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Neuroprotection by Exogenous and Endogenous Neuregulin-1 in Mouse Models of Focal Ischemic Stroke

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Abstract

Identifying novel neuroprotectants that can halt or reverse the neurological effects of stroke is of interest to both clinicians and scientists. We and others previously showed the pre-clinical neuroprotective efficacy of neuregulin-1 (NRG-1) in rats following focal brain ischemia. In this study, we examined neuroprotection by exogenous and endogenous NRG-1 using a mouse model of ischemic stroke. C57BL6 mice were subjected to middle cerebral artery occlusion (MCAO) followed by reperfusion. NRG-1 or vehicle was infused intra-arterially (i.a.) or intravenously (i.v.) after MCAO and before the onset of reperfusion. NRG-1 treatment (16 μg/kg; i.a.) reduced cerebral cortical infarct volume by 72% in mice when delivered post-ischemia. NRG-1 also inhibited neuronal injury as measured by Fluoro Jade B labeling and rescued NeuN immunoreactivity in neurons. Neuroprotection by NRG-1 was also observed in mice when administered i.v. (100 μg/kg) in both male and female mice. We investigated whether endogenous NRG-1 was neuroprotective using male and female heterozygous NRG-1 knockout mice (NRG-1+/−) compared with wild-type mice (WT) littermates. NRG-1+/− and WT mice were subjected to MCAO for 45 min, and infarct size was measured 24 h following MCAO. NRG-1+/− mice displayed a sixfold increase in cortical infarct size compared with WT mice. These results demonstrate that NRG-1 treatment mitigates neuronal damage following cerebral ischemia. We further showed that reduced endogenous NRG-1 results in exacerbated neuronal injury in vivo. These findings suggest that NRG-1 represents a promising therapy to treat stroke in human patients.

Keywords Cell death · erbB receptor · Ischemia · Neuroprotection · Inflammation · Transgenic

Introduction

Stroke remains a leading cause of death in the USA and the most frequent cause of long-term disability. Ischemic stroke occurs as a result of an obstruction within a blood vessel supplying blood to the brain. The neuronal death that ensues results from the induction of genes and the activation of pathways associated with a number of cellular functions including apoptosis, inflammation, and oxidative stress (Iadecola and Anrather 2011). Currently, the tissue plasminogen activator (t-PA) is the only approved drug treatment for ischemic stroke. Unfortunately, t-PA has a limited time window for therapeutic use, and only 3–5% of stroke patients arriving at the hospital will qualify for treatment (Fisher et al. 2009). Thus, there is a strong need to understand the molecular mechanisms associated with ischemic stroke so that more effective modes of treatment can be investigated.

Multiple neuroprotective compounds have been reported to be effective against cerebral ischemia in animal and cell models, but none of these drugs were shown to be clinically effective (Bosetti et al. 2017; Fisher et al. 2009; Saver et al. 2009; Savitz and Fisher 2007). Neuregulins have been implicated in normal brain function, as well as in neuroprotection following cerebral ischemia. Previous studies from our laboratory and others demonstrated that neuregulin-1 (NRG-1) reduced ischemia-induced neuronal death in a rat focal stroke model by ~90% with a therapeutic window of > 13 h (Guo et al. 2006; Li et al. 2007; Shyu et al. 2004; Wang et al. 2015, 2018; Xu et al. 2004, 2005, 2006). NRG-1 administration also resulted in a significant improvement of neurological function (Iaci et al. 2010; Xu et al. 2006). NRG-1 administration

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resulted in a significant improvement of neurological function when administered 3 days following ischemia, suggesting a role in neuronal repair (Iaci et al. 2010, 2016). Recent clinical studies demonstrated the utility of NRG-1 in human patients (Gao et al. 2010; Jabbour et al. 2011; Lenihan et al. 2016) (ClinicalTrials.gov identifiers NCT01258387; NCT01251406). Systemic administration of NRG-1 improved cardiac function in heart failure patients. The doses used in these clinical trials were comparable to the effective dose in stroke models; thus, NRG-1 has potential therapeutic value in treating individuals after acute ischemic stroke in clinical studies.

In this study, we examined the neuroprotective efficacy of exogenous and endogenous NRG-1 on cerebral injury after the induction of ischemia by middle cerebral artery occlusion (MCAO) in mice. NRG-1 homozygous knockout mice are embryonic lethal at embryonic day 10.5. However, heterozygous knockout mice lacking one functional copy of the gene for NRG-1 (NRG-1+/−) express reduced levels of brain NRG-1 (Erickson et al. 1997; Gerlai et al. 2000; Sandrock et al. 1997) and display hyperactive and schizophrenia-like phenotypes during neurobehavioral testing (O'Tuathaigh et al. 2007, 2008, 2010; Stefansson et al. 2002). We demonstrated that exogenously administered NRG-1-protected neurons from cerebral ischemia in both male and female mice. In addition, NRG-1+/− mice displayed significantly larger infarcts than wild-type littermates, suggesting a neuroprotective role for endogenous NRG-1. Our data demonstrate that NRG-1 is a potent neuroprotectant against ischemic brain injury with high clinical potential. These findings may aid in identifying a novel therapeutic strategy for the treatment of stroke.

Materials and Methods

Animals All animals used in these studies were treated humanely and with regard for alleviation of suffering and pain, and all protocols involving animals were approved by the IACUC of Morehouse School of Medicine and University of California-Riverside prior to the initiation of experimentation. Male and female C57BL6 mice (8–10 weeks old) were purchased from Charles River (Wilmington, MA) and housed with a 12-h daily light/dark cycle. Male and female (10 months old) NRG-1 heterozygous transgenic mice (NRG-1+/−) and wild-type littermates (WT; NRG-1+/+) were also used in this study (Meyer and Birchmeier 1995). The NRG-1 gene knockout mouse was generated by deletion of exons 7, 8, and 9, which encode the carboxy terminal of the EGF domain of all known NRG-1 isoforms. Mice with homozygous knockout of NRG-1 (NRG-1−/−) are embryonic lethal. However, the NRG-1+/− mice display reduced brain NRG-1 levels which are viable but display some abnormal neurobehavioral functions (Erickson et al. 1997; Gerlai et al. 2000; Sandrock et al. 1997; Stefansson et al. 2002). Food and water were provided ad libitum. All surgical procedures were performed by sterile/aseptic techniques in accordance with institutional guidelines.

Transient Middle Cerebral Artery Occlusion Animals were subjected to left MCA occlusion (MCAO). Mice were anesthetized with 1.5% isoflurane in 68.5% N_2O and 30% O_2. MCA occlusion was induced by the intraluminal suture transient MCAO method as previously described (Barber et al. 2004). The left common carotid artery (CCA) was exposed through a midline incision and was carefully dissected free from surrounding nerves and fascia. The occipital artery branches of the external carotid artery (ECA) were then isolated, and the occipital artery and superior thyroid artery branches of the ECA were coagulated. The ECA was dissected further distally. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated close to its origin with a 6–0 silk suture. Then, a 12-mm 7–0 surgical monofilament nylon suture with rubber silicon (Doccol, Sharon, MA) was inserted from the external carotid artery (ECA) into the internal carotid artery (ICA) then into the circle of Willis to occlude the origin of the left middle cerebral artery. The nylon suture was withdrawn 1 h following ischemia, and the brain tissues were reperfused for 23 h before sacrifice. Mice within the intravenous treatment group underwent filament occlusion for 90 min, and the brain tissues were reperfused for 22.5 h before sacrifice because of variability in baseline infarct size between male and female mice with 60 min of MCAO. Regional cerebral blood flow (CBF) was measured by continuous laser Doppler flowmetry (LDF) with a laser Doppler probe (Perimed, Kings Park, NY) placed in the ipsilateral skull close to the middle cerebral artery from the beginning of the MCAO surgery until 15 min after reperfusion. For NRG-1+/− ($n = 6$) and WT ($n = 6$) knockout mice, MCAO occurred for 45 min and the brains were reperfused for 23.25 h before sacrifice. The 45-min time point was chosen so that infarction was submaximal which allowed us to determine if infarcts grew larger in the transgenic animals. The surgical procedure was considered successful if CBF was reduced by ≥ 70% immediately after placement of the occluding suture; otherwise, mice were excluded.

To determine the effects of NRG-1 on ischemic stroke, mice were randomly divided into two treatment groups and injected intra-arterially (i.a.) with either a single-bolus 5-μL dose of vehicle (0.1% BSA in PBS) or NRG-1β (10 μmol/L NRG-1 (EGF-like domain, R&D Systems, Minneapolis, MN) in 1% BSA in PBS) through a Hamilton syringe as previously described (Xu et al. 2004, 2005, 2006). This resulted in the administration of 16 μg of NRG-1/kg body weight. NRG-1 ($n = 9$) or vehicle (PBS; $n = 9$) was administered by bolus injection into the ICA through the ECA immediately before reperfusion. A second group of female ($n = 6$) and male ($n = 4$)

mice were injected intravenously (i.v.) into the tail vein with either a single-bolus 100-μL dose of vehicle (0.1% BSA in PBS) or NRG-1β (100 μg/kg) with a 30-G needle immediately after filament insertion. All NRG-1 and vehicle treatment studies were performed in a blinded manner. Core body temperature was closely monitored with a rectal probe and maintained at 36.1 ± 0.1 °C with a Homeothermic Blanket Control Unit (Harvard Apparatus, Holliston, MA) during anesthesia to prevent hyperthermia as a confounding factor (Barber et al. 2004).

Infarct Size Measurement and Neurobehavioral Testing Twenty-four hours after MCAO, 1.0- or 2.0-mm-thick brain sections were stained with 2–5% triphenyltetrazolium chloride (TTC) solution and then transferred into a 4% formaldehyde solution for fixation. The infarcted region appears white, whereas the normal non-infarcted tissue appears red. The infarct area was identified by using five to seven slices of coronal sections from each brain. The infarct area was calculated in a blinded manner by capturing the images with a digital camera. Percentages of cortical and striatal infarct were calculated as a percentage of cortical and striatal volume, respectively (percentage of total infarct = infarct volume/(hemispheric volume) × 100%.). Infarct volumes were analyzed by *t* test; $p < 0.05$ was regarded as significant. Mice were assessed for neurobehavioral deficits 24 h after MCAO. The neurobehavioral score evaluation system is as follows: 0, normal; 1, consistent forelimb and axial flexion toward the contralateral to the lesion side when lifted by the tail; 2, observations of score 1 with consistently reduced resistance to lateral push and gait toward the paretic side; 3, observations of score 2 plus large or weak circling toward the paretic side; and 4, score 2 plus circling toward the paretic side (Barber et al. 2004).

Histology and Immunohistochemistry After MCAO, mice were deeply anesthetized with 2% isoflurane and perfused transcardially with saline followed by cold 4% paraformaldehyde (PFA) solution in PBS for 30 min ($n = 4$–5 animals/group). Brains were quickly removed and cryoprotected in 30% sucrose. The brains were then frozen in OCT compound and stored at − 80 °C until sectioning. Coronal sections of 10–20-μm thickness were cryosectioned and mounted on slides which were then stored at − 80 °C until further processed. Fluoro Jade B (FJB; AG310, Millipore, Billerica, MA) labeling was performed according to the manufacturer's protocol with minor modifications. Briefly, after 30 min drying at 50 °C, sections were post-fixed with 4% paraformaldehyde for 15 min, washed with distilled water, and then directly incubated in 0.06% potassium permanganate ($KMnO_4$) for 10 min on a shaker table followed by distilled water for 2 min. Sections were then incubated in a freshly prepared solution of 0.0004% FJB for 20 min, rinsed in distilled water, and then dried at 50 °C. Dried slides were cleared by immersion in xylene for 2 min before cover slipping with DPX mounting medium.

TUNEL labeling was performed using the in situ death detection kit from Roche Molecular Biochemicals (Indianapolis, IN). Briefly, sections were incubated in 20 μg/ml proteinase K solution for 10 min at room temperature to increase permeability. After 2 washes in PBS, sections were immersed in the TUNEL reaction mixture, containing biotinylated dUTP and terminal deoxynucleotidyl transferase (TdT) conjugated with fluorochrome tetramethyl rhodamine red for 60 min at 37 °C in a dark, humidified atmosphere. The process was terminated by washing sections twice with a blocking buffer (PBS, 0.1% Triton X-100, and 5 mg/mL BSA). In each assay, negative controls were included using the same incubation procedure but omitting TdT in the process, whereas positive controls were performed by incubating the permeated sections with DNase (1 μg/mL) to induce DNA strand breakage.

For immunohistochemical studies, sections were dried at room temperature for 30 min. After rinsing with 0.01 M PBS, sections were blocked in PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 h at 4 °C and then incubated overnight at 37 °C with primary antibodies of monoclonal mouse anti-NeuN (1:200, MAB314, Millipore, Billerica, MA). Sections were washed with PBS and incubated with a FITC-conjugated goat anti-mouse IgG antibody (1:400, Cat#115-095-146, Jackson ImmunoResearch Laboratory, West Grove, PA) for 1 h at room temperature. A Zeiss microscope equipped with a CCD camera (Carl Zeiss Microimaging Inc., Thornwood, NY) was used to capture all digital images of sections at ×20 magnification.

Quantification FJB- and NeuN-Immunopositive Cells The number of FJB- and NeuN-positive cells was determined using Image Pro Plus and ImageJ software (Media Cybernetics, Inc., Bethesda, MD) by an individual who was blinded to the experimental treatments. Only normal morphological profiles of NeuN-positive cells were counted, and shrunken NeuN-positive signals were excluded. A mean value of FJB-positive or NeuN cells per unit area from five brain sections within the brain regions was obtained for each individual mouse brain. Data were expressed as mean ± SEM. These mean values from each individual mouse were used as the statistical unit of measure for analysis by *t* test to determine statistically significant treatment effects.

Results

NRG-1 Protects Against Ischemia-Induced Brain Injury We examined the neuroprotective effects of NRG-1 following MCAO in mice. NRG-1 was administered immediately after reperfusion. Brain tissues were analyzed in mice treated with

NRG-1 or vehicle i.a. immediately after reperfusion and sacrificed 24 h after MCAO. Figure 1a illustrates a typical TTC staining of brain sections treated with vehicle or NRG-1 after MCAO. Compared with controls, i.a. administration of NRG-1 drastically reduced infarct volume after MCAO in mice as we and others previously showed in rats (Guo et al. 2006; Li et al. 2007; Shyu et al. 2004; Wang et al. 2015, 2018; Xu et al. 2004, 2005, 2006). The percentage of total infarct volume to the ipsilateral hemisphere in vehicle-treated animals was 41%. NRG-1 significantly reduced the total infarct volume to 15% of hemispheric volume representing a 67% reduction in infarct size (from 78.9 to 25.6 mm^3) (Fig. 1b). The relative reduction in cortical and subcortical neuronal injury was also calculated. The infarct volume of control animals represented 56% of the total size of the ipsilateral cortex. Treatment with NRG-1 resulted in an infarct that was reduced to 16% of the ipsilateral cortex, which corresponds to a 72% reduction in cortical infarct size (from 53.0 to 15.0 mm^3) (Fig. 1c). NRG-1 treatment reduced infarction by 50% in subcortical regions (Fig. 1d). Regional CBF was measured by LDF for each mouse during MCAO and after reperfusion. NRG-1 did not significantly affect the CBF reduction after MCAO ($p = 0.8695$) or recovery after reperfusion ($p = 0.8722$) (Fig. 2; $n = 9$ in each group). NRG-1 also did not affect the baseline body temperature, body temperature 24 h post-MCAO, or body weight loss (Fig. 3). No significant difference in neurological outcome was observed between controls and NRG-1-treated animals at this time point (data not shown).

NRG-1 Blocks Ischemia-Induced Neuronal Death Neuronal injury and the neuroprotective effects of NRG-1 treatment were also assessed by FJB staining. Brain tissues from mice treated with either NRG-1 or vehicle were examined 24 h after MCAO/reperfusion, and extensive FJB staining was found within the ischemic cortical and subcortical regions (Fig. 4). Ischemia-induced FJB staining was reduced by 64% NRG-1 treatment in the areas within the cerebral cortex. NRG-1 also dramatically reduced TUNEL labeling after MCAO (Fig. 5). MCAO reduced NeuN immunoreactivity in cortical neurons (Fig. 6). These injured areas showed high numbers of FJB labeling in adjacent sections as seen in Fig. 4. Neighboring neurons that were not injured showed relatively higher levels of NeuN immunoreactivity. NRG-1 post-treatment rescued NeuN immunoreactivity following MCAO.

Neuroprotection was observed following i.v. administration of NRG-1 in male and female mice (Fig. 7a, b). Vehicle-treated mice exhibited an average total infarct volume of 24.9% and NRG-1 reduced that percentage to 13.3%, a 46.6% reduction (Fig. 7c; 2 males + 3 females per treatment group). Vehicle-treated mice resulted in an average cortical infarct volume of 26.8% that NRG-1 significantly reduced to an average of 7.5%, a 72.1% reduction specifically within the ipsilateral cortex. No difference in lesion size was seen within the ipsilateral subcortical region.

Endogenous NRG-1 Protects Neurons from Ischemic Injury To determine if endogenous NRG-1 is involved in ischemic brain injury, we examined cerebral infarction in male and female (3

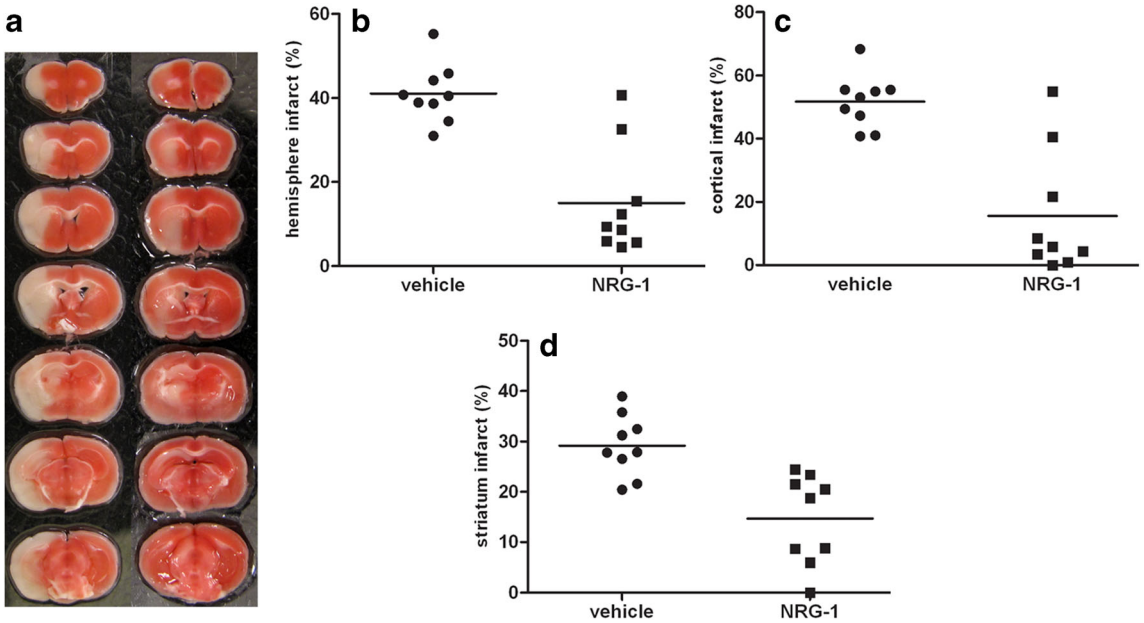

Fig. 1 NRG-1 reduced infarct volume following MCAO/reperfusion. Mice were treated with NRG-1 (16 μg/kg) or vehicle immediately after MCAO followed by 23 h of reperfusion. Brains were removed, sliced into seven coronal sections (1 mm thick), and treated with 2% TTC (**a**). NRG-1 reduced total infarct volume by 67% (**b**; $p < 0.0001$). Treatment with NRG-1 resulted in a 72% reduction in cortical infarct size (**c**; $p < 0.0001$) and 50% in subcortical regions (**d**; $p = 0.001$). $N = 9$ mice per treatment group

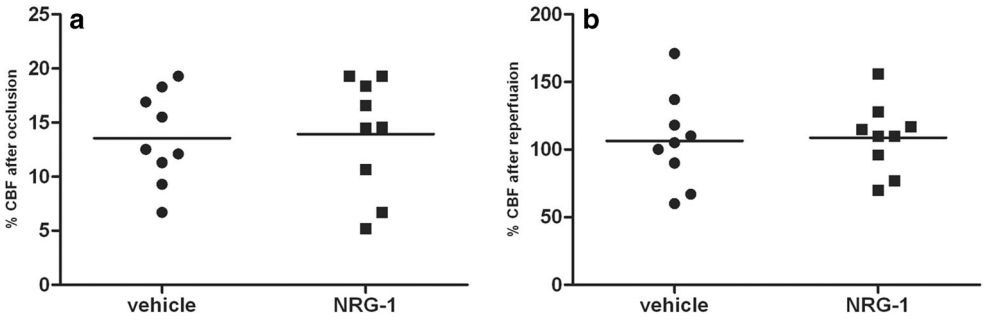

Fig. 2 NRG-1 does not affect regional CBF. Regional CBF was measured by LDF for each mouse during MCAO and after reperfusion. NRG-1 did not significantly affect the CBF reduction after MCAO (**a**; $p = 0.8695$) or recovery after reperfusion (**b**; $p = 0.8722$) ($n = 9$ in each group)

males and 3 females per group) NRG-1+/− mice and WT littermates. MCAO was performed for 45 min rather than 1 h to produce smaller, submaximal infarcts and examine whether reduced endogenous NRG-1 could result in increased neuronal damage. There was no difference in CBF reductions and recovery between WT and NRG-1+/− mice (Fig. 8a, b); however, the infarct volume in the cerebral cortex of NRG-1+/− mice was sixfold larger than that observed in WT mice (Fig. 8c–f). However, there was no difference in subcortical infarct volume between the two groups and thus total hemisphere infarct volume was not statistically significant when compared.

Discussion

NRG-1 belongs to a family of multipotent neuroprotective and anti-inflammatory growth factors that include acetylcholine receptor–inducing activities (ARIAs), glial growth factors (GGFs), heregulins, and neu differentiation factors (NDFs) (Falls et al. 1993; Ho et al. 1995; Holmes et al. 1992; Marchionni et al. 1993; Wen et al. 1992). We have shown that NRG-1 reduced ischemic brain injury when administered either before or 13.5 h after MCAO (Xu et al. 2006). NRG-1 has also been shown to promote functional recovery in a rat stroke model when administered intravenously up to 7 days after ischemia (Iaci et al. 2010). In this study, we examined if exogenously applied or endogenous NRG-1 was neuroprotective in a mouse MCAO model. We observed that both i.a. and i.v. administration of NRG-1 reduced neuronal injury in mice. The neurobehavioral test we used to examine the acute effects of ischemic stroke and NRG-1 did not correlate with the histological protection as we observed in the rat stroke model (Xu et al. 2004, 2006). Future studies will employ more sensitive tests to examine the long-term neurobehavioral consequences of NRG-1 treatment following ischemia in mice beyond the 24-h time point.

We also observed that ischemic brain injury was more severe in NRG-1+/− knockout mice compared with WT mice suggesting that endogenously expressed NRG-1 is involved in

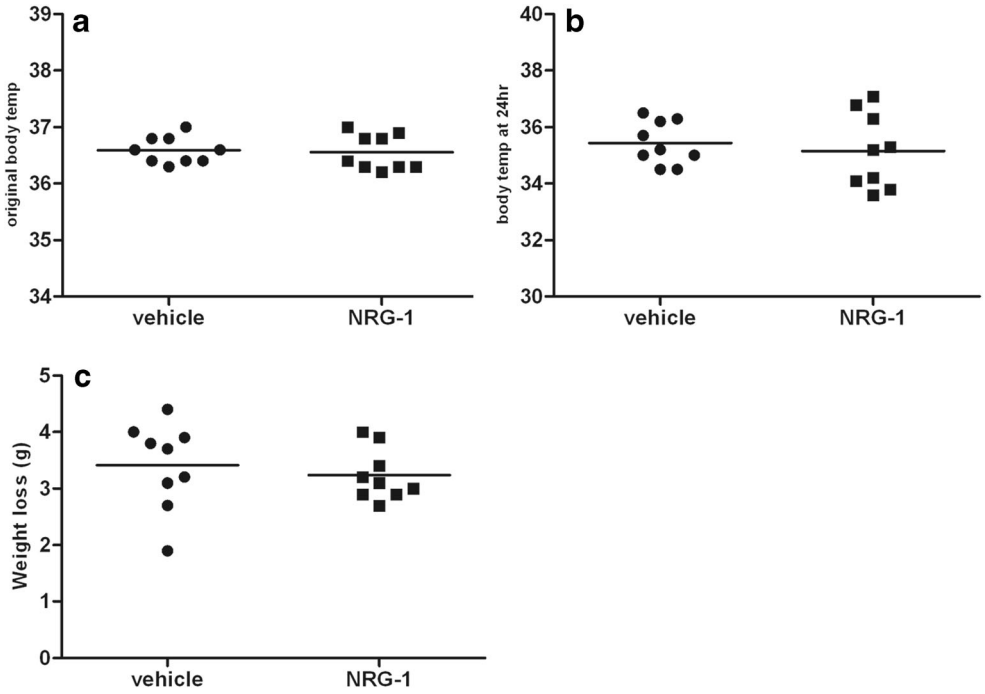

Fig. 3 NRG-1 does not affect animal body temperature weight. NRG-1 or vehicle was administered immediately after reperfusion. There was no statistical significance between vehicle and NRG-1-treated animals for the baseline body temperature (**a**; $p = 0.8020$); body temperature 24 h post-MCAO (**b**; $p = 0.5944$), or body weight loss (**c**; $p = 0.5584$) ($n = 9$ in each group)

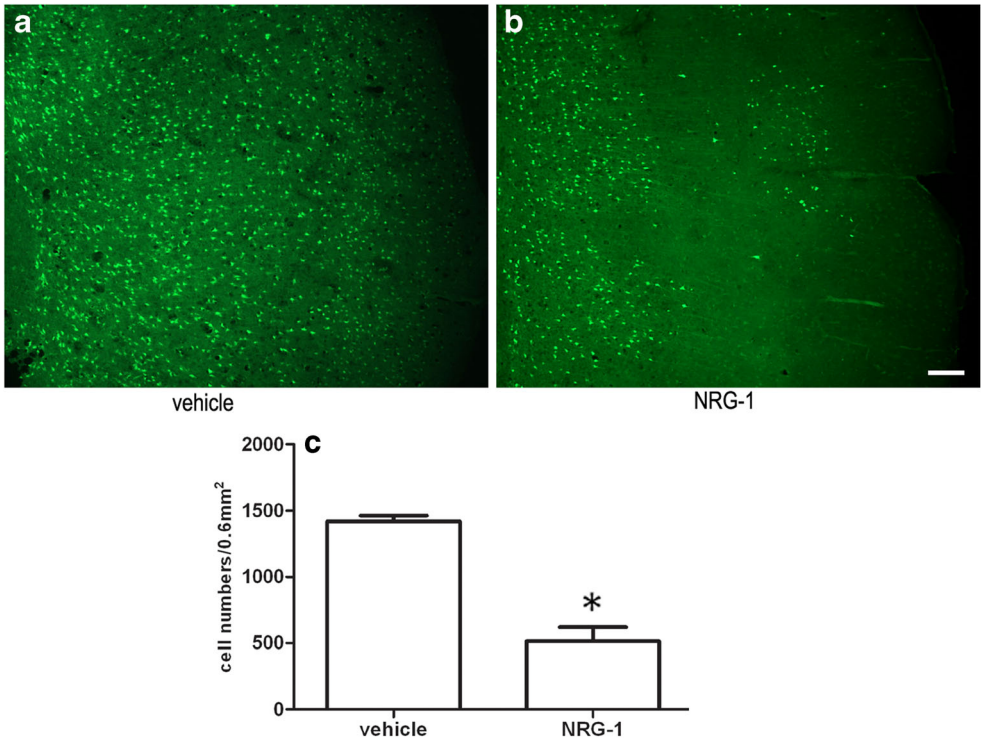

Fig. 4 NRG-1 reduced FJB staining in the brain following MCAO/reperfusion. Brains from mice treated with either vehicle or NRG-1 were examined 24 h after MCAO/reperfusion. Extensive FJB staining was found in brain tissues following ischemia (**a**). Ischemia-induced FJB staining was significantly reduced by NRG-1 (**b**). A mean value of FJB-positive or per unit area from five brain sections within the brain regions was obtained for each individual mouse brain (**c**; $p = 0.0001$). Data are expressed as mean ± SEM. Scale bar = 100 μm

the function of neurons under normal conditions. NRG-1−/− homozygous knockout mice die in utero, but heterozygous NRG-1+/− mice are viable and with reduced amounts of NRG-1 in the brain (Erickson et al. 1997; Gerlai et al. 2000; Sandrock et al. et al. 1997; Stefansson et al. 2002). Heterozygous NRG-1 knockout mice were shown to have a "schizophrenia-like" phenotype including alterations in pre-pulse inhibition and anxiety-like behaviors (Erickson et al. 1997; Gerlai et al. 2000; O'Tuathaigh et al. 2007, 2008, 2010; Sandrock et al. 1997; Stefansson et al. 2002). Polymorphisms in the NRG-1 gene have also been associated with increased risk for schizophrenia in human patients (Stefansson et al. 2002, 2004). Similar results were shown with erbB4 knockout mice following stroke. Neuroprotection by NRG-1 against cerebral ischemia was prevented in the mice with erbB4 deleted in parvalbumin (PV)-positive interneurons (Guan et al. 2015).

An important finding in this study is that NRG-1 was neuroprotective in both male and female mice. Pre-menopausal women are protected from ischemic stroke compared with males (Murphy et al. 2004). However, this neuroprotective capacity is lost in post-menopausal women, where estrogen levels reduced. Experimental studies have shown that pre-menopausal female rodents and non-human primates were protected from ischemia-induced brain injury compared with males (Alkayed et al. 1998; Murphy et al. 2004, 2008; Toung

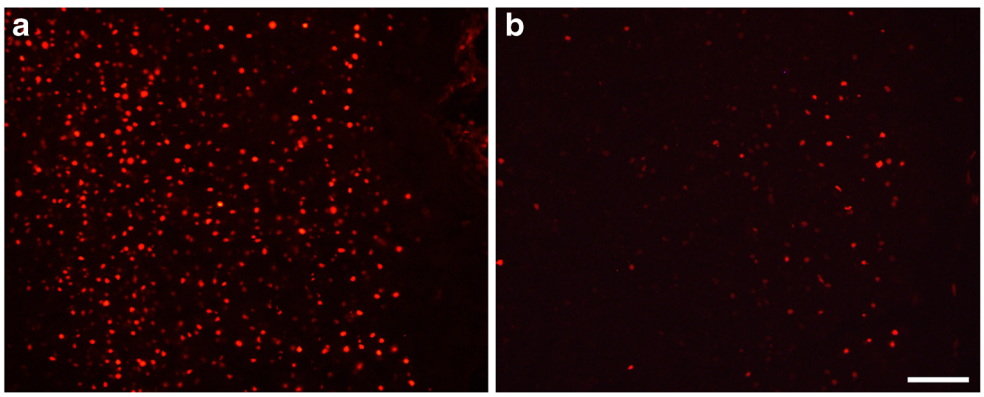

Fig. 5 NRG-1 blocks ischemia-induced apoptosis. Brains from mice treated with either vehicle or NRG-1 were examined 24 h after MCAO/reperfusion using TUNEL labeling. Extensive TUNEL labeling was found within the brain following ischemia (**a**). Ischemia-induced TUNEL labeling was blocked by NRG-1 treatment in the areas within the cerebral cortex (**b**). Scale bar = 50 μm

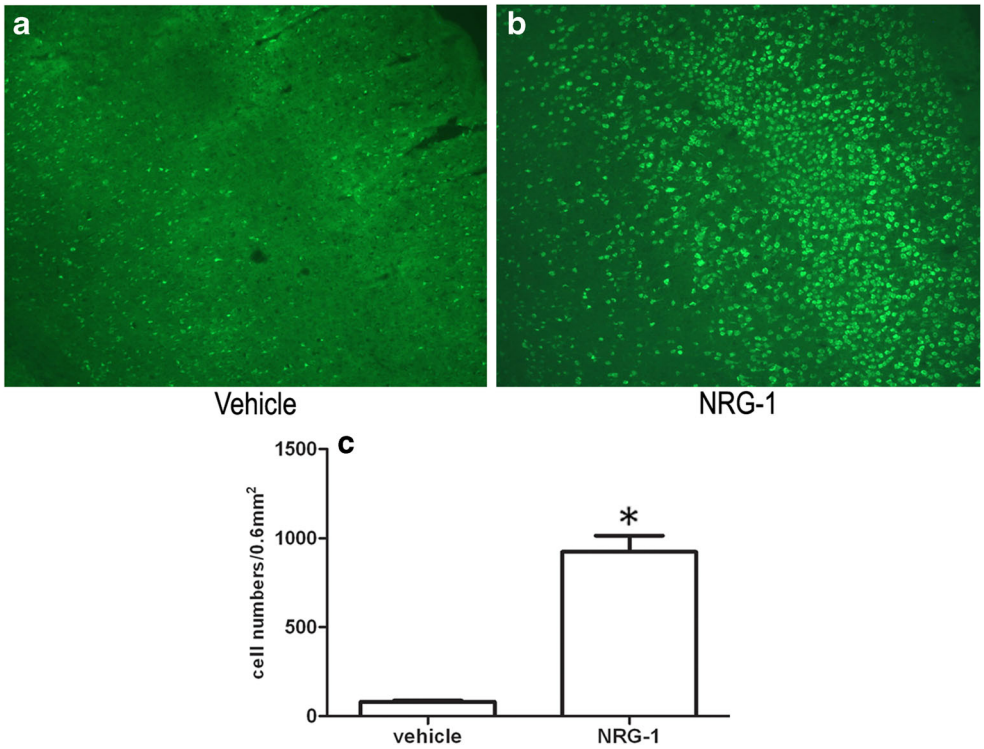

Fig. 6 NRG-1 protects neurons from ischemic damage following MCAO/reperfusion. MCAO/reperfusion resulted in reduced NeuN immunoreactivity in cortical neurons (**a**). NRG-1 treatment rescued NeuN immunoreactivity (**b**, **c**; $p = 0.0001$). Scale bar = 100 μm

et al. 2004). The observation that the neuroprotective effect of NRG-1 was gender-independent makes it a more attractive clinical candidate for neuroprotective therapy.

The STAIR report produced in response to the multiple failed stroke neuroprotection clinical trials suggested that pre-clinical stroke studies be performed in multiple species.

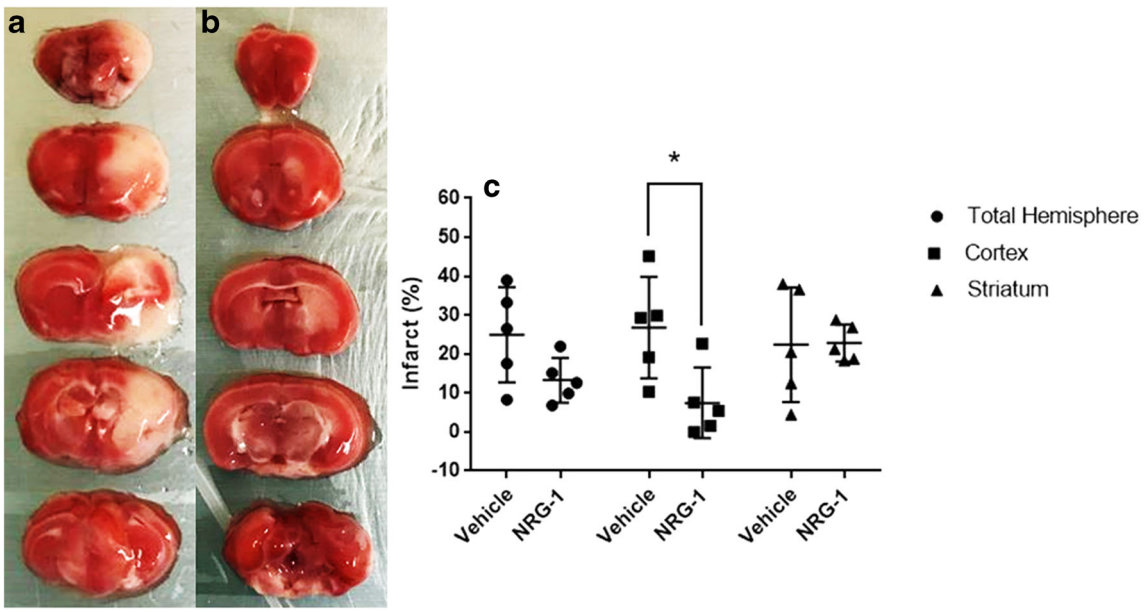

Fig. 7 NRG-1 reduced infarct volume via intravenous administration in male and female mice. **a** Vehicle or **b** NRG-1 (100 μg/kg) was administered immediately after filament insertion. MCAO occurred for 90 min followed by 23 h of reperfusion. Brains were removed, sliced into five coronal sections (2 mm thick), and treated with 2% TTC. **c** NRG-1 reduced total infarct volume by 46.6% (**b**; $p < 0.05$). Treatment with NRG-1 resulted in a 72.1% reduction in cortical infarct size (**c**; $p < 0.05$) and no significant difference in subcortical regions. $N = 5$ mice (2 males + 3 females) per treatment group

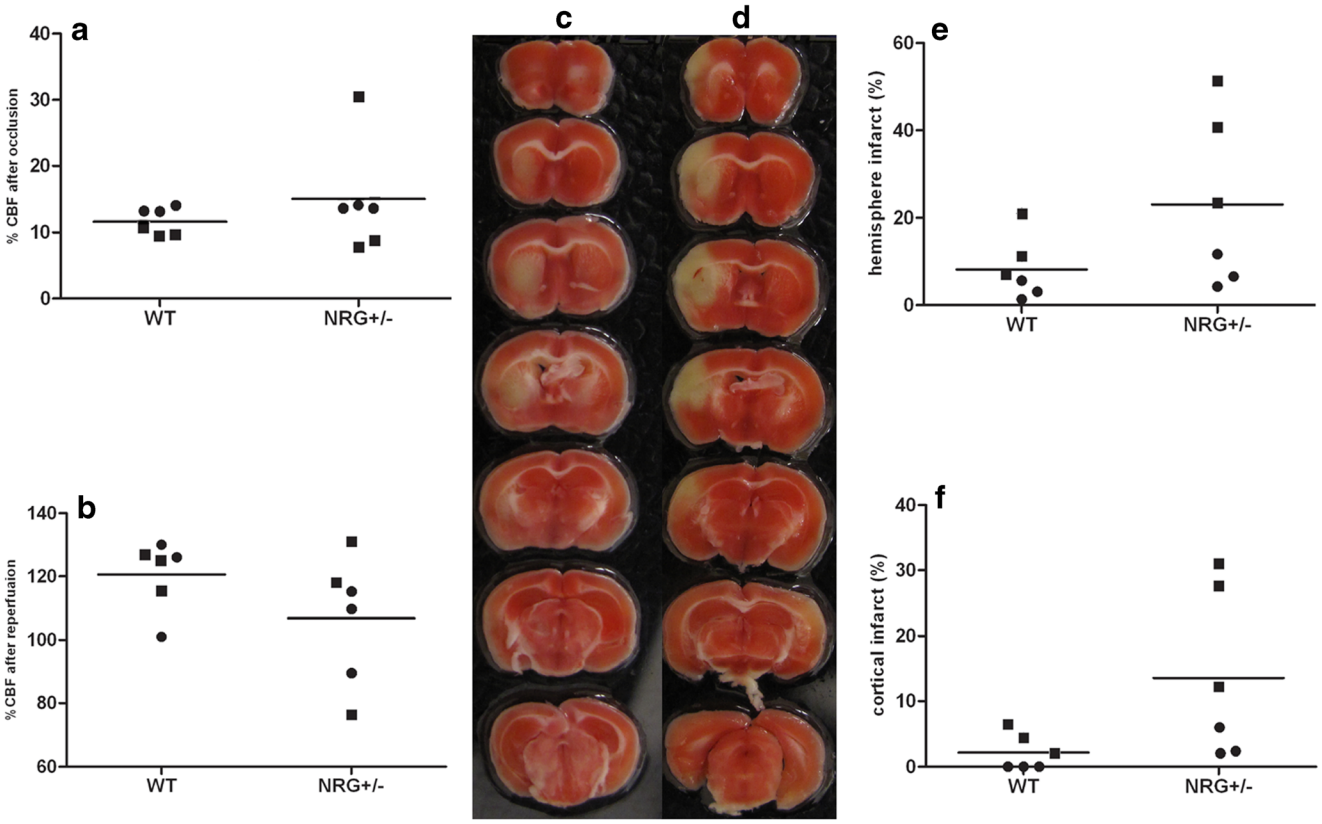

Fig. 8 Endogenous NRG-1 protects neurons following MCAO/reperfusion. NRG-1+/− mice and WT littermates were subjected to 45 min MCAO followed by 23.25 h of reperfusion. There was no difference in CBF reduction (**a**, $p = 0.3494$) or recovery (**b**, $p = 0.1645$) between WT and NRG+/− mice. TTC labeling was used to measure brain infarction in WT (**c**) and NRG-1+/− mice (**d**). There was no difference in subcortical infarct volume between the two groups (not shown), and thus, total hemisphere infarct volume was not statistically significant when compared (**e**; $p = 0.0549$). However, the infarct volume in the cerebral cortex of NRG-1+/− mice was 6-fold larger than that observed in WT mice (**f**; $p = 0.0406$) $N = 6$ per experimental group

Therefore, it is clinically significant that NRG-1 is equally neuroprotective in both mice and rats. Recently, we developed a novel, minimally invasive ischemic stroke model with young male rhesus macaques (Rodriguez-Mercado et al. 2012) which has been replicated with similar results in aged female monkeys (Zhang et al. 2015; Li et al. 2018). This procedure reliably produced infarcts and resulted in discrete and limited neurobehavioral deficits, indicating the potential of this stroke model for chronic neuroprotection studies in the future. Therefore, in future studies we will be able to directly examine the neuroprotective capacity of NRG-1 following stroke in males and female non-human primate stroke models.

Thrombolysis is the only approved therapy for acute ischemic stroke and can be only be used in a limited patient population. The present study demonstrates that NRG-1 is a viable candidate neuroprotectant to treat acute ischemic stroke. NRG-1 (Neucardin; Zensun Biotechnology, Shanghai, China) is currently in phase III human clinical trials for the treatment of heart failure and showed significant efficacy for improving cardiac function in phase II patient studies in China (Chinese Clinical Trial: ChiCTR-TRC-00000414) and Australia (Australian New Zealand Clinical Trials Registry: ACTRN12607000330448). Three additional clinical trials to determine the ability of NRG-1 to improve cardiac function after heart failure have been initiated in the USA (ClinicalTrials.gov identifiers NCT01258387; NCT01541202; NCT01251406). These clinical studies demonstrated that NRG-1 significantly improved heart function in patients and the effective doses were shown to be safe and tolerable (Gao et al. 2010; Jabbour et al. 2011). This suggests rationale for future clinical adjunctive treatment with NRG-1 and tPA or endovascular thrombectomy. We recently developed a partnership with Zensun to pursue FDA approval to use Neucardin in clinical trials for stroke treatment (https://news.ucr.edu/articles/2019/05/09/hope-horizon-treating-stroke).

Recent studies showed that erbB4 prevented neuronal injury and improved blood-brain barrier integrity in animal models of subarachnoid hemorrhage (Qian et al. 2018; Yan et al. 2017). This suggests that NRG-1 would not induce

hemorrhagic transformation after ischemia and could protect patients from tPA-mediated toxicity. These findings could support the development of clinical studies using NRG-1 alone or in conjunction with other therapies for the treatment of patients with acute ischemic stroke.

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Conflict of Interest The corresponding author (B.D.F.) holds patents related to the work being reported without direct corporate involvement at the time.

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