Genes & Diseases (2019) 6, 448–454

Available online at www.sciencedirect.com

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FULL LENGTH ARTICLE

NDRG4 prevents cerebral ischemia/reperfusion injury by inhibiting neuronal apoptosis

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Received 17 September 2018; accepted 10 January 2019
Available online 17 January 2019

KEYWORDS
Apoptosis;
BDNF;
Ischemia;
NDRG4;
Reperfusion injury

Abstract Cerebral ischemia is a major cause of mortality and long-term morbidity worldwide. NDRG4 has been shown to protect against cerebral ischemia, although the underlying mechanisms remain largely unclear. Here we found that NDRG4 expression was decreased in the brain tissues of ischemia/reperfusion (IR) rats, indicating increased apoptosis rates among cerebral cells. NDRG4 restoration via an adenovirus significantly attenuated cerebral infarct sizes and impairments in IR rats. Furthermore, adenovirus-mediated NDRG4 inhibited cell apoptosis in the brains of IR rats and regulated the expression of Bcl-2, Bax, caspase-3, and c-Fos. Moreover, we found that NDRG4 increased expression of BDNF, which is strongly related to cerebral ischemia and cellular apoptosis. Altogether, our findings demonstrate that NDRG4 protects cerebral IR injury by inhibiting cell apoptosis and regulates cerebral cell apoptosis by increasing BDNF expression. These results suggest that NDRG4 may be a therapeutic target for IR treatment.

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Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2019.01.001

Introduction

Cerebral ischemia is a major contributor to brain tissue damage that can lead to death and long-term disability.[1] The effectiveness of current therapies is limited by a therapeutic time window.[2] Additionally, prognoses can be further complicated if the process of cerebral ischemia recovery and reperfusion aggravates preexisting brain injuries; the result of this is known as ischemia/reperfusion (IR) injury.[3,4] Apoptosis is the main molecular biological mechanism underlying IR injury, and involves members of the B-cell lymphoma/leukemia-2 (Bcl-2) family, such as Bcl-2 (an anti-apoptotic protein) and Bcl-2-associated X protein (Bax) (a pro-apoptotic protein).[5] However, the precise trigger for cellular apoptosis in cerebral IR injury remains unclear.

The N-Myc downstream-regulated gene (NDRG) family includes NDRG1-4, which is involved in brain development, the immune system, and cancer metastasis.[6] NDRG4 is widely distributed in the human brain[7] and NDRG4-deficient mice exhibit poor spatial learning and cerebral ischemia vulnerability, as well as growth retardation and postnatal lethality.[8,9] Furthermore, NDRG4 knockdown induces glioblastoma cell apoptosis independent of mitotic defects or DNA damage.[10] Cerebral cell apoptosis such as this contributes significantly to neurological damage.[11] However, it remains unclear whether the effects of NDRG4 on cerebral ischemia are related to cerebral cell apoptosis.

Brain-derived neurotrophic factor (BDNF) is a major neurotrophin which regulates neuronal proliferation and differentiation in the central nervous system during development, as well as influencing synaptic structure and function postnatally.[12] Activation of BDNF and its receptor TrkB in neurogenic regions has also been shown to have neuroprotective effects in cerebral ischemia.[13] Furthermore, recombinant BDNF enhances infarct tolerance, while BDNF deletion aggravates ischemic injury.[14,15] Collectively, these results suggest a crucial role of BDNF in cerebral ischemia. Recent findings in mouse models showed that BDNF inhibited intestinal epithelial cell apoptosis,[16] while NDRG4 deletion decreased BDNF expression.[17]

In the present study, we utilized an IR rat model to investigate the role of NDRG4 in cerebral IR injury and potential underlying mechanisms.

Materials and methods

Reagents

Rabbit anti-NDRG4 antibody (cat. no. 9039), anti-Bax antibody (cat. no. 14796), anti-Bcl-2 antibody (cat. no. 2876), anti-caspase-3 antibody (cat. no. 9662) (Cell Signaling Technology, USA); Rabbit anti-BDNF antibody (cat. no. ab108319), anti-c-Fos antibody (cat. no. ab134122) and anti-β-actin (cat. no. 4044) (Abcam, USA); TRIzol buffer, radioimmune precipitation buffer (RIPA), 4′6-diamidino-2-phenylindole (DAPI), bicinchoninic acid protein assay kit (BCA), enhanced chemiluminescence (ECL) (Beyotime Technology, Shanghai, China); anti Alexa Fluor 647-goat anti-rabbit IgG (Abcam, USA); anti-rabbit FITC tagged secondary antibody (Abcam, USA); terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) kit (Roche, Basel, Switzerland); horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. no. sc-2004, Santa Cruz Biotechnology Inc, USA).

Animals

In total, 191 male Sprague—Dawley (S-D) rats (250—300 g, 8—10 weeks) were included in this study, which was approved by the Experimental Animal Center of Chongqing Medical University according to the "Guiding Principles for Research Involving Animals and Human Beings." Rats were housed at 21—22 °C on a 12 h light/dark cycle. Fifty-five S-D rats were excluded due to developing no neurologic abnormalities (12) or dying (43). The remaining 132 S-D rats were randomly divided into four groups: a sham operated group (Control, n = 33), an IR group (IR, n = 33), an IR with AAV-C group (IR + AAV-C, n = 33), and an IR with AAV-NDRG4 group (IR + AAV-NDRG4, n = 33).

Transient middle cerebral artery occlusion (MCAO) in rats

To induce transient focal cerebral ischemia in the rats, an MCAO surgery was performed using the three-vessel occlusion technique described previously.[18] Briefly, we anesthetized rats with 3.5% chloral hydrate (350 mg/kg, intraperitoneally) and exposed and distally ligated the right common carotid artery (CCA) via a midline neck incision. We then isolated the external and internal carotid arteries. MCAO sutures (Guangzhou Jia Ling Biotechnology Co, Ltd, Guangzhou, China) were used for IR modeling. One and a half hours after ischemia induction, reperfusion was established by removing the suture. Rats in the sham group underwent identical surgical procedures without occlusion of the right CCA.

Adenovirus-mediated NDRG4 overexpression

The open reading frame of NDRG4 was inserted into a pAAV-CMV bGlobin-MCS-eGFP-3Flag vector (AAV-NDRG4). The empty control vector was pAAV-CMV bGlobin-MCS-eGFP-3Flag (AAV-C). The synthesized AAV-NDRG4 and AAV-C were packaged in an adenovirus using GENECHEM Technology Company techniques (Shanghai, China). This adeno-associated virus vector is broadly used for gene delivery to the mouse brain.[19,20] The adenovirus titers were AAV-C and AAV-NDRG4 with 1×10^{12} and 2×10^{12} copies per milliliter, respectively. A 4 μL aliquot of the suspension was injected into the brain, and after three weeks, MCAO surgery was performed (ML: 1.5 mm, AP: 1.0 mm, DV: 4 mm) in IR + AAV-C and IR + AAV-NDRG4 groups.

Evaluation of neurological deficits

Neurological deficits were evaluated by a blinded investigator. Neurological grading scores were analyzed before rats were executed, as described previously.[21] Neurological deficits were graded on a scale of 0—4 as follows: 0 - observable deficits, 1 - torso flexion to the left, 2 - spontaneous circling to the left, 3 - falling to the left, and 4 - no spontaneous movement.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

Twenty-four hours after reperfusion, the ipsilateral ischemic penumbra region was harvested and underwent TTC staining, per the manufacturer protocol. Briefly, rats were randomly selected for TTC staining (n = 6 per group). After anesthetization, the rats' brains were rapidly removed and sectioned (2 mm coronal sections). Viable tissues were stained with 2% TTC for 20 min at 37 °C. Sections were then photographed and the area of infarcted tissue that appeared pale was calculated using ImageJ software (v. 1.48, National Institutes of Health, Bethesda, MD, USA).

RNA isolation and real-time quantitative PCR (RT-qPCR)

Animals were executed at 1 h, 3 h, 5 h, 7 h, 12 h, 24 h, and 48 h after reperfusion respectively. Total RNA was extracted from ipsilateral ischemic penumbra tissues with TRIzol (Beyotime Biotechnology, China). Total RNA concentrations were analyzed using an ultraviolet spectrophotometer. Reverse transcriptase (Western Biotechnology, China) was used for the reverse transcription of RNA. qPCR was performed using 2 × SYBR Green (Western Biotechnology, China) and the Bio-Rad iQ5 Gradient Real-Time PCR system (Bio-Rad, USA), per manufacturer instructions. qPCR parameters were as follows: 94 °C for 4 min, 35 cycles at 94 °C for 20 s then 60 °C for 30 s, then 72 °C for 30 s to extend the transcripts. Primer sequences were as follows: NDRG4 forward primer: 5′-tcttccctgatttggtggag-3′, NDRG4 reverse primer: 5′-ccagaagagctgaaggttgg-3′; BDNF forward primer: 5′-gtcccggtatcaaaaggcca-3′, BDNF reverse primer: 5′-atccttatgaaccgccagcc-3′; β-actin forward primer: 5′-cccatctatgagggttacgc-3′, and β-actin reverse primer: 5′-tttaatgtcacgcacgatttc-3′. Relative gene expression was normalized to β-actin using the $2^{-\Delta\Delta CT}$ method.

Western blot

Animals were executed at 1 h, 3 h, 5 h, 7 h, 12 h, 24 h and 48 h after reperfusion, respectively. Approximately 50—100 mg of rat brain tissue, including the ipsilateral ischemic penumbra, were lysed using the RIPA buffer per the manufacturer protocol. Total protein concentration was measured using a BCA kit per manufacturer instructions. A total of 50 μg of protein was separated via SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were then incubated with 5% nonfat dry milk at room temperature for 1 h, then with the following antibodies at 4 °C overnight (1:1000 for all): anti-NDRG4, anti-BDNF, anti-Bax, anti-caspase-3, anti-Bcl-2, anti-c-Fos, and anti-β-actin. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-Rabbit) (1:5000) at room temperature for 2 h. Finally, bands were visualized using ECL solution and a gel imaging system (Tannon, China) according to the manufacturer instructions. Bands were quantified using the Quantity One software (Labworks Analysis Software, USA), and normalized to β-actin.

Immunofluorescence

Brain tissues containing the ipsilateral ischemic penumbra were isolated and quickly frozen at −80 °C, coated with a layer of optimum cutting temperature compound for 10 min, then sectioned at 10 μm. After blocking with goat serum, sections were incubated with anti-NDRG4 rabbit polyclonal antibody (1:100) and anti-BDNF mouse monoclonal antibody (1:750) overnight at 4 °C. Next, slides were incubated with Alexa Fluor 647-goat anti-rabbit IgG (1:800) and FITC-labeled anti-rabbit secondary antibody (1:800) for 1 h at 37 °C. Slides were also incubated with DAPI at room temperature for 15 min in the dark. Finally, a confocal laser scanning microscope was used to detect section fluorescence.

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay

Apoptosis was quantified using a TUNEL kit per the manufacturer instructions. Briefly, slides were incubated with TUNEL reaction mixture for 60 min at 37 °C and then DAB-H2O2 solution at room temperature for 10 min. Cell nuclei were stained using hematoxylin for 10 min at room temperature. Five non-overlapping fields per slide were randomly imaged using a light microscope (OLYMPUS, Japan) at 400× magnification.

Statistical analyses

All experiments were performed at least three times using independent samples. Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). One-way analyses of variance (ANOVAs) were used to analyze differences among more than two groups, followed by Tukey's post hoc tests. $P < 0.05$ was considered to indicate statistical significance.

Results

NDRG4 was dysregulated in rat cerebral tissues in a temporally-specific manner

To investigate the role of NDRG4 in cerebral IR injury, we used the IR rat model described previously. At variable post-operative times, rats were sacrificed and their brain tissues were collected. qRT-PCR revealed that NDRG4 mRNA expression was increased most at 5 h ($P < 0.01$), then decreased at 24 h and 48 h in tissues with cerebral IR injury ($P < 0.01$) (Fig. 1A). Moreover, western blotting revealed that NDRG4 protein expression followed the same trend (Fig. 1B).

NDRG4 reduced cerebral infarction and neurological deficits

To further determine the role of NDRG4 in IR injury, we constructed an adenovirus containing a pAAV-CMV bGlobin-MCS-eGFP-3Flag (AAV-C) or AAV-NDRG4 vector. We injected this adenovirus into rats that were subjected to IR three

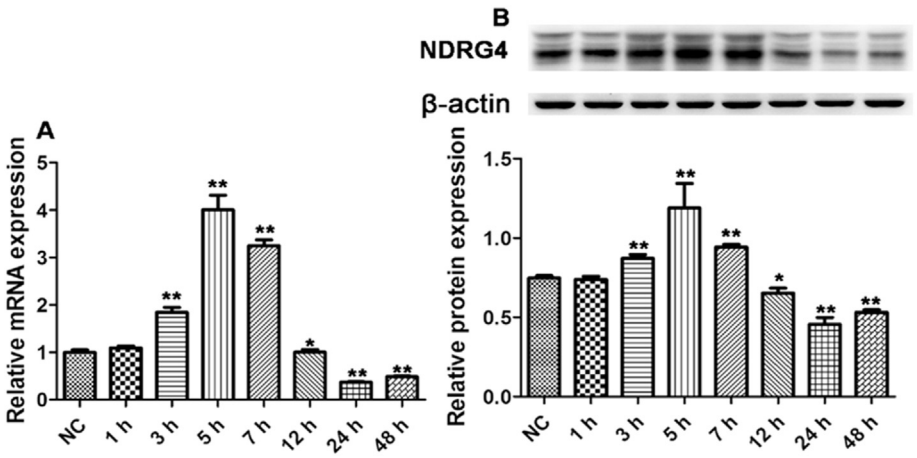

Figure 1 Expression pattern of NDRG4 in rat brain tissues after ischemia/reperfusion (IR) injury. A. NDRG4 mRNA was detected by RT-qPCR, normalizing to β-actin. B. NDRG4 protein was measured by western blot. The relative band density was calculated by normalization to β-actin. Compared with the control group, *P < 0.05, **P < 0.01.

weeks later. TTC staining revealed that NDRG4 overexpression significantly reduced cerebral infarct lesion sizes (P < 0.01) (Fig. 2A, B). Moreover, cerebral function testing revealed that NDRG4 significantly attenuated neurological deficits (P < 0.01) (Fig. 2C).

NDRG4 attenuated neuronal apoptosis in cerebral IR injury

Next, we sought to determine whether NDRG4 protected against cerebral IR injury by regulating cell apoptosis. As expected, the TUNEL assay revealed that IR rats exhibited significantly increased apoptosis rates in brain tissues (P < 0.01). Moreover, NDRG4 significantly reduced brain apoptosis rates as compared to controls (P < 0.01) (Fig. 3A, B). Next, we analyzed the expression of apoptosis-related genes, including Bax, Bcl-2, Caspase-3, and c-Fos. Western blotting revealed increased protein expression of Bax, Caspase-3, and c-Fos in the brain tissues of IR rats, which were decreased when NDRG4 was upregulated (P < 0.01). Bcl-2 protein expression exhibited the opposite changes (P < 0.01) (Fig. 4). Together, these data suggest that cerebral IR injury causes brain cell apoptosis while NDRG4 prevents brain cell apoptosis.

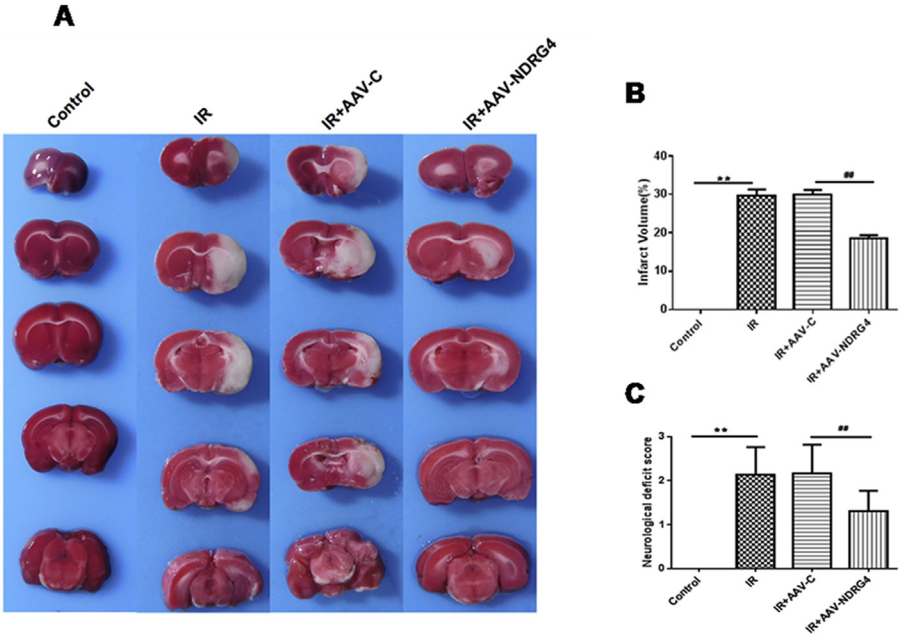

Figure 2 NDRG4 inhibits infarct volume and mitigates neurological deficits 24 h after ischemia/reperfusion (IR) injury. A.2, 3, 5-triphenyltetrazolium chloride staining (white) indicating infarct sizes in rats with different treatments and in normal controls (NC). B. Histogram depicting infarct volumes. C. Histogram depicting neurological deficit scores. Compared with the control group, **P < 0.01; compared with the IR + AAV-C group, ##P < 0.01.

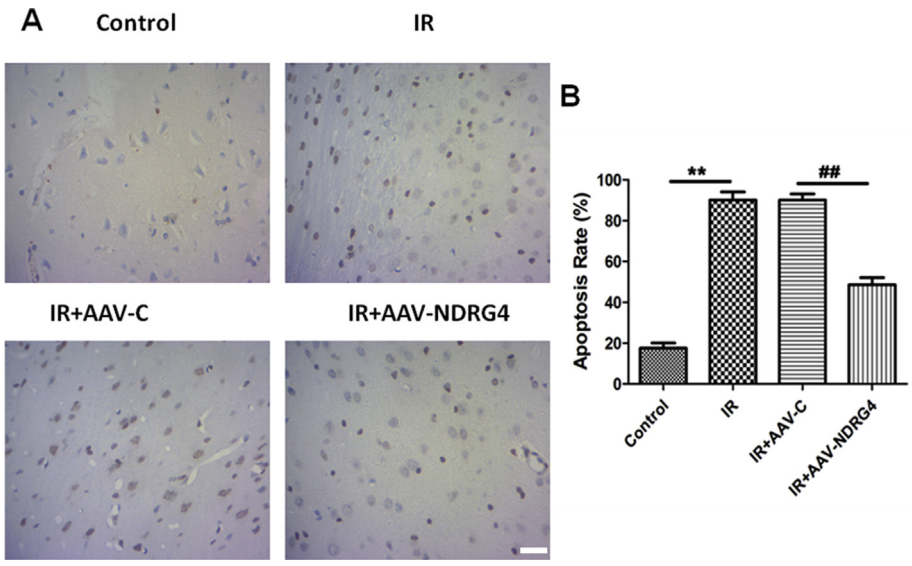

Figure 3 NDRG4 inhibits neuronal apoptosis. A. Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining revealed cerebral cellular apoptosis following ischemia/reperfusion (IR) injury. B. Histogram depicts the number of TUNEL-positive cells. Compared with the Control group, **P $<$ 0.01; compared with IR $+$ AAV-C group, ##P $<$ 0.01, bar $=$ 20 μm.

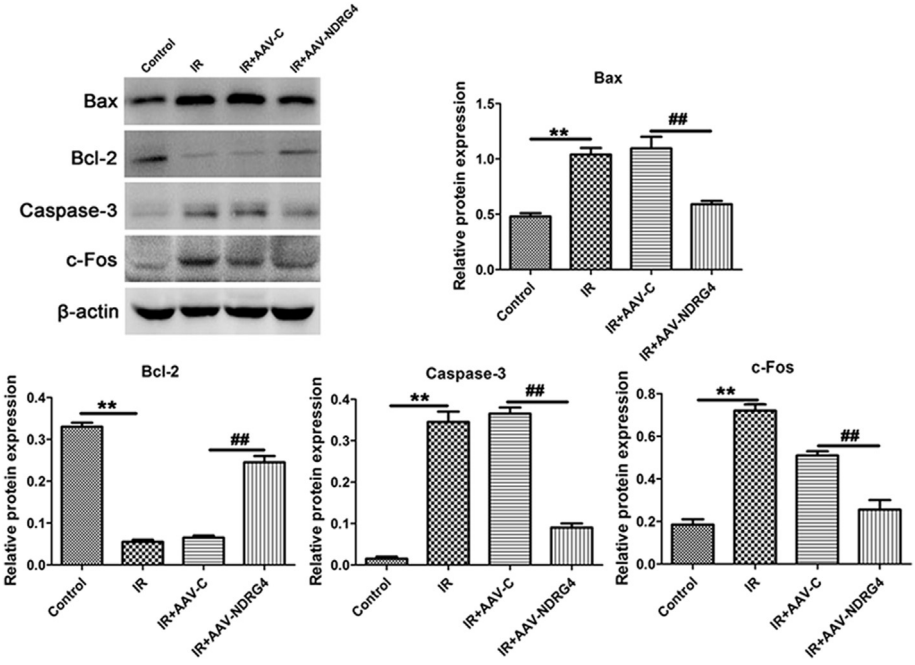

Figure 4 NDRG4 regulates the expression of apoptosis-related proteins. The relative protein levels of Bax, Bcl-2, caspase-3, and c-Fos, detected via western blotting and normalized to β-actin. Compared with the Control group, **P $<$ 0.01; compared with the IR $+$ AAV-C group, ##P $<$ 0.01.

NDRG4 increased BDNF expression

Immunofluorescence revealed that the expression of NDRG4 and BDNF was significantly decreased in the brain tissues of IR rats and that BDNF expression was restored when NDRG4 was upregulated (Fig. 5A). Moreover, qRT-PCR and western blotting also confirmed these immunofluorescence results via mRNA and protein expression of NDRG4 and BDNF, respectively (P $<$ 0.01) (Fig. 5B, C). Collectively, these data suggest that NDRG4 may promote cell apoptosis by driving increased expression of BDNF.

Discussion

In the present study, we found that downregulation of NDRG4 protected against cerebral IR injury and was associated with increased apoptosis of cerebral cells in an IR rat

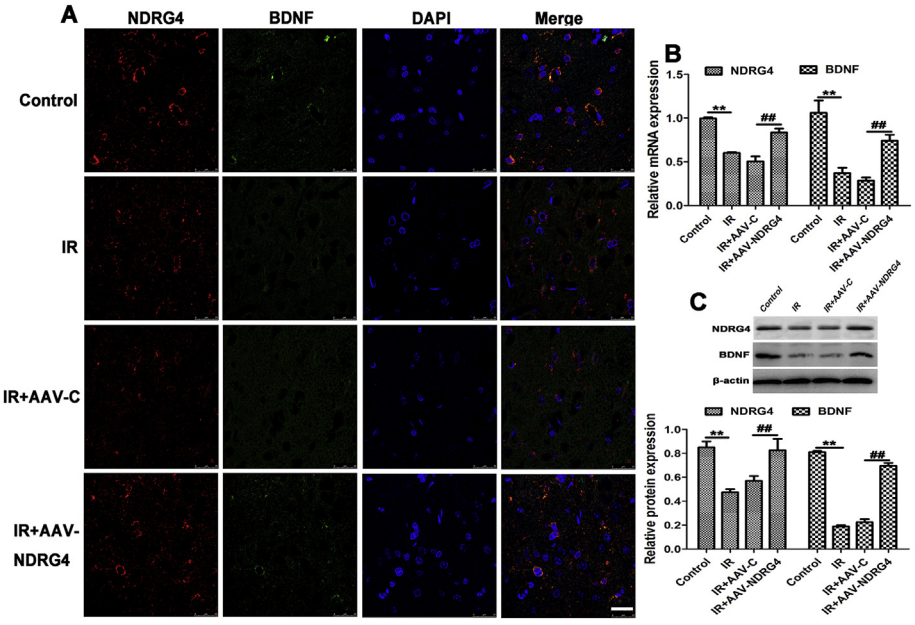

Figure 5 NDRG4 promotes the expression of BDNF at 24 h after ischemia/reperfusion (IR) injury. A. The expression of BDNF and NDRG4 was measured via immunofluorescence. Red refers to NDRG4 and green refers to BDNF. Cell nuclei were stained with DAPI. B. NDRG4 and BDNF mRNA was detected via RT-qPCR. C. NDRG4 and BDNF protein levels were measured via western blotting. Relative mRNA expression was normalized to β-actin. Compared with the Control group, **P $<$ 0.01; compared with the IR + AAV-C group, ##P $<$ 0.01, bar = 25 μm.

model. We also found that increasing NDRG4 rescued the apoptosis of cerebral cells. These data suggest that NDRG4 is protective against cerebral ischemia in a rat model.

The NDRG4 protein expression patterns are orthologous to humans and variety of animals. For example, NDRG4 is highly expressed in both the human brain and heart but not in other organs, and is exclusively expressed in the embryonic zebra fish heart and central nervous system.[7,22] NDRG4 has been reported to be involved in the cell cycle and the survival of glioblastoma cells, and its deficiency can led to impaired spatial learning and increased vulnerability to cerebral ischemia in a mouse model.[10,17] These studies suggest that NDRG4 may inhibit or buffer against cerebral ischemia in vivo. Pursuant to this, in the present study, we established an IR rat model with decreased expression of NDRG4 in the brain at 24 h and 48 h; however, via compensatory mechanisms, it was overexpressed at 3 h, 5 h and 7 h. We speculate that this compensation is a response to early cerebral cellular apoptosis.

Cerebral ischemic injury often involves cellular apoptosis.[23] For instance, we observed increased apoptosis in cerebral cells in the IR rat model used here, as well as aberrant expression of apoptosis-related genes, including Bax, Bcl-2, Caspase-3, and c-Fos, all of which have reported roles in ischemic injury-caused neuronal cell apoptosis.[24–28] Previous studies have also shown that NDRG4 is involved in oncogenic (e.g. glioblastoma, malignant meningioma) cellular apoptosis.[10,29] However, whether this is associated with cerebral cellular apoptosis remains unclear. To address this question, we artificially increased expression of NDRG4 in the brain tissues of the IR rats using an adenovirus, as has been described previously.[30] We also found that restoration of NDRG4 inhibited cerebral cell apoptosis and attenuated cerebral infarct sizes and impairments in IR rats. These findings suggest that NDRG4 may serve as a potential target for IR therapy.

Several studies have reported that BDNF mitigates severe ischemia-caused brain injury,[14] and also inhibits ischemia-induced neuronal apoptosis via regulation of the expression of Bcl-2, Bax, and Caspase-3.[31,32] These reports are consistent with our finding that increased NDRG4 levels in IR rats are linked to greater levels of cortical BDNF and the inhibition of neuronal apoptosis. Consistent with our finding that levels of BDNF were increased in the brains of NDRG4-overexpressed rats, a previous study reported that the cortices of NDRG4-deficient mice (in a MCAO model) contained decreased levels of BDNF.[17] This prior work, as well as that presented here, suggests that NDRG4 alters BDNF expression. However, the mechanism(s) by which NDRG4 influences cerebral BDNF levels remain unknown.

In conclusion, our findings suggest that NDRG4 prevents cerebral IR injury through inhibiting cerebral cell apoptosis. Further investigation of NDRG4 may provide insight into effective therapies for ischemia reperfusion injury.

Acknowledgements

Funding: This work was supported by the National Natural Science Foundation of China [No. 81271306].

Declarations of interest

none

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2019.01.001.

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