu, Z. Chen and G. Xu participated in study concept and design, acquisition of raw data and critical revisions of the paper. Y. Jiang and N. Wei contributed equally to this work.

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