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Neuregulin-1 Attenuates Hemolysis- and Ischemia Induced-Cerebrovascular Inflammation Associated with Sickle Cell Disease

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Abstract

Objectives: Individuals with sickle cell disease (SCD) are at severely heightened risk for cerebrovascular injury and acute cerebrovascular events, including ischemic and hemorrhagic stroke, potentially leading to impaired development and life-long physical and cognitive disabilities. Cerebrovascular injury specific to SCD includes inflammation caused by underlying conditions of chronic hemolysis and reduced cerebrovascular perfusion. The objectives of this study were to investigate whether expression of neuregulin-1β (NRG-1), an endogenous neuroprotective polypeptide, is increased in SCD or experimental conditions mimicking the hemolysis and ischemic conditions of SCD, and to determine if treatment with exogenous NRG-1 reduces markers of cerebrovascular inflammation.

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Authors' Contributions

CC, JS, and BG designed the experiments, CC performed the experiments, CC and BG wrote the manuscript, CC, JS and BG reviewed the manuscript. BG serves as the senior author. All authors revised the manuscript and gave final approval of the current version.

Ethics Approval and Consent to Participate:

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Emory University School of Medicine, Atlanta, Georgia.

Consent for Publication

Not applicable

Competing Interests

The authors declare that they have no competing interests

Materials and Methods: Plasma and brain-specific NRG-1 levels were measured in transgenic SCD mice. Endogenous NRG-1 levels and response to experimental conditions of excess heme and ischemia were measured in cultured human brain microvascular cells and astrocytes. Pre-treatment with NRG-1 was used to determine NRG-1's ability to ameliorate resultant cerebrovascular inflammation.

Results: Plasma and brain-specific NRG-1 were elevated in transgenic SCD mice compared to healthy controls. Neuregulin-1 expression was significantly increased in cultured human microvascular cells and astrocytes exposed to excess heme and ischemia. Pre-treatment with NRG-1 reduced inflammatory chemokine (CXCL-1 and CXCL-10) and adhesion molecule (ICAM-1 and VCAM-1) expression and increased pro-angiogenic factors (VEGF-A) in microvascular cells and astrocytes exposed to excess heme and ischemia.

Conclusions: Elevated NRG-1 in SCD is likely a protective endogenous response to ongoing cerebrovascular insults caused by chronic hemolysis and reduced cerebrovascular perfusion. Administration of NRG-1 to reduce cerebrovascular inflammation may be therapeutically beneficial in SCD and warrants continued investigation.

Keywords

Astrocytes; Endothelium; Hemolysis; Cerebrovascular; Neuroinflammation; Ischemia; Neuregulin; Sickle Cell Disease

Background

Sickle cell disease (SCD) is a group of inherited red blood cell disorders caused by a mutation in the human betaglobin gene. The most common and severe form, Hemoglobin SS (also known as sickle cell anemia, SCA), results from homozygous inheritance of the hemoglobin S mutation of beta globin ($\beta^{6\,(glu\,->\,val)}$) on chromosome 11. This mutation leads to non-covalent hydrophobic polymerization of hemoglobin molecules into rod-like structures within erythrocytes and the hallmark "sickle" shaped red blood cells under conditions of low oxygen saturation, intracellular dehydration, and/or acidosis [1]. Sickled red blood cells are rigid, adhesive, and prone to breakage, and have lower oxygen-affinity when compared to normal red blood cells. This leads to conditions of chronic hemolytic anemia and excess free-heme, reduced oxygen delivery, endothelial dysfunction, and frequent vaso-occlusion especially within cerebrovascular tissues.

Cerebrovascular disease leads to disabling complications in children with SCD, exemplified by high prevalence of elevated Transcranial Doppler (TCD) velocity, cerebral artery stenosis, silent cerebral infarction, and/or overt stroke. Without preventive treatment, approximately 11% of children with SCD experience a stroke before the age of 20, roughly 200 to 400 times higher risk than that experienced by children without SCD [2]. In addition to overt stroke, as many as 37% of children with SCD by the age of 14 may experience what are known as "silent" cerebral infarcts, identifiable by magnetic resonance imaging [3–5]. Both symptomatic and subclinical cerebral infarctions can impair normal brain development and are associated with poor school performance, lessened cognitive functioning, life-long physical disabilities, and heightened risk for recurrent or progressive stroke [4, 6].

While the pathophysiology of cerebrovascular disease in SCD is not well understood, ongoing cerebrovascular injury due to increased hemolysis and ischemic conditions is a known contributor [1, 7–10]. Chronic hemolysis in SCD produces toxic circulating "free" heme resulting in oxidative injury to surrounding cerebrovascular tissues including endothelial cell dysfunction and RBC membrane instability, further perpetuating hemolysis, pro-inflammatory responses, and vaso-occlusive factors (Figure 1) [7, 10–12]. Ischemic conditions, both chronic and acute, occur in SCD due to a number of frequently occurring factors including vaso-occlusion of microvasculature by sickled red cells, arterial stenosis, anemia, and reduced heme-oxygen saturation (Figure 1) [8, 13–16].

Neuregulin-1β is an endogenous neuroprotective peptide with demonstrated ability to reduce cerebral and cerebrovascular injury in an array of *in-vitro* and *in-vivo* models. Activation of the NRG-1/ ERBB4 receptor cascade has been shown to be involved in nervous system development and recovery from injury and is expressed in human brain microvascular endothelial cells, neurons, astrocytes, and microglia [17–20]. Neuregulin/ERBB activity results in increased rates of angiogenesis in microvascular tissue, decreased endothelial cell apoptosis, and shorter recovery times of endothelial cells from ischemic injury [20–24]. Previously, our lab has demonstrated NRG-1's ability to reduce heme-induced inflammation associated with experimental cerebral malaria and attenuate apoptosis and tight junction dysregulation in co-cultured astrocytes and brain microvascular endothelial cells exposed to experimental conditions of excess heme [23, 25–27]. Furthermore, intravenously delivered NRG-1 is able to cross the intact blood brain barrier to act on the ERBB cascade [28]. In animal stroke models, NRG-1 administration has been shown to reduce infarct size and post-ischemic inflammation [18, 20, 29, 30]. We have previously identified platelet derived growth factor type-AA (PDGF-AA) and brain derived neurotrophic factor (BDNF) as markers of cerebral ischemia and predictors of cerebral infarct and stroke risk in children with SCA [31]. We have also identified elevated neuregulin-1β (NRG-1) in children with SCD and positive association of NRG-1 with PDGF-AA and BDNF levels [32].

These results lead us to hypothesize that NRG-1 is elevated in children with SCD as a neuroprotective response to ongoing exposure to circulating free heme and subclinical ischemia, as is suggested by elevation of hypoxia-inducible angiogenic growth factors and BDNF [31], and that exogenous NRG-1 may reduce cerebrovascular inflammation. [33, 34] .Therefore, we designed the *in vitro* studies to test how cells in the blood-brain barrier which have immediate proximity to hemolysis- and ischemia (human brain microvascular endothelial cells and astrocytes) will respond to experimental conditions of excess heme and ischemia. These cell types are known to produce and respond to NRG-1 [33–37]. In addition, we measure NRG-1 in the brains of transgenic SCD mice to validate that the brain may be a source of elevated plasma NRG-1.

Since NRG-1 may have therapeutic effects on both acute and chronic cerebrovascular disease seen in SCD, we test the effect of NRG-1 pre-treatment on cerebrovascular inflammation caused by excess heme and ischemia

Methods

1. Overview

In brief, we first determined if SCD influences in-vivo NRG-1 production using plasma and brains of the Townes sickle cell mouse model (Jackson Laboratories, Stock #013071) [35]. The Townes mouse model of SCD is a well-established animal model of human SCD, in which we measured circulating plasma NRG-1 levels as well as total brain NRG-1 levels.

Next, we determined if conditions of excess heme and experimental ischemia influence endogenous NRG-1 expression in human brain microvascular endothelial cells and astrocytes. To achieve this, human astrocytes and brain microvascular cells were cultured and treated with excess heme, ischemia, or a combination of the two. After treatment, cells were collected and analyzed for differential gene expression of NRG-1 and its ERBB4 receptor, along with positive controls to confirm experimental conditions.

Lastly, we determined if administration of exogenous NRG-1 can mitigate the pro-inflammatory effects of excess heme and experimental ischemia in human brain microvascular endothelial cells and astrocytes. To achieve this, human astrocytes and brain microvascular endothelial cells were cultured and treated as in the earlier experiments with the addition of exogenous NRG-1. Gene expression analysis was conducted to assess the effects of NRG-1 on neuroinflammation markers, including chemokines, cellular adhesion molecules, and angiogenic response. Additionally, ERBB4 receptor blockade studies were conducted to confirm the involvement of NRG-1/ERBB4 activation on neuroinflammatory responses. Detailed methods for each experiment are listed below.

2. Cell Culture

Human astrocytes (Sciencell, Cat #1800) were cultured on 2 μg/cm2 poly-L-Lysine (Sciencell Cat #0403) coated culture vessels in complete astrocyte medium (Sciencell Cat #1801) containing 500 ml basal media, 10 mL fetal bovine serum (Sciencell, Cat #0010), 5 mL astrocyte growth supplement (Sciencell, Cat #1852), and 5 mL penicillin/streptomycin solution (Sciencell Cat #0503). Human brain microvascular endothelial cells (HBMVECs) (Sciencell Cat #1000) were cultured on 2 μg/cm2 fibronectin (Sciencell Cat #0903) coated culture vessels in complete endothelial cell medium (Sciencell Cat #1001) containing 500 mL basal media, 25 mL fetal bovine serum (Sciencell Cat #0025), 5 mL endothelial cell growth supplement (Sciencell Cat #1052), and 5 mL penicillin/streptomycin solution (Sciencell Cat #0503). All cells were seeded at density 0.5×10^6 in 9.6 cm^2 wells with 2 mL media. All cells were cultured under 37 °C, 5% CO2 incubation and allowed to reach confluency before undergoing 24-hour serum starvation and respective experimental treatment. All cells were received at passage-1 and experiments were conducted between passages-2–4.

3. Experimental Conditions

Conditions of excess heme were achieved by first preparing Hemin (Sigma Aldrich Cat #51280) stock solution according to manufacturer's protocol (stock: hemin dissolved in DMSO at 1 mM). Hemin stock was then diluted in cell specific complete medium to prepare

a 50 μM hemin/media solution (concentration similar to that seen in people with SCD and other hemolytic diseases and based on previously published dose-response experiments)[26, 36]. Control groups contained equivalent amounts of DMSO vehicle and were added to cell specific complete media with no hemin.

Conditions of experimental ischemia/ oxygen glucose deprivation were used to mimic ischemia [39–41]. This condition was chosen over hypoxia alone as is more closely recapitulates the internal enviormental changes caused by vaso-oclusive events in SCD. Glucose free media consisted of 500 mL glucose-free Dulbecco's Modified Eagle Medium (ThermoFisher Cat #11966025) with 50 mL dialyzed fetal bovine serum (ThermoFisher Cat #A3382001) for HBMVECs, and 500 mL glucose-free Roswell Park Memorial Institute 1640 Medium (ThermoFisher Cat #11879020) with 50 mL dialyzed fetal bovine serum (ThermoFisher Cat #A3382001) for astrocytes. ThermoScientific Heracell 150i CO2 Incubator at 37 °C, 5% CO2, and 93% N2 was used to achieve hypoxic conditions (2% O2). Similar techniques have been used widely to mimic ischemia [37–39]. For those groups exposed to experimental reperfusion, 5.5 mM D-glucose was reintroduced to the media after experimental ischemia, and cells were transferred to normoxic incubation at 37 °C, 5% CO2, 0% added N2. Control groups were incubated under normoxic conditions of 37 °C, 5% CO2, 0% added N2.

Conditions of combined excess heme and experimental ischemia were achieved by diluting hemin stock in cell-specific glucose-negative media to prepare a 50 μM hemin/ media solution. Cells were additionally exposed to hypoxic incubation. For those groups exposed to experimental reperfusion, 5.5 mM D-glucose was reintroduced to the media after experimental ischemia, and cells were transferred to normoxic incubation at 37 °C, 5% CO2, 0% added N2. Control groups were incubated under normoxic conditions of 37 °C, 5% CO2, 0% added N2, with no added hemin.

Neuregulin-1β treatments were achieved by pre-treating cells with 100 ng/mL (12.5 nM) human recombinant NRG-1 (hrNRG1 (RnD Systems Cat #396-HB-050) for 30 minutes as previously described [23, 26]. Cells were then washed three times with phosphate buffered saline (PBS) before being immediately exposed to experimental conditions according to respective experimental group.

Anti-ERBB4 competitive NRG-1/ERBB-4 inhibition was achieved by pre-treating cells with 3.34 μg/mL ERBB4 polyclonal antibody (Proteintech Cat# 19943–1-AP) and 100 ng/mL (12.5 nM) hrNRG-1 for 30 minutes. Cells were then washed three times with PBS before being exposed to experimental conditions according to respective experimental group. Controls were treated with equal volumes PBS.

All experiments utilizing conditional exposures were carried out in triplicate.

4. Real-Time Polymerase Chain Reaction Analysis

For the cultured cell experiments, cells were washed post-treatments thoroughly in PBS and lysed directly in-vessel using Buffer RLT (Qiagen Cat#79216) and repeated passage through a 21-gauge needle before collection. Total RNA was isolated form cell lysates

using the RNeasy Mini Kit (Qiagen Cat# 74106). Total RNA was quantified using the Nanodrop N-1000 (Agilent Biosystems). One-step quantitative reverse transcription PCR from RNA was performed using iTaq Universal SYBR Green One-Step Kit (Bio-Rad Cat# 1725151). Uniform sample RNA concentrations (200 ng/8.25 μL in 20 μL reactions) were used in all assays. Quantitative thermocycling and data analysis was performed using a CFX96 Real-Time PCR System Bio-Rad set to 10 min reverse transcription at 50° C, 1 min polymerase activation and DNA denaturation at 95° C, and 10 second 95° C + 40 second 60° C amplification for 40 cycles. PrimePCR SYBR Green Assay primers (Bio-Rad Cat# 10025636) were used for all reactions. Unique Bio-Rad primer IDs are as follows: (Target, Bio-Rad Assay ID) ERBB4, qHsaCID0017862; HO1, qHsaCID0022141; CXCL10, qHsaCED0046619; NRG-1, qHsaCID0010926; PGK-1, qHsaCED0003721; VEGF-A, qHsaCED0006937; VCAM-1, qHsaCID0016779; GAPDH, qHsaCED0038674; ICAM-1, qHsaCED0004281; CXCL-1, qHsaCID0010973.

5. Animals

All mice were maintained and studied in accordance with the US National Research Council's Guide for the Care and use of Laboratory Animals [40]. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University. Equivalent numbers of male (n=4) and female (n=4) homozygous humanized sickle cell mice (Townes) aged 8–12 weeks (equivalent to human young adults), expressing human beta-sickle globin (SS) were used [35]. Similar age-matched male (n=5) and female (n=4) Townes mice expressing normal human hemoglobin (AA) were used as controls. No mice used in this study were pregnant or used for breeding at any point in their lifespan.

6. Immunoassays

For plasma immunoassays, mice were anesthetized to minimize stress (isoflurane: 3% induction and 2% maintenance with 100% O2 carrier), and blood was collected via cardiac puncture using an ethylenediaminetetraacetic acid (EDTA) coated syringe. Blood was immediately transferred to a K3 EDTA microtube (Sarstedt Inc. Cat #41.1395105) to prevent clotting. Whole blood was centrifuged at 1500 g for 10 minutes to separate plasma for collection. Enzyme-linked immunosorbent assay (ELISA) for NRG-1 (Novus Bio Cat# NBP2–68070) was performed on undiluted plasma samples overnight at 4° C + 1 hour at 37° C. Samples were read at 450 nm on a Tecan microplate reader. All assays were performed in duplicate for statistical analysis. For brain immunoassays, mice were anesthetized as above to minimize stress, and blood was collected via cardiac puncture. Mice were perfused with saline to remove any excess blood and the brain was immediately extracted. Tissues were placed in gentleMACS M Tubes (Miltenyi Biotec Cat# 130–093-236) with 1.5 mL PBS and homogenized using a gentleMACS Dissociator (Miltenyi Biotec). Enzyme-linked immunosorbent assay for NRG-1 (Novus Bio Cat# NBP2–68070) was performed on diluted homogenate (1:50) overnight at 4° C + 1 hour at 37° C. Samples were read at 450 nm on a Tecan microplate reader. All assays were performed in duplicate for statistical analysis. Cardiac puncture was selected and approved by the IACUC for blood collection to maximize plasma collection as NRG-1 is a molecule found in relatively low concentrations and requires assessment by immunoassays using large undiluted volumes of sample [41].

Though a level of stress is involved in this terminal procedure, standardizing its use across experimental groups assist in eliminating any potential inter-genotypic variation.

7. Statistical Analysis

Data analysis and generation of graphics were performed using Prism statistical software (GraphPad Prism 7.0, San Diego, CA, USA). Analysis of statistical difference was conducted using two-tailed Mann-Whitney tests to compare non-parametric groups and Student's t-test to compare parametric groups, where applicable. For data comparing mean variance over time, one-way analysis of variance (ANOVA) was applied. In assessment of multiple groups across varied factors (i.e., treatment/time), two-way ANOVA was applied with Turkey's or Dunnett's post-hoc analysis. Gene expression data was reported as 2-ΔΔCt with ΔCT = Ct (gene of interest) – Ct (GAPDH) and ΔΔCt = ΔCt (treated sample) – ΔCt (untreated control). Assays were performed in duplicate unless otherwise denoted. All p-values resulted from two-sided statistical tests with α=0.05 and significant threshold set at $p < 0.05$ denoted by (*). Highly significant p-values were further denoted as follows: $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data were reported as mean ± standard deviation or median with interquartile range (IQR) where appropriate. All analyses were reviewed and approved by the Biostatistics Core at Morehouse School of Medicine.

Results

Animal Studies:

1. Neuregulin-1 Levels in the Townes Sickle Cell Mouse Model—Plasma was collected and analyzed for eight Townes SS (SCD) and nine Townes AA mice (healthy controls) (Figure 2A). Immunoassays identified elevated plasma NRG-1 levels in Townes SS mice (138.5 ± 29.05 pg/mL) compared to healthy controls ((75.76 ± 33.37 pg/mL) p-value = 0.0009). Brain tissue was collected from six Townes SS and seven Townes AA mice (Figure 2B). Immunoassays identified elevated brain NRG-1 levels in SS mice (13.54 ± 2.86 ng/mL) compared to healthy controls ((10.40 ± 1.53 ng/mL) p-value = 0.0283). Brain tissue NRG-1 levels were approximately 100-fold higher than plasma concentrations.

Cell Studies:

1. Experimental Conditions—Conditions of excess heme were created by utilizing 50 μM hemin/complete media solutions, a clinically relevant concentration previously shown to stimulate maximal responses in endothelial cells and astrocytes and exposing the cells for different durations of time [10, 23, 26, 27]. Upon treatment with 50 μM hemin, we saw a significant increase in heme oxygenase-1 (HO-1) expression in the HBMVECs at 6-hours (p value < 0.0001), 12-hours (p value < 0.0001), and 24-hours (p value < 0.0001) when compared to the untreated 0-hour control. Similar results were seen in astrocytes at 6-hours (p value < 0.0001) and 12-hours (p value < 0.0001) but decreased back towards control levels at 24-hours (p value > 0.05). For both cell types, we saw the largest increase in HO-1 expression at 6-hours of excess heme exposure with a steady decrease during the following timepoints (Figure 3 A, B). With HO-1 being a known heme responsive gene, these results indicate that our experimental conditions of excess heme have an active effect on cellular response in both HBMVECs and astrocytes [42].

Experimental conditions of ischemia were created using a combination of two methods. First, cells were deprived of glucose, an essential nutrient lost during physiologic ischemia. Additionally, cells were deprived of oxygen using a CO2, N2 incubator. This created a condition of oxygen-glucose deprivation similar to what is seen in ischemia. Similar techniques are often used in other published studies [37, 38]. Upon treatment with oxygen deprivation (2% O2) and glucose negative media, we observed an upward trend in PGK-1 expression in HBMVECs and astrocytes which reached significance at 24-hours (Figure 3 C, D; p value < 0.001; < 0.0001 respectively). Ischemia for 24 hours followed by 6 hours of reperfusion (24/6 I.R.) decreased PGK-1 expression compared to the 24-hour time point, however remained significantly higher than the untreated control. With PGK-1 being a known ischemia responsive gene, these results indicate that our experimental conditions of ischemia caused the expected effect on cellular response in both HBMVECs and astrocytes [43–45].

2. NRG-1 and ERBB-4 Response to Excess Heme and/or Ischemia—Upon exposure to conditions of excess heme, NRG-1 expression increased significantly in HBMVECs ($p < 0.05$) at 6-hours compared to the untreated control and reduced to baseline at the 12 and 24-hour timepoints (Figure 4A). Heme did not significantly increase NRG-1 expression in astrocytes and decreased at the 24-hour timepoint ($p < 0.01$) (Figure 4B). Exposure to ischemic conditions caused a spike in NRG-1 expression at 6- hours ($p < 0.0001$) in both HBMVECs and astrocytes, which returned to baseline levels throughout the 12, 24, and 24/6 I.R.-hour timepoints (Figure 4 C, D). When combining excess heme exposure and ischemic conditions we observe a similar 6-hour spike in NRG-1 levels in both HBMVECs and astrocytes in comparison to our untreated controls, which returned to low baseline levels throughout the 12, 24, and 24/6 I.R.-hour timepoints (Figure 4 E, F). Neuregulin-1 expression in HBMVECs was stimulated by either heme or ischemia. Astrocyte NRG-1 responded more to ischemia than heme.

Neuregulin −1's receptor, ERBB-4, showed variable responses to experimental conditions (Figure 5). ERBB-4 expression was unresponsive to conditions of excess heme in both HBMVECs and astrocytes (Figures 5 A -B) but did spike in HBMVECs exposed to ischemic conditions at 12-hours and reduced at 24-hours ($p < 0.0001$, $p < 0.01$) (Figure 5 C). Astrocytes showed a significant increase in ERBB-4 expression at 6-hours ($p < 0.0001$) of ischemia but decreased at each time point following (Figure 5D). Similar results in HBMVECs were observed upon exposure to excess heme and ischemic conditions as we saw an increase in ERBB-4 expression at 12-hours ($p < 0.001$). There was an overall decrease in expression in astrocytes at 12, 24, and 24/6 I.R- hours exposure ($p < 0.001$, $p < 0.01$, $p < 0.0001$ respectively) (Figure 5 E, F)). ERBB-4 expression in both HBMVECs and astrocytes were most responsive to ischemia conditions.

3. NRG-1 Effect on Chemokine Expression Under Experimental Conditions —C-X-C motif chemokines CXCL-1 and CXCL-10 are pro-inflammatory chemokines which act as chemo-attractants for several immune and non-hematopoietic cells. We have previously shown CXCL-10 to be elevated in brain microvascular cells exposed to conditions of excess heme and both chemokines are known to respond under conditions of

vascular injury [30]. Chemokine induced-leukocyte recruitment is known to contribute to vaso-occlusion and hemolysis in SCD, potentially perpetuating cerebral and cerebrovascular injury when occurring in the brain.

In HBMVECs exposed to conditions of excess heme, ischemia, and excess heme in combination with ischemia, we observed significant increases in CXCL-1 expression throughout several durations of exposure (Figure 6 A, C, E), solid lines). Pretreatment of cells with 100 ng/mL NRG-1 significantly reduced CXCL- 1 expression induced by all conditions. In astrocytes, excess heme treatment resulted in significant increase in CXCL-1 expression at 6-hours exposure. This increase was also reduced by NRG-1 pretreatment (Figure 6B). Similar results were observed in astrocytes exposed to ischemic conditions for 6 and 12 hours (Figure 6D). CXCL-1 expression spiked under conditions of excess heme and ischemia at 6 and 24/6 I.R. -hour timepoints, however NRG-1 pretreatment appeared to significantly reduce expression only at the 24/6 I.R.-hour timepoint (Figure 6F).

To assess whether NRG-1 binding to its receptor ERBB4 was necessary for reduction in chemokine expression, we pre-treated a sub-group of cells exposed to our experimental conditions of excess heme, ischemia, or excess heme and ischemia for 6-hours with either NRG-1 or NRG-1 + anti-ERBB4 antibody (or vehicle control). This allowed observation of NRG-1 activity under conditions of competitive ERBB-4 inhibition. In these subgroups we observed NRG-1-induced reduction of CXCL-1 expression in response to our experimental conditions, as seen previously. However, upon competitive inhibition of the ERBB-4 receptor, NRG-1 pretreatment no longer had significant effect on CXCL-1 expression (Figure 7).

In HBMVECs, CXCL-10 expression increased steadily throughout exposure to our experimental conditions (Figure 8 (A, C, E)). In astrocytes, similar to CXCL-1, we saw significant spikes in CXCL-10 expression between 6- and 12-hours exposure. NRG-1 treatment significantly reduced increased CXCL-10 expression in both cell types, showing the strongest effect in conditions of ischemia and excess heme with ischemia (Figure 8 (C, D; E, F). NRG-1's effect on experimental conditions of excess heme reached significance at 24- hours exposure in HBMVECs and 6- and 12- hours in astrocytes. As in previous experiments, competitive ERBB4 inhibition significantly impaired NRG-1's ability to reduce CXCL-10 expression (Figure 9).

4. NRG-1 Effect on Angiogenic Response Under Experimental Conditions— Pro-angiogenic responses can be induced by oxygen and nutrient deprivation as well as vascular injury and surrounding cell dysfunction. Vascular Endothelial Growth Factor-A (VEGF-A) is an essential growth factor in the angiogenic response that induces proliferation and migration of endothelial cells. Its expression has been seen in both endothelial cells and astrocytes and is known to be HO-1 responsive and increased under conditions of hypoxia and ischemia [46, 47].

In HBMVECs and astrocytes exposed to conditions of excess heme, ischemic conditions, and excess heme with ischemic conditions, we saw a significant increase in VEGF-A expression with the highest effect being seen during ischemia (Figure 10). Under conditions

of excess heme, NRG-1 pretreatment resulted in increased VEGF-A response, however NRG-1 had no effect on VEGF-A expression under ischemic conditions. When excess heme was combined with ischemic condition, NRG-1 was able to again, significantly increase VEGF-A expression. A subset of HBMVECs and astrocytes were exposed to our experimental conditions of excess heme, ischemia, or excess heme and ischemia for six-hours (Figure 11) and either NRG-1 or NRG-1 + antiERBB-4 pretreatment. Competitive inhibition of the ERBB-4 receptor resulted in a loss of NRG-1 mediated VEGF-A increases in cells exposed to excess heme and excess heme with ischemia. Neither NRG-1 nor competitive inhibition of the ERBB-4 receptor had any significant effect on VEGF-A expression under ischemic conditions.

5. NRG-1 Effect on Cellular Adhesion Molecules Under Experimental Conditions—Adhesive interactions between the endothelial lining, red blood cells, platelets, and immune-responsive cells are known to contribute to vaso-occlusive events in SCD. In addition to their role in immune modulation, astrocytes are known to express many adhesion molecules which facilitate lymphocyte interactions in response to CNS injury and cerebral vascular inflammation. Increased cellular adhesion molecule production has been well documented in SCD, including elevated Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Endothelial Cell Adhesion Molecule-1 (VCAM-1) expression. Increases in soluble forms of VCAM-1 have also been documented in circulating plasma of individuals with SCD.

We observed significant increases in ICAM-1 expression in our HBMVECs exposed to experimental conditions of excess heme and excess heme with ischemia (Figure 12). We did not observe significant increases under exposure to ischemic conditions alone. In astrocytes, exposure to heme, ischemia, and heme with ischemia all caused significant increases in ICAM-1 in comparison to our untreated control. In both cell types, NRG-1 pre-treatment decreased ICAM-1 expression under conditions of excess heme and excess heme with ischemia. Under conditions of ischemia alone, NRG-1 treatment had no significant effect (Figure 12).

We saw similar expression patterns with VCAM 1 (Figure 14). In HBMVECs and astrocytes exposed to conditions of excess heme and excess heme with ischemia, we observed significant increases in VCAM-1 expression at 6-hours exposure before returning towards baseline and decreased through the following timepoints. Ischemic treatment caused an increase in VCAM-1 in endothelial cells, while it caused decreased expression in astrocytes. In all cases where we saw increases in VCAM 1 expression, NRG-1 treatment was able to return that expression back to baseline.

As with previous studies, a subset of HBMVECs and astrocytes were exposed to our experimental conditions of excess heme, ischemia, or excess heme and ischemia for six-hours with pre-treatment with either NRG-1 or NRG-1 + antiERBB-4 pretreatment (Figures 13 and 15). Competitive inhibition of the ERBB-4 receptor resulted in a loss of NRG-1 mediated ICAM-1 and VCAM-1 responses in both cell types exposed to excess heme and excess heme with ischemia. Neither NRG-1 nor competitive inhibition of the ERBB-4

receptor had any significant effect on ICAM-1 expression under ischemic conditions but showed ability to reduce VCAM-1 significantly.

Discussion

1. Summary

We measured NRG-1 in transgenic mice with SCA (expressing human hemoglobin SS) equivalent in age to human young adults in order to validate that elevated plasma levels in human children with SCD may originate in the brain [32]. The SCD mice had elevated plasma NRG-1 levels in comparison to healthy controls (mice with human Hemoglobin AA). Additionally, perfused whole brain homogenates of SCA mice had elevated levels of NRG-1 compared to controls, suggesting a tissue specific response. These mice were younger than 13 months of age, when they have been shown to have spontaneous cortical microinfarcts and intravascular pathologies [48].

We then performed in *vitro* experiments to test whether cells which are part of the blood brain barrier react to conditions of excess heme and ischemia used to mimic chronic hemolysis and reduced cerebrovascular perfusion in SCD by increasing NRG-1 production. We utilized human brain microvascular endothelial cells and astrocytes, two principal components of the blood brain barrier which are most likely to encounter these conditions *in vivo* and serve as the first line of defense from further cerebral injury. Excess heme and ischemia increased NRG-1 expression in both HBMVECs and astrocytes (Figure 4). Expression of ERBB4 was not affected by excess heme but showed acute increases in conditions of ischemia or excess heme with ischemia (Figure 5).

Lastly, we tested whether administration of exogenous NRG-1 would have neuroprotective effects, as has been shown in models with neurons and other cerebrovascular cell lines in response to heme or ischemic injury [37, 43, 66, 68, 69]. Similar to our previous studies which identified NRG-1's ability to reduce heme-induced apoptosis and tight-junction dysfunction in HBMVECs and astrocytes, the current studies demonstrated that exogenous NRG-1 reduced chemokine expression, increased pro-angiogenic response, and decreased adhesion molecule expression in HBMVECs and astrocytes exposed to excess heme and ischemia (Figure 6, 8, 10, 12, 14) [23, 26]. Neuregulin-1, in combination with competitive inhibition of its receptor, ERBB-4, reduced anti-inflammatory responses, demonstrating ERBB4 signal transduction to be necessary for the ameliorative effects of NRG-1 administration (Figure 7, 9, 11, 13, 15). Of note, proangiogenic responses can prove beneficial in ischemic and inflammatory environments, however dysregulation can lead to neovascularization under certain conditions. Figure 16 summarizes the known effects of NRG-1 on cerebral injury as well as our lab's contributions and contributions made by the current studies.

2. Limitations

Due to study constraints, only gene expression data were measured and reported in the *in vitro* studies. A number of assessed targets have intracellular, membrane bound, and/or cleaved-extracellular localization. These targets were additionally assessed with in multiple

cell types, with differing treatment groups, at numerous timepoints. Protein quantitation of all subcellular and extracellular fractions would provide further insights to NRG-1-related responses and will be collected as assessed as availability of resources allow. Human brain microvascular endothelial cells and astrocytes were specifically selected for use in these studies for their role in the blood-brain barrier and immediate proximity to hemolysis- and ischemic- induced insults in SCD. These cells are also known to produce and respond to NRG-1 [49–53]. Individual isolated cell types are an incomplete model of the blood brain barrier. Future studies could be expanded to 3-dimensional culture systems or organoids, neuronal and/or microglial cells to assess their roles and responses to NRG-1 in SCD.

3. Conclusion

Individuals with SCD are at a severely heightened risk for cerebrovascular injury and acute cerebrovascular events including ischemic and hemorrhagic stroke. Research and development of molecular targets which may provide protection from such injury in SCD is critical in improving health outcomes.

We have previously identified elevated circulating NRG-1 in children with SCD compared to healthy controls and found elevations in NRG-1 to be positively correlated with stroke risk predictors in SCD [32]. In these current studies, we identified NRG-1 elevations in both plasma and brain homogenates of the Townes SCD mouse model compared to healthy controls. Because NRG-1 has proven to be neuroprotective in a number of cerebral injury models, we postulate that elevations of NRG-1 in children and mice with SCD may be a result of the subclinical cerebrovascular injury caused by chronic hemolysis and reduced cerebral perfusion. This hypothesis is supported by our *in vitro* studies which demonstrate elevated NRG-1 expression in HBMVECs and astrocytes exposed to experimental conditions of excess heme and ischemia.

Though we investigated a number of specific pathways known to interact with NRG-1/ERBB4 signaling and the reduction of hemolytic/ischemic outcomes, interactions with additional pathways including the nitric oxide and carbon monoxide systems may play a role in reduction of disease related phenotypes as previously demonstrated in SCD [54–57]. Of note, it has recently been indicated that administration of endothelium-derived NRG1 can compensate for endothelial-derived nitric oxide synthase deficiency in the heart and kidneys and that endothelial cells compensate for eNOS deficiency by increasing the secretion of NRG-1 [55]. Additionally, alteration of the carbon monoxide system shows similar protection from ischemic/reperfusion injury in rodent models of SCD to that observed by NRG-1/ERBB-4 [54]. Such interactions can be further expounded upon and elucidated in continued research efforts.

We have previously demonstrated that administration of exogenous NRG-1 acts through the ERBB4/ STAT3/AKT pathway to reduce heme-induced apoptosis and tight-junction dysfunction in HBMVECs and astrocytes [23, 26]. We have now demonstrated that addition of exogenous NRG-1 ability reduces chemokine expression, increases pro-angiogenic response, and decreases adhesion molecule expression in an ERBB4 dependent manner in HBMVECs and astrocytes exposed to conditions of excess heme and ischemia. We believe future pre-clinical studies in the SCD mouse model are warranted. If NRG-1 is found to

reduce stroke severity in SCD mice, translation to people with SCD would be facilitated by its current use in clinical trials for ischemic heart disease. Recombinant human NRG-1β has already passed safety and efficacy testing to be administered intravenously to humans [58]. Neuregulin-1β is also slated to begin clinical trials for treatment of post ischemic injury for non-SCA stroke [59]. Our findings strongly support the NRG-1/ERBB4 pathway as a potential therapeutic target for preventing or treating SCD cerebrovascular disease.

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Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

List of Abbreviations

AA	Hemoglobin AA
AB	Antibody
AC	Hemoglobin AC
AKT	Protein kinase B
ARC	Absolute reticulocyte count
AS	Hemoglobin AS
BDNF	Brain derived neurotrophic factor
CT	Cycle of threshold
CXCL-1	C-X-C motif chemokine ligand 1
CXCL-10	C-X-C motif chemokine ligand 10
DAPI	4′,6-diamidino-2-phenylindole

DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ERBB4	Erythroblastic oncogene B receptor tyrosine kinase 4
eNOS	Endothelial derived nitric oxide synthase
GFAP	Glial fibrillary acidic protein
HB	Hemoglobin
HBMVEC	Human brain microvascular endothelial cells
HO-1	Heme oxygenase 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLU	Glutamine
ICAM-1	Intracellular adhesion molecule 1
IQR	Interquartile range
LYS	Lysine
MCAO	Middle cerebral artery occlusion
N/A	Not applicable
NEUN	Neuronal nuclei
NRG-1	Neuregulin-1β
PBS	Phosphate buffered saline
PDGF-AA	Platelet derived growth factor type-aa
PGK-1	Phosphoglycerate kinase 1
RBC	Red blood cell
RPM	Rotations per minute
SCA	Sickle cell anemia
SCD	Sickle cell disease
SS	Hemoglobin SS
STAT	Signal transducer and activator of transcription proteins
TCD	Transcranial Doppler
VAL	Valine
VEGFA	Vascular endothelial growth factor a

VCAM-1	Vascular cell adhesion molecule 1
WBC	White blood cell

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Highlights:

- Mice with SCD show elevated plasma NRG-1 levels in comparison to healthy controls
- Experimental conditions mimicking hemolysis and ischemia increase NRG-1 production
- NRG-1 reduced inflammatory cytokine and adhesion molecule expression
- NRG-1 expression in SCD is likely a protective endogenous response

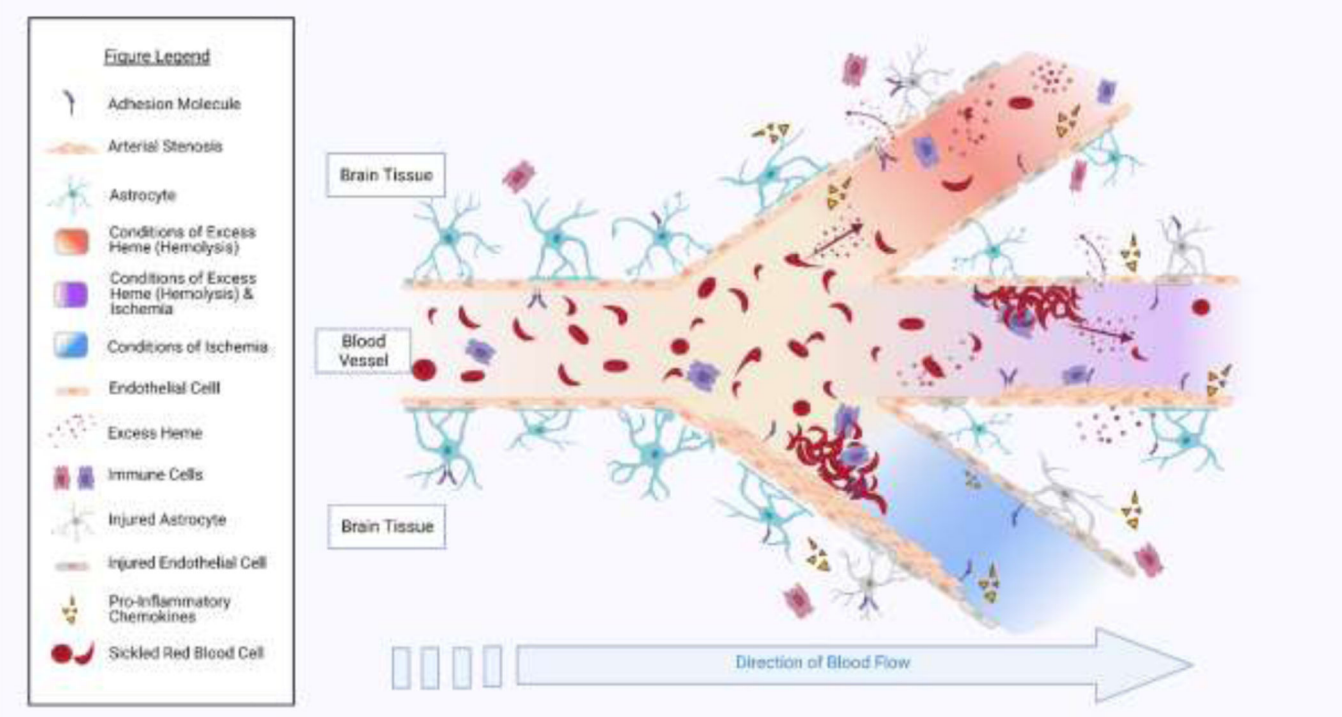

Figure 1. Hemolysis- and ischemia induced-cerebrovascular inflammation associated with sickle cell disease.

Chronic hemolysis and reduced cerebrovascular perfusion are common to SCD. These pathologies generate internal conditions of excess circulating “free” heme (upper branch (red)), systemic ischemia (lower branch (blue)), and often times a combination of the two (middle right branch (purple)). Hemolysis and excess free heme in SCD result in oxidative injury to surrounding cerebrovascular tissues including astrocyte and endothelial cell dysfunction and RBC membrane instability, further perpetuating hemolysis, pro-inflammatory responses, and production of vaso-occlusive factors. Ischemic conditions, both chronic and acute, occur in SCD due to a number of frequently occurring factors including vaso-occlusive crises, arterial stenosis, anemia, and reduced heme-oxygen saturation. These factors, as with excess heme, result in astrocyte and endothelial cell dysfunction as well as hemoglobin polymerization and further sickling/instability of RBC’s.

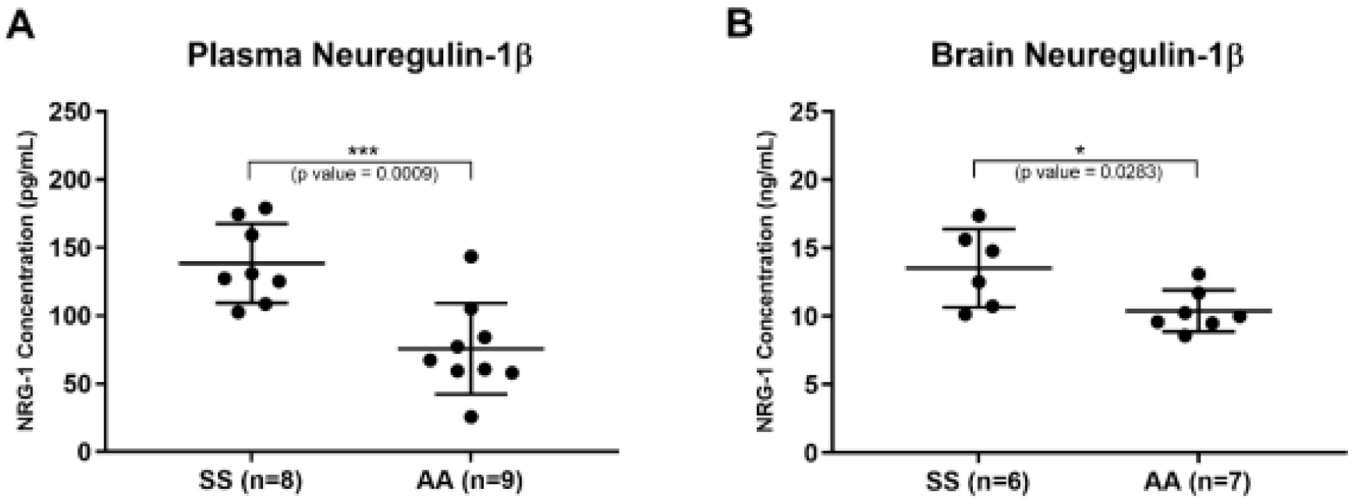

Figure 2. Neuregulin-1 levels were elevated in the plasma and brains of SCD mice

(A) Plasma NRG-1 concentrations and (B) Brain NRG-1 levels in Townes SS (SCD) and AA (healthy control) mice, analyzed using ELISA. Differences between SCD and control mice were statistically significant. Values are reported as Mean ± Standard Deviation.

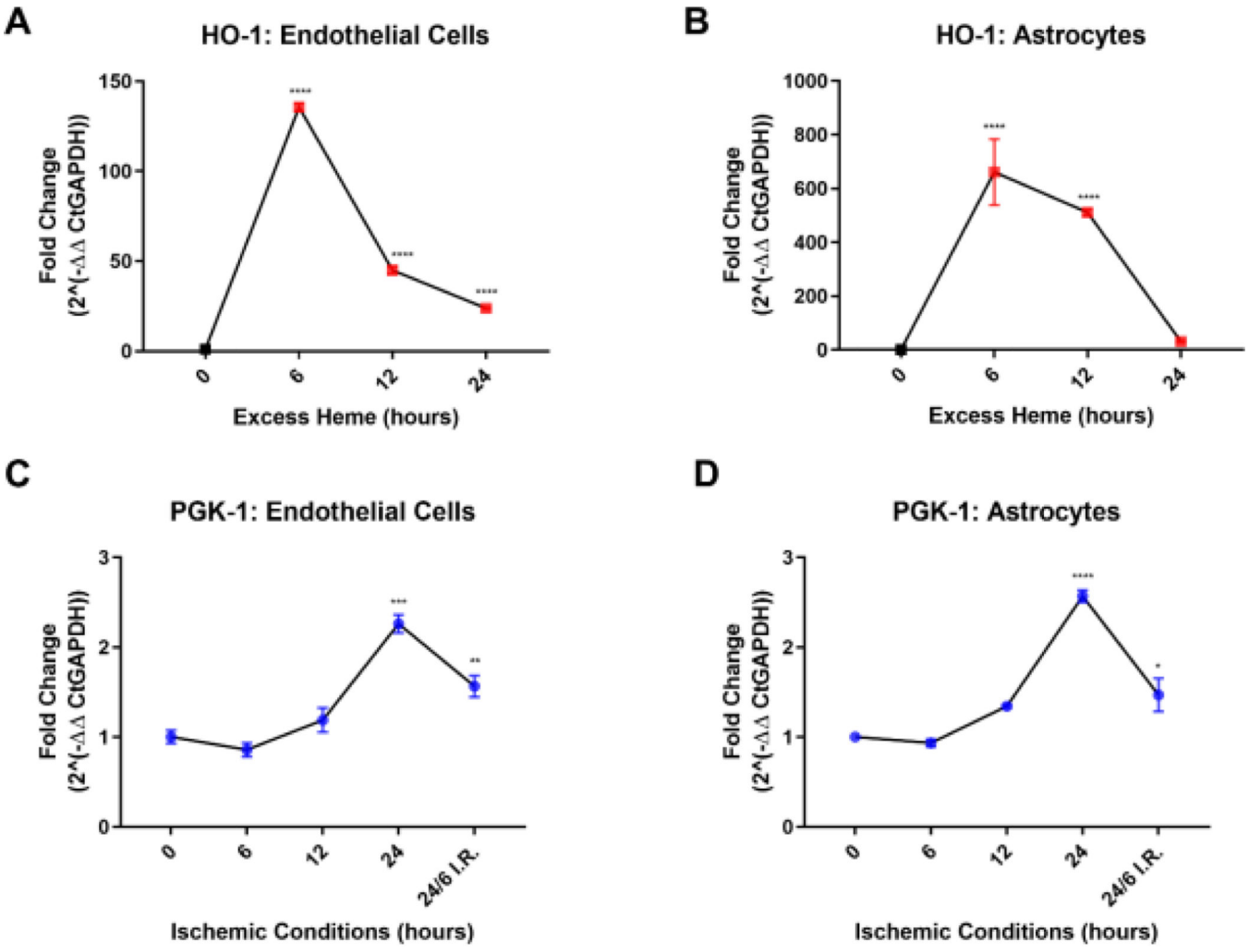

Figure 3. Experimental conditions generate expected cellular responses

HBMVECs (A) and astrocytes (B) treated with 50 μM heme in complete media had significantly increased HO-1 expression compared to our 0-hour untreated control, indicating a positive cellular response to excess heme. Peak response occurred at 6 hours post-treatment. Upon exposure to oxygen deprivation (2% O2) and glucose depleted media, HBMVECs (C) and astrocytes (D) had increased PGK-1 expression which reached significance at 24-hours in both cell types. Ischemia for 24 hours followed by 6 hours of reperfusion (24/6 I.R.) decreased PGK-1 expression compared to the 24-hour time point, however remained significantly higher than the untreated control. P-values were denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data were reported as mean ± standard deviation.

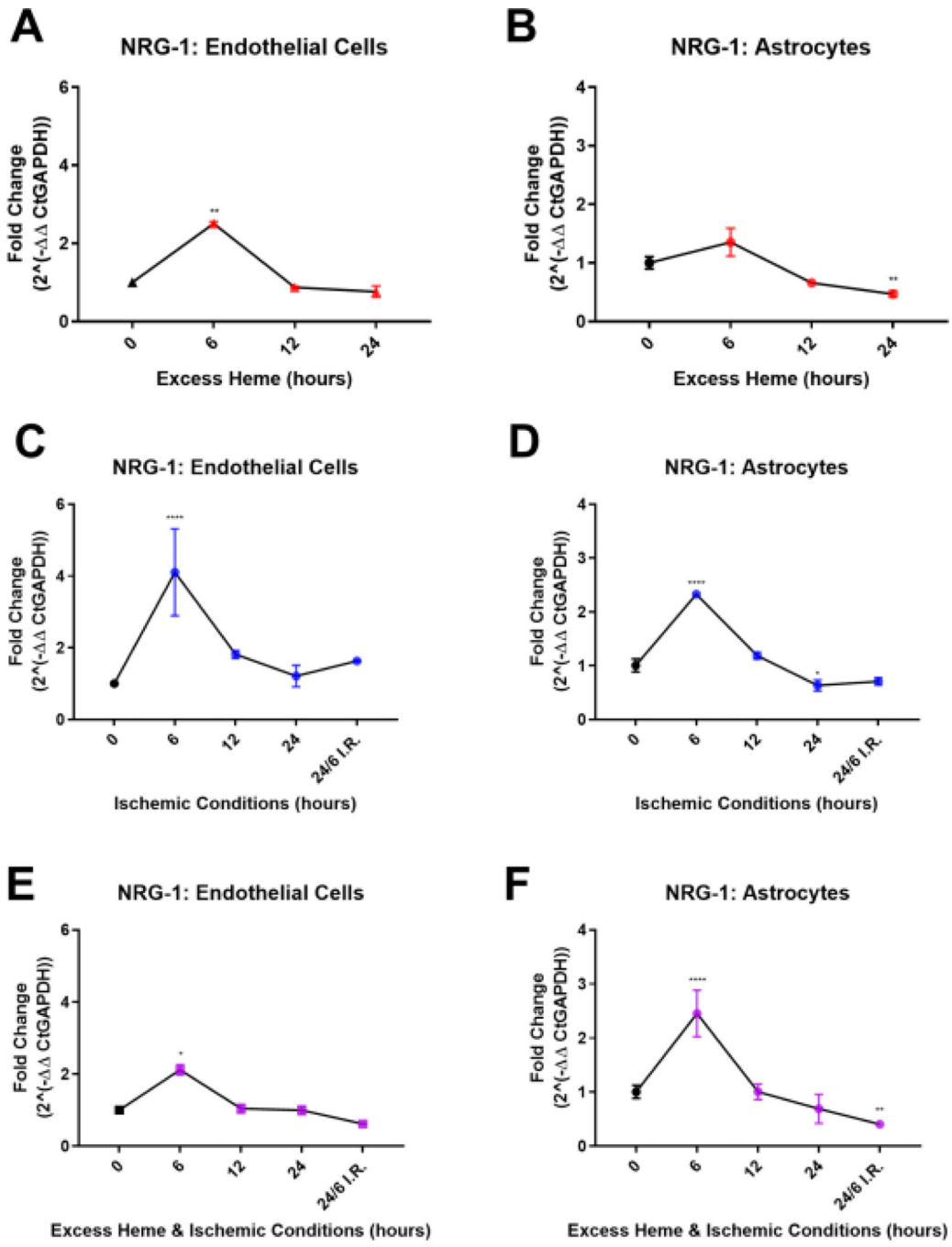

Figure 4. Neuregulin-1 Increases Under Experimental Conditions of Excess Heme and Ischemia

Exposure of HBMVEC's to excess heme (A), ischemia (C), and excess heme and ischemia (E) resulted in spikes in NRG-1 expression at 6-hours. Similar effects were seen in astrocytes exposed to ischemia (D) and excess heme and ischemia (F), However NRG-1 in astrocytes remained relatively constant throughout exposure to excess heme alone (B). P-values were denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

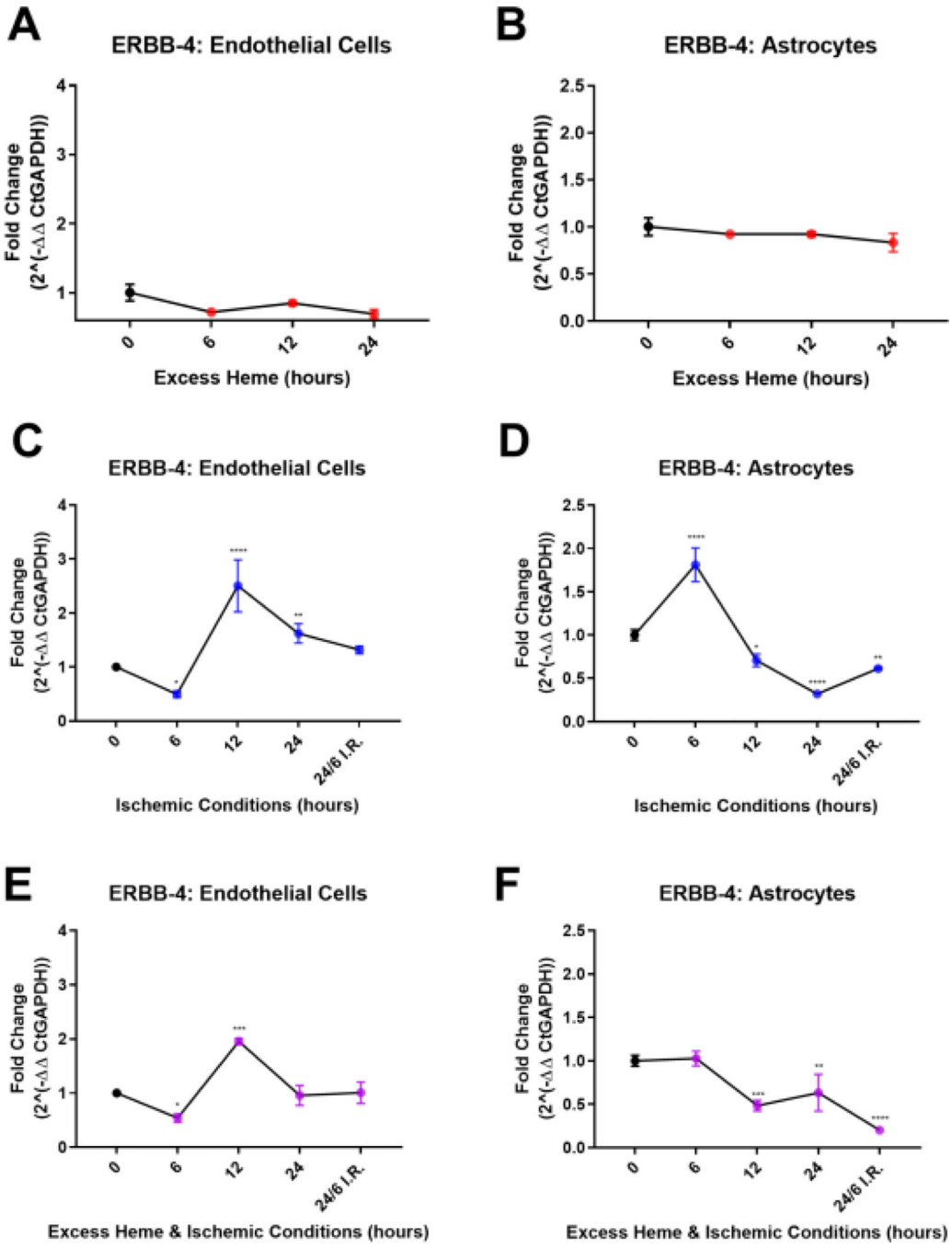

Figure 5. ERBB-4 receptor shows variable response to experimental conditions of excess heme and ischemia

Expression of NRG-1's receptor, ERBB-4, in HBMVECs treated with excess heme (A), ischemia (C), heme and ischemia (E); and astrocytes treated with excess heme (B), ischemia (D), or heme and ischemia (F). P-values were denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

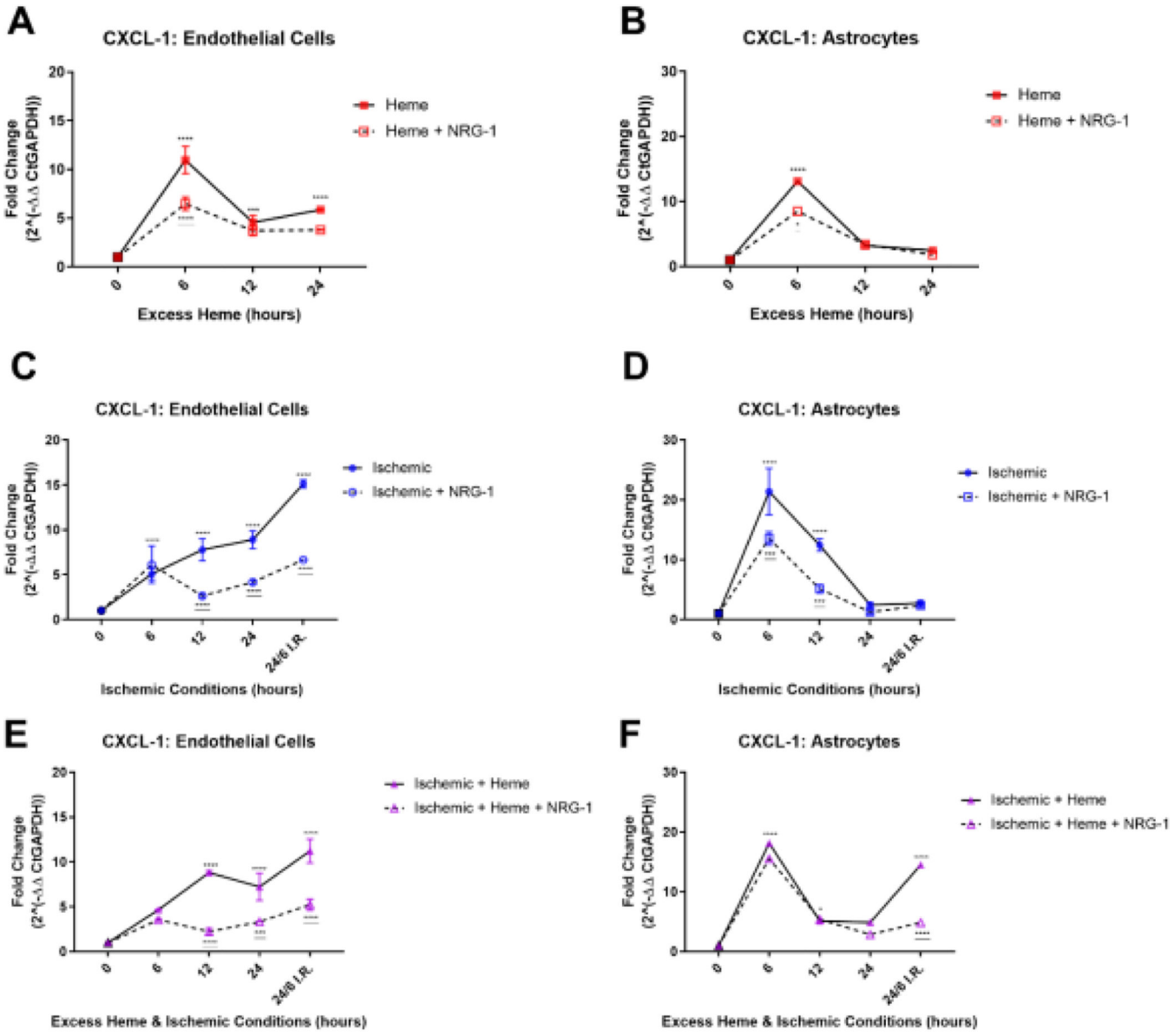

Figure 6. NRG-1 reduces CXCL-1 expression in HBMVECs and astrocytes exposed to experimental conditions

Overall, CXCL-1 expression tended to increase significantly upon exposure to experimental conditions of excess heme (A, B), ischemia (C, D), and excess heme combined with ischemia (E, F) in both HBMVECs and astrocytes. Cells pre-treated with 100ng/mL hrNRG-1 showed significant reductions in resultant CXCL-1 expression (dotted lines) in HBMVECs and astrocytes exposed to conditions of excess heme and ischemia independently. When these treatments were combined in our excess heme and ischemic treatment group, NRG-1 reduced resultant CXCL-1 expression in HBMVECs but had no significant effect in astrocytes. Significant differences in CXCL-1 expression between non-NRG-1 treated groups and 0-hour untreated controls were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Significant differences in CXCL-1 expression between NRG-1 treated and non-NRG-1 treated groups were denoted as: $p < 0.05$ (*) $p <$

0.01 (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

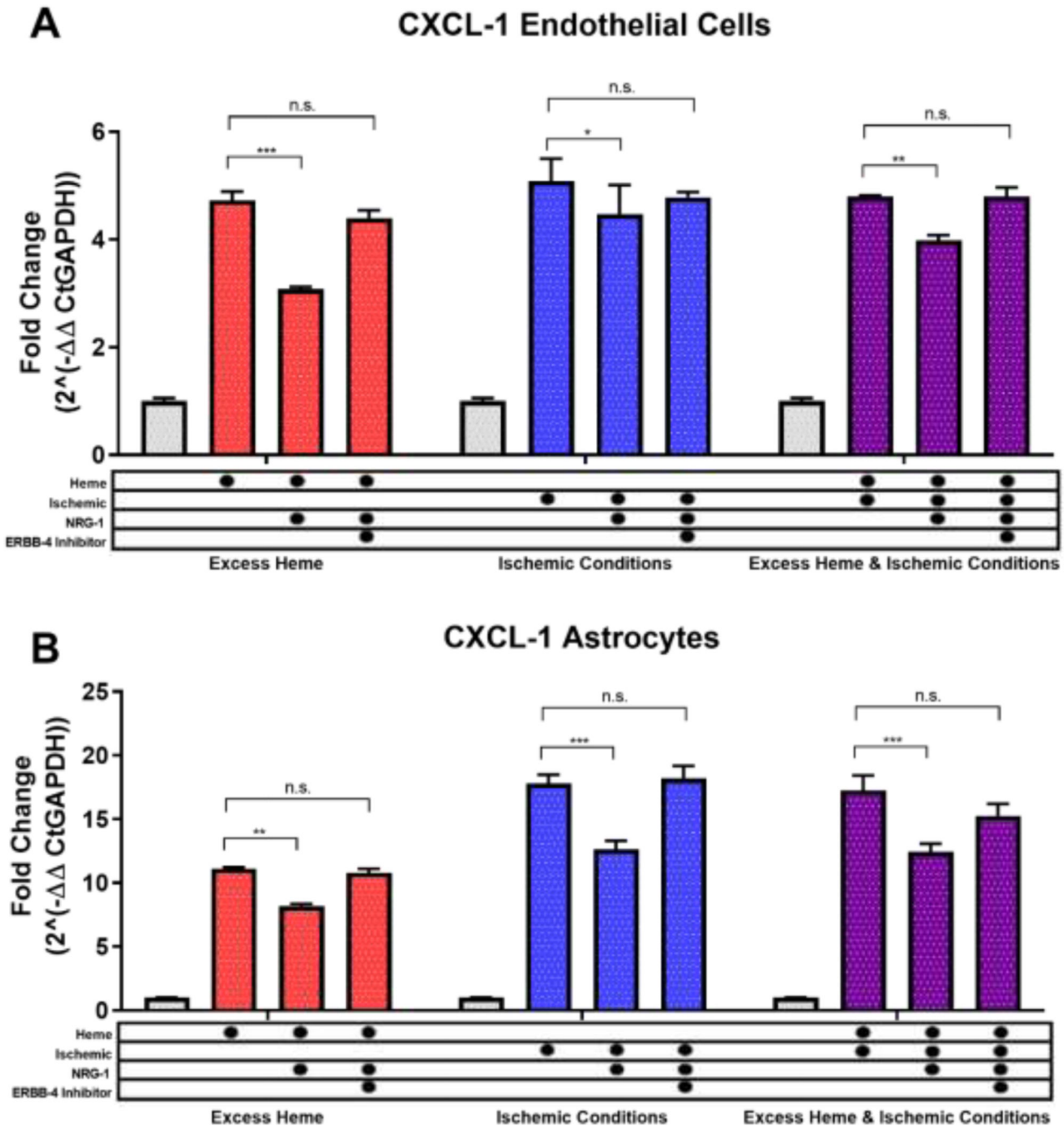

Figure 7. Competitive Inhibition of the ERBB-4 Receptor Limits NRG-1's Ability to Reduce CXCL-1 Expression Resulting from Experimental Conditions.

A subset of HBMVECs and astrocytes were pretreated with either NRG-1 or NRG-1 + anti-ERBB-4 antibody (competitive ERBB-4 inhibition) before being exposed to experimental conditions of excess heme, ischemia, or excess heme combined with ischemia for six hours. As previously demonstrated, NRG-1 pretreatment was able to reduce increases in CXCL-1 expression resulting from exposure to experimental conditions, however upon competitive inhibition of the ERBB-4 receptor, NRG-1 showed no significant effect on resultant CXCL-1 expression. P-values were denoted as follows: $p > 0.05$ (n.s.), $p < 0.05$

(*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to untreated controls shown in grey. Data were reported as mean ± standard deviation.

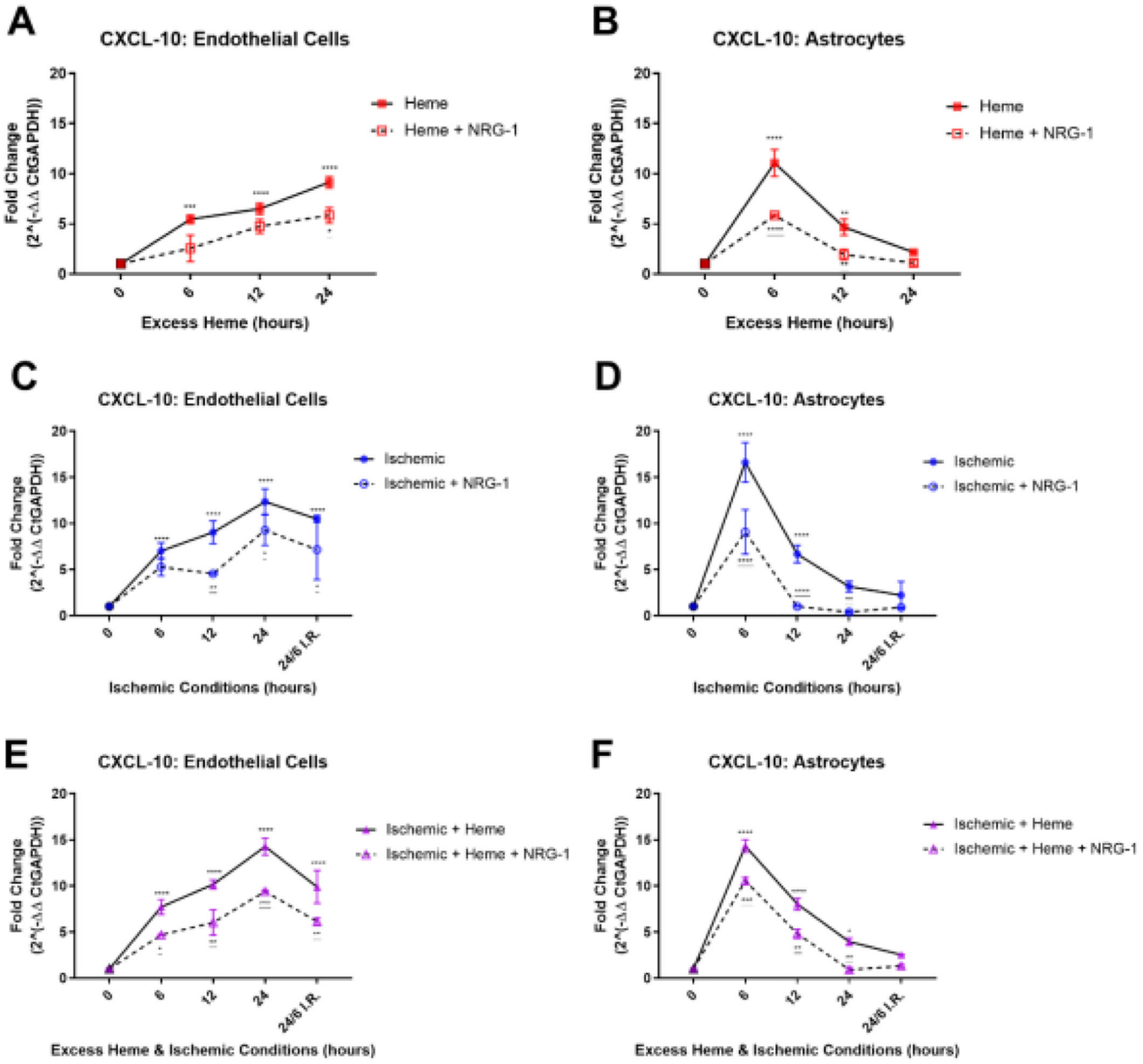

Figure 8. NRG-1 reduces CXCL-10 expression in HBMVECs and astrocytes exposed to experimental conditions

CXCL-10 expression increased throughout exposure of HBMVECs to experimental conditions of excess heme (A), ischemia (C), and excess heme with ischemia (E). In astrocytes, CXCL-10 expression tended to spike between 6- and 12- hours exposure (B, D, F). In both cell types, NRG-1 treatment significantly reduced CXCL-10 expression, showing the largest effect in conditions of ischemia and excess heme with ischemia ((C-F) dotted lines). NRG-1 treatment also reduced CXCL-10 expression under conditions of heme, reaching significance at 24- hours in HBMVECs and 6- and 12- hours in astrocytes ((A, D) dotted lines). Significant differences in CXCL-10 expression between non-NRG-1 treated groups and 0- hour untreated controls were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p <$

0.001 (***), and $p < 0.0001$ (****). Significant differences in CXCL-10 expression between NRG-1 treated and non-NRG-1 treated groups were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

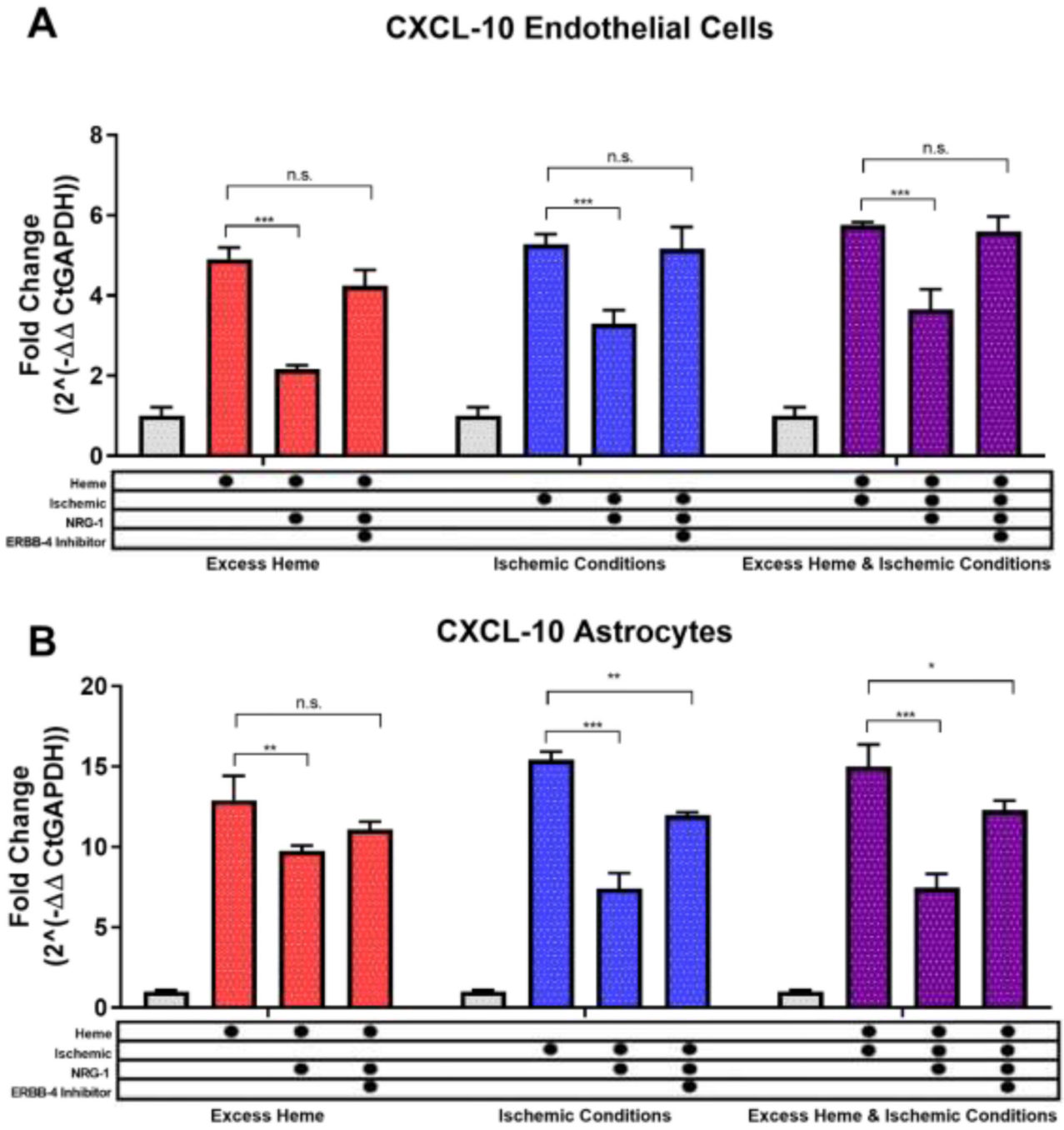

Figure 9. Competitive inhibition of the ERBB-4 receptor limits NRG-1's ability to reduce CXCL-10 expression resulting from experimental conditions.

A subset of HBMVECs and astrocytes were pretreated with either NRG-1 or NRG-1 + anti-ERBB-4 antibody (competitive ERBB-4 inhibition) before being exposed to experimental conditions of excess heme, ischemia, or excess heme combined with ischemia for six hours. As previously demonstrated, NRG-1 pretreatment was able to reduce increases in CXCL-10 expression resulting from exposure to experimental conditions, however upon competitive inhibition of the ERBB-4 receptor, NRG-1 showed no significant effect on resultant CXCL-10 expression. P-values were denoted as follows: $p > 0.05$ (n.s.), $p < 0.05$

(*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to untreated controls shown in grey. Data were reported as mean ± standard deviation.

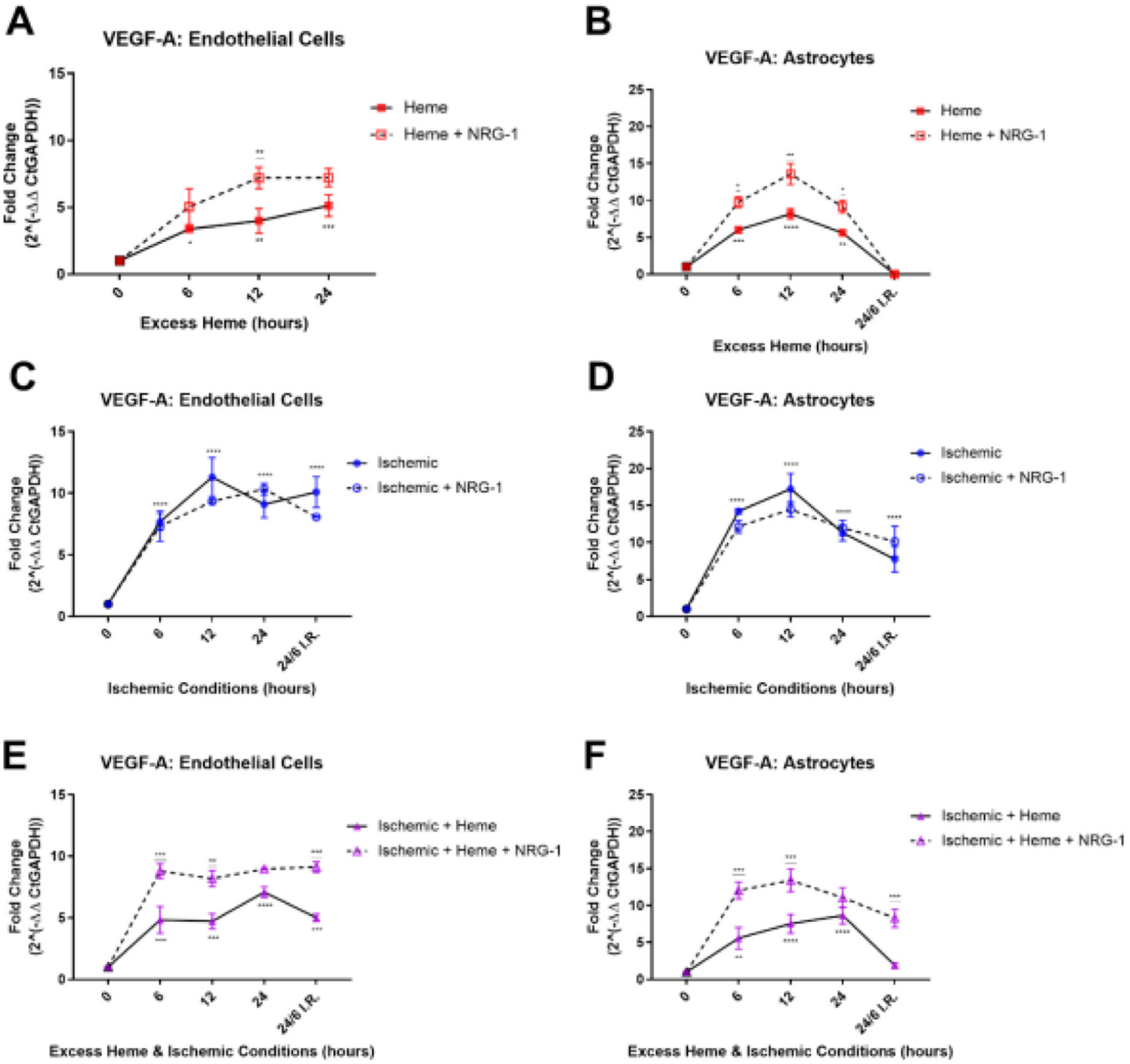

Figure 10. NRG-1 increases VEGF-A expression in HBMVECs and astrocytes exposed to conditions of excess heme but not experimental ischemia alone

Experimental conditions of excess heme (A, B), ischemia (C, D), and excess heme with ischemia (E, F) lead to an increase in VEGF-A expression. The largest increase in VEGF-A expression was observed under ischemic conditions. Neuregulin-1 treated cells (dotted lines) further increased VEGF-A expression under conditions of excess heme but had no significant effect under ischemic conditions alone. When excess heme was combined with ischemic conditions NRG-1, again, was able to significantly increase VEGF-A expression. Significant differences in VEGF-A expression between non-NRG-1 treated groups and 0-hour untreated controls were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Significant differences in VEGF-A expression between NRG-1 treated

and non-NRG-1 treated groups were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

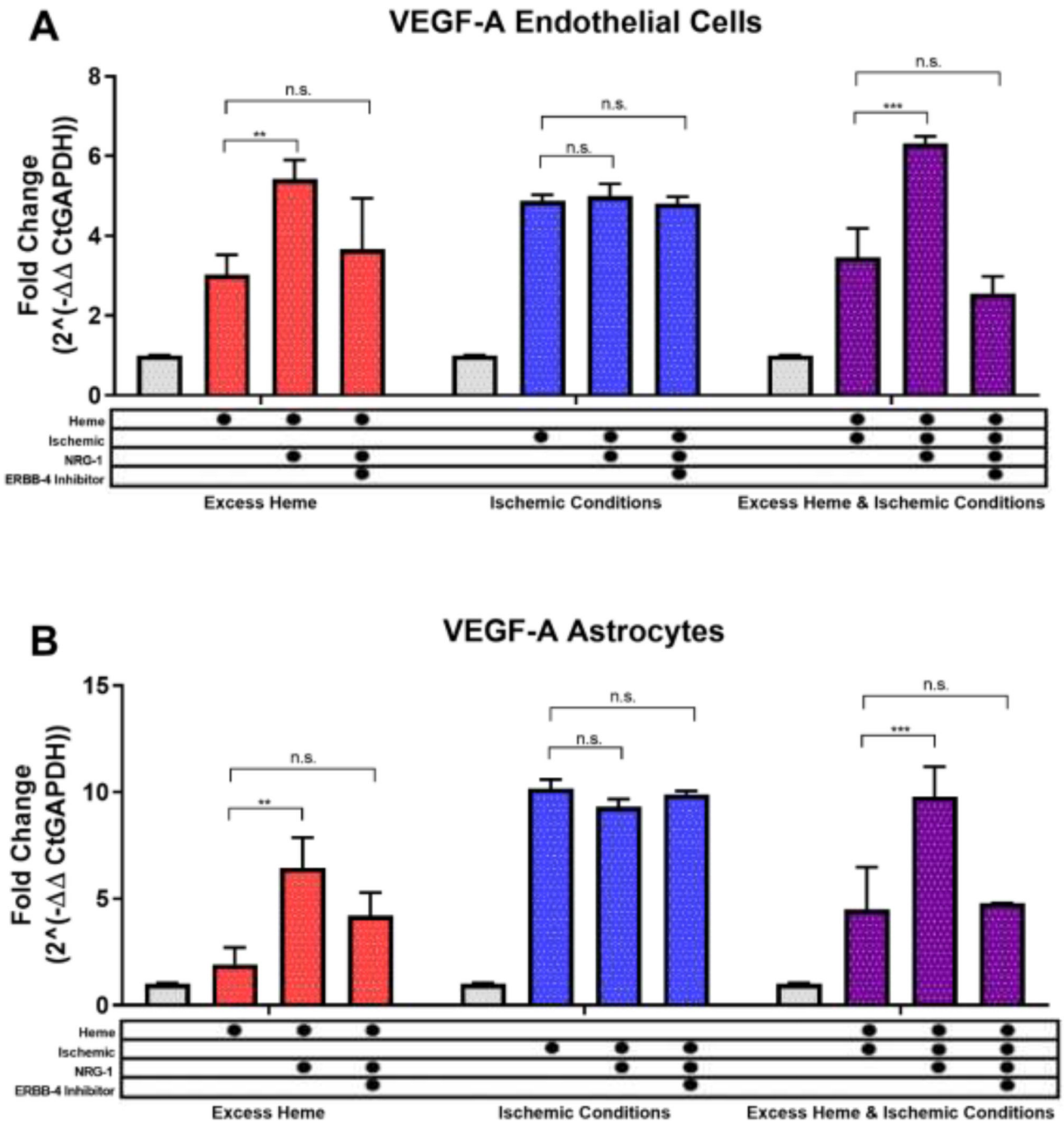

Figure 11. Competitive inhibition of the ERBB-4 receptor limits NRG-1's ability to reduce VEGF-A expression resulting from experimental conditions

A subset of HBMVECs and astrocytes were pretreated with either NRG-1 or NRG-1 + anti-ERBB-4 antibody (competitive ERBB-4 inhibition) before being exposed to experimental conditions of excess heme, ischemia, or excess heme combined with ischemia for six hours. As previously demonstrated, NRG-1 pretreatment was able to further increase VEGF-A expression resulting from exposure to experimental conditions of excess heme and excess heme with ischemia, however upon competitive inhibition of the ERBB-4 receptor, NRG-1 showed no significant effect on resultant VEGF-A expression. P-values were denoted as

follows: $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared untreated controls shown in grey. Data were reported as mean ± standard deviation.

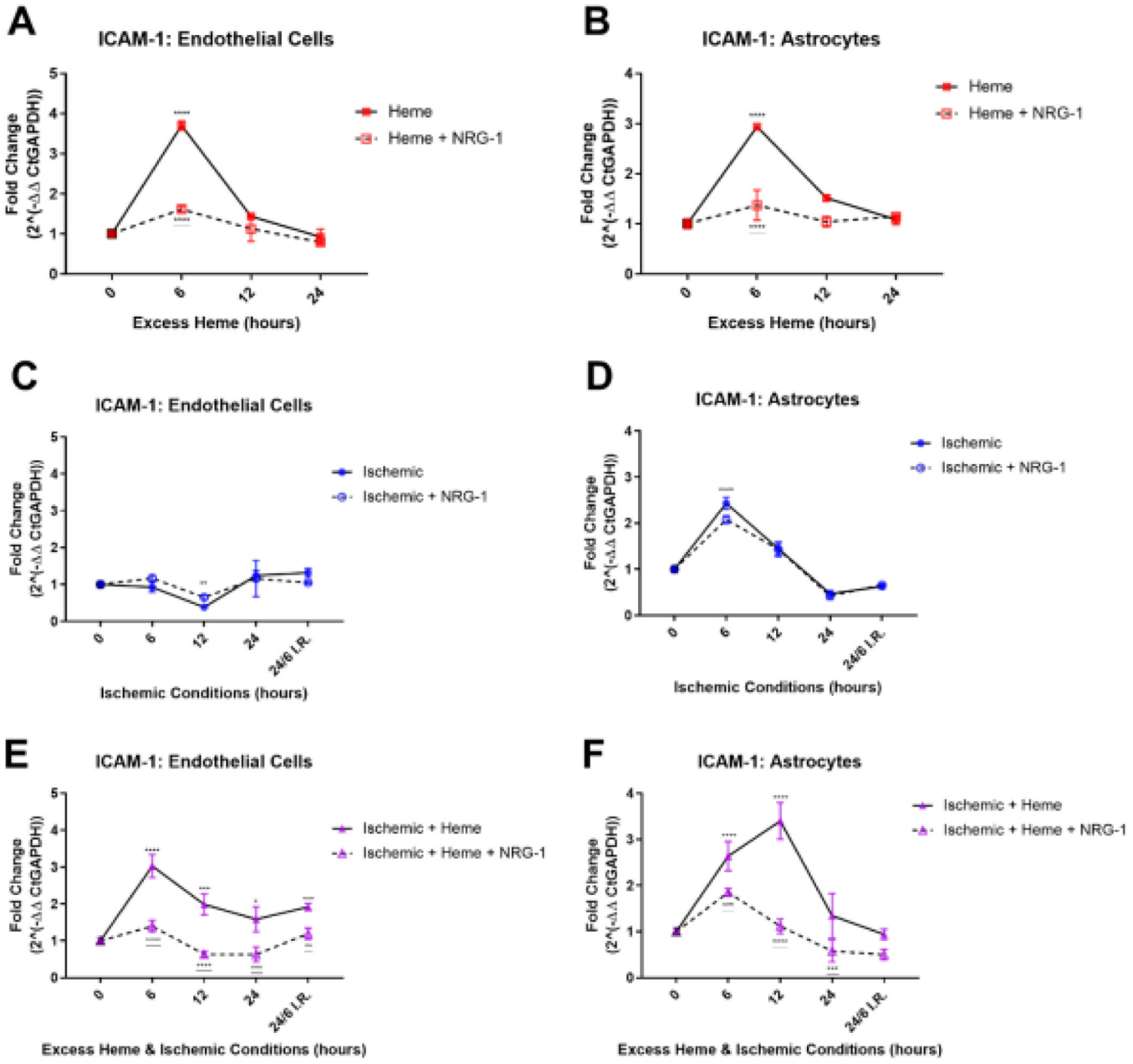

Figure 12. NRG-1 treatment decreased ICAM-1 expression in conditions of excess heme but not experimental ischemia alone.

Experimental conditions of excess heme (A, B) and excess heme with ischemia (E, F) resulted in increased ICAM-1 expression in both HBMVECs and astrocytes. Ischemic conditions resulted in increased ICAM-1 expression in astrocytes (D) but not endothelial cells (C). Pretreatment of cells with NRG-1 was able to significantly reduce resultant increases in ICAM-1 expression but had no effect under ischemic conditions (dotted lines). Significant differences in ICAM-1 expression between non-NRG-1 treated groups and 0-hour untreated controls were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Significant differences in ICAM-1 expression between NRG-1 treated and non-NRG-1 treated groups were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***),

and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

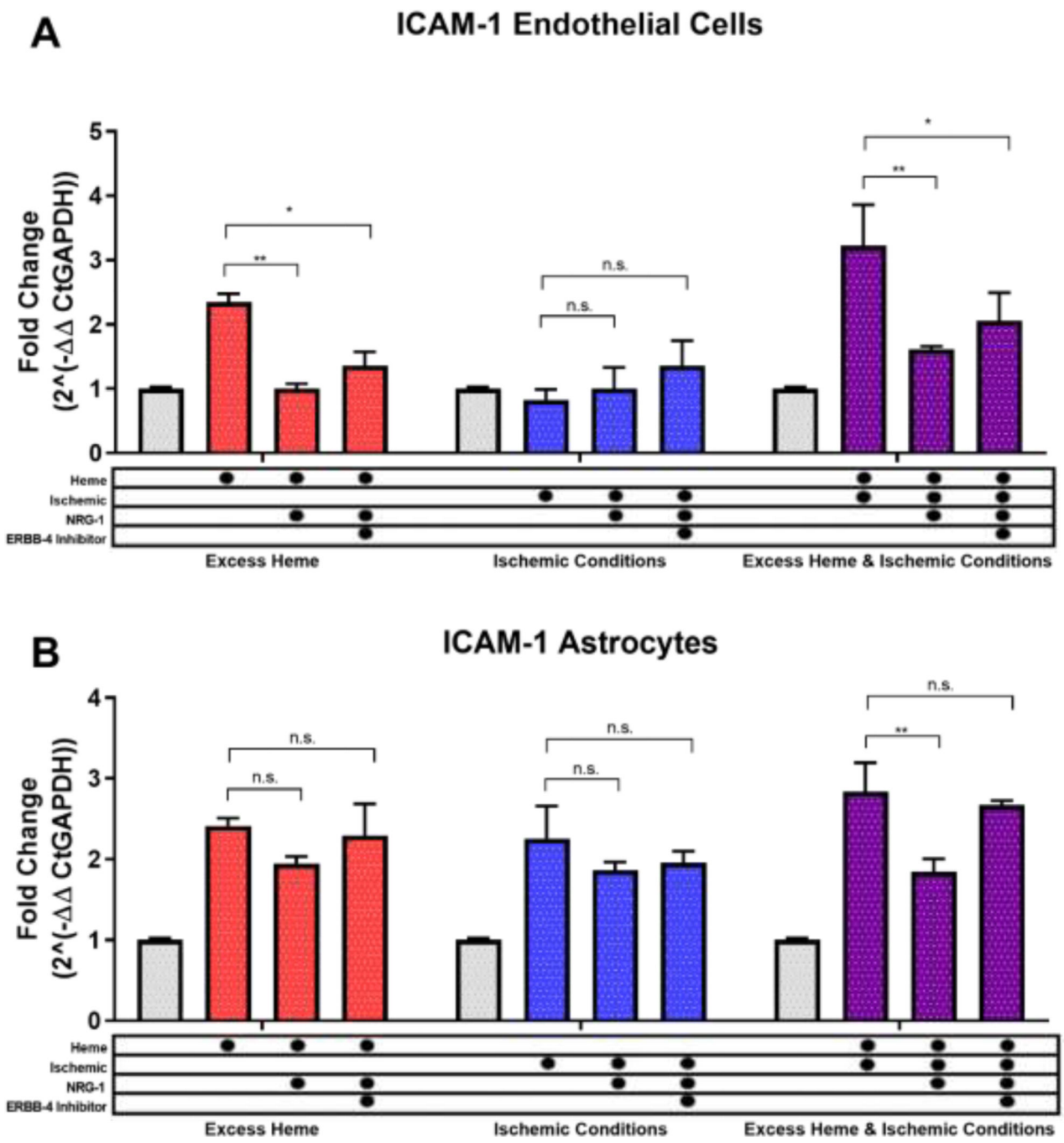

Figure 13. Competitive inhibition of the ERBB-4 receptor limits NRG-1's ability to reduce ICAM-1 expression resulting from experimental conditions.

A subset of HBMVECs and astrocytes were pretreated with either NRG-1 or NRG-1 + anti-ERBB-4 antibody (competitive ERBB-4 inhibition) before being exposed to experimental conditions of excess heme, ischemia, or excess heme combined with ischemia for six hours. As previously demonstrated, NRG-1 pretreatment was able to reduce ICAM-1 expression resulting from exposure to experimental conditions of excess heme and excess heme with ischemia, however under competitive inhibition of the ERBB-4 receptor, NRG-1 showed no ability to significantly reduce resultant increases in ICAM-1 expression. Neither NRG-1

nor competitive inhibition of the ERBB-4 receptor had any significant effect on ICAM-1 expression under ischemic conditions. P-values were denoted as follows: $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to untreated controls shown in grey. Data were reported as mean ± standard deviation.

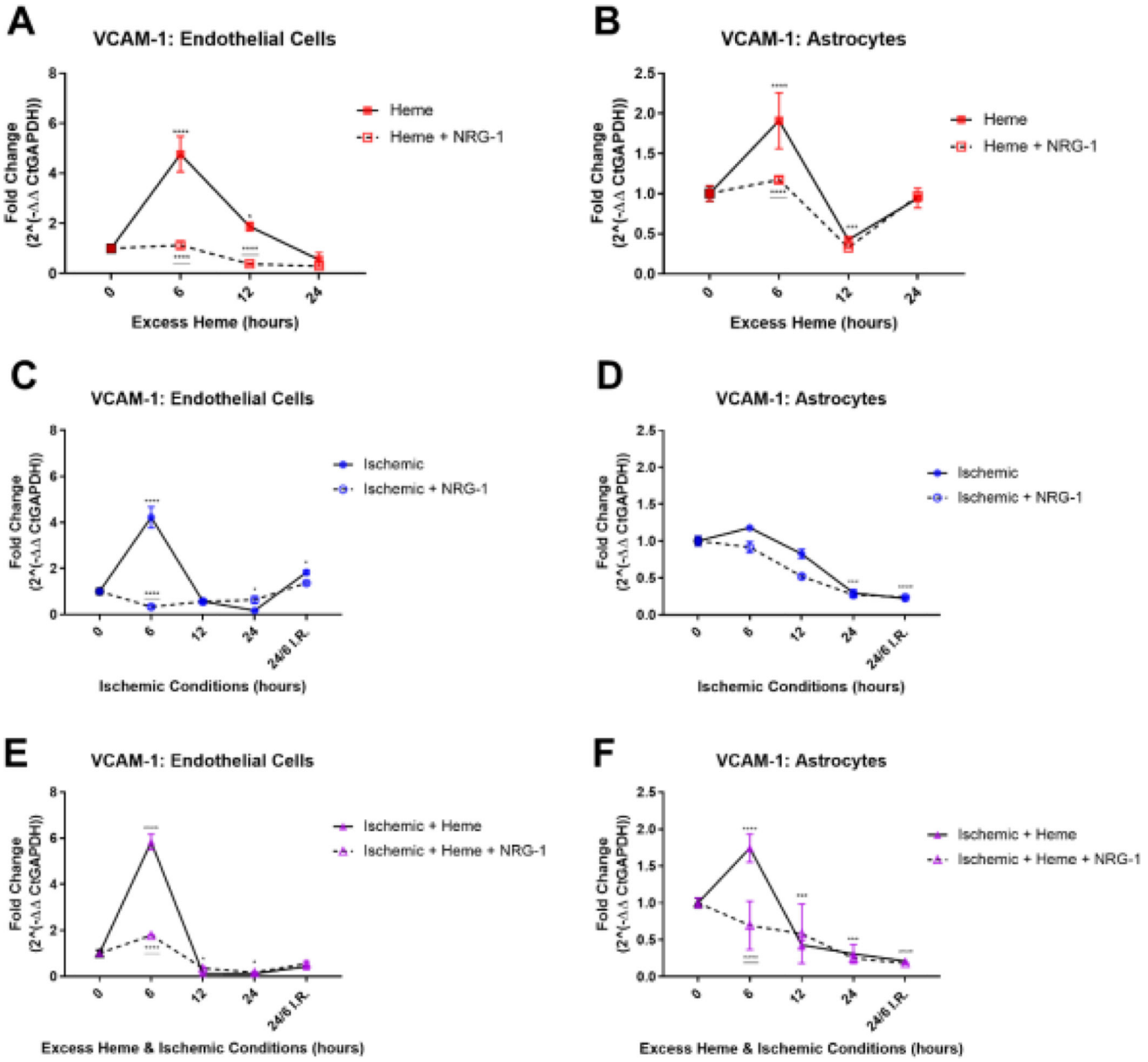

Figure 14. NRG-1 treatment decreased VCAM-1 expression in conditions of excess heme and ischemia

Experimental conditions of excess heme (A, B), ischemia (C, D), and excess heme with ischemia (E, F) resulted in increases in VCAM-1 expression in both HBMVECs and astrocytes which spiked at the 6-hour timepoints. Neuregulin-1 treatment (dotted lines) was able to reduce resultant VCAM-1 expression towards baseline in all conditions. Significant differences in VCAM-1 expression between non-NRG-1 treated groups and 0-hour untreated controls were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Significant differences in VCAM-1 expression between NRG-1 treated and non-NRG-1 treated groups were denoted as: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***),

and $p < 0.0001$ (****). Delta expression is compared to 0-hour untreated controls shown in black. Data were reported as mean ± standard deviation.

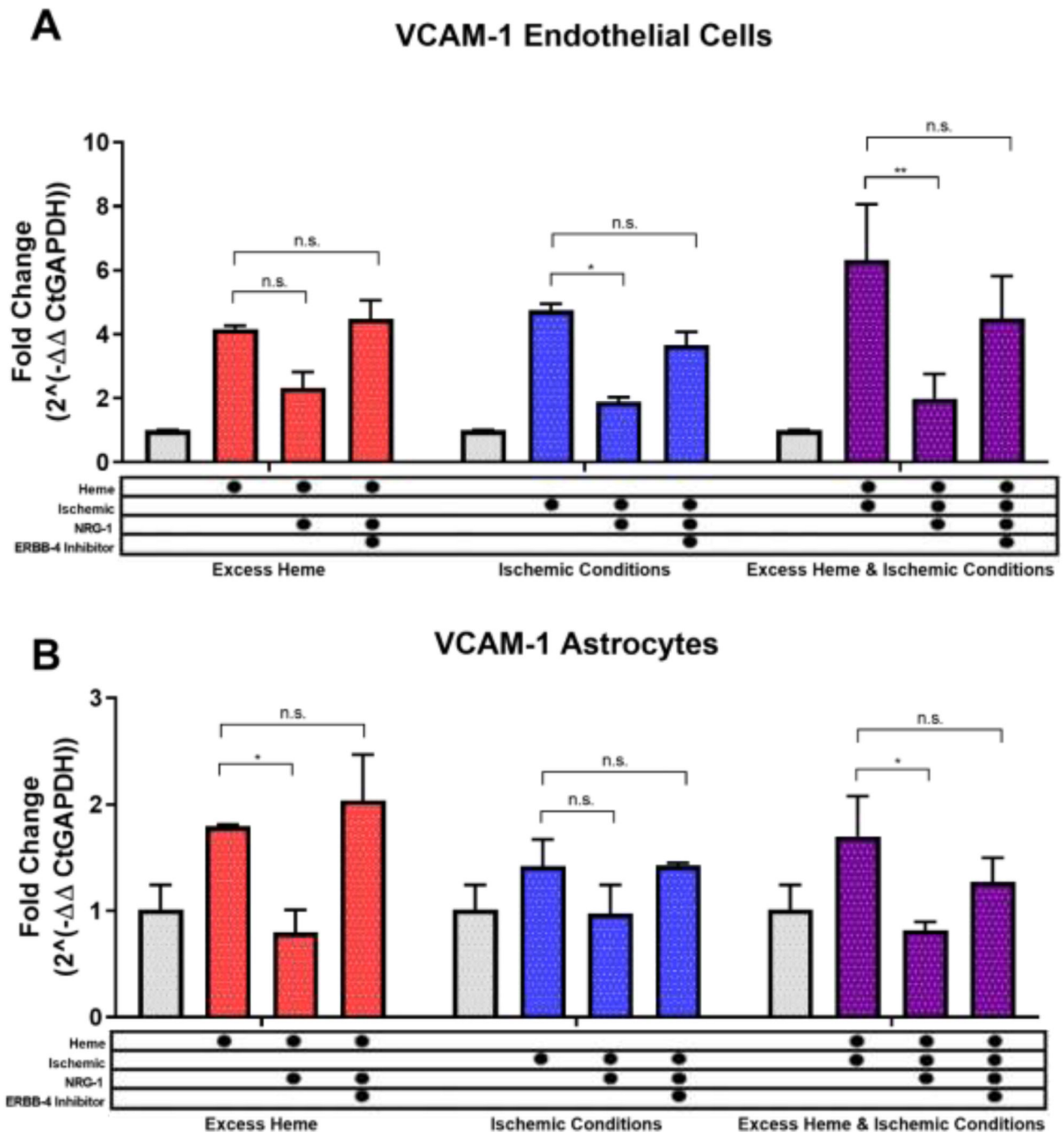

Figure 15. Competitive inhibition of the ERBB-4 receptor limits NRG-1's ability to reduce VCAM-1 expression resulting from experimental conditions.

A subset of HBMVECs and astrocytes were pretreated with either NRG-1 or NRG-1 + anti-ERBB-4 antibody (competitive ERBB-4 inhibition) before being exposed to experimental conditions of excess heme, ischemia, or excess heme combined with ischemia for six hours. As previously demonstrated, NRG-1 pretreatment was able to reduce VCAM-1 expression resulting from exposure to experimental conditions of excess heme, ischemia, and excess heme with ischemia, however under competitive inhibition of the ERBB-4 receptor, NRG-1 showed no ability to significantly reduce resultant increases in ICAM-1 expression. P-values

were denoted as follows: $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Delta expression is compared to untreated controls shown in grey. Data were reported as mean ± standard deviation.

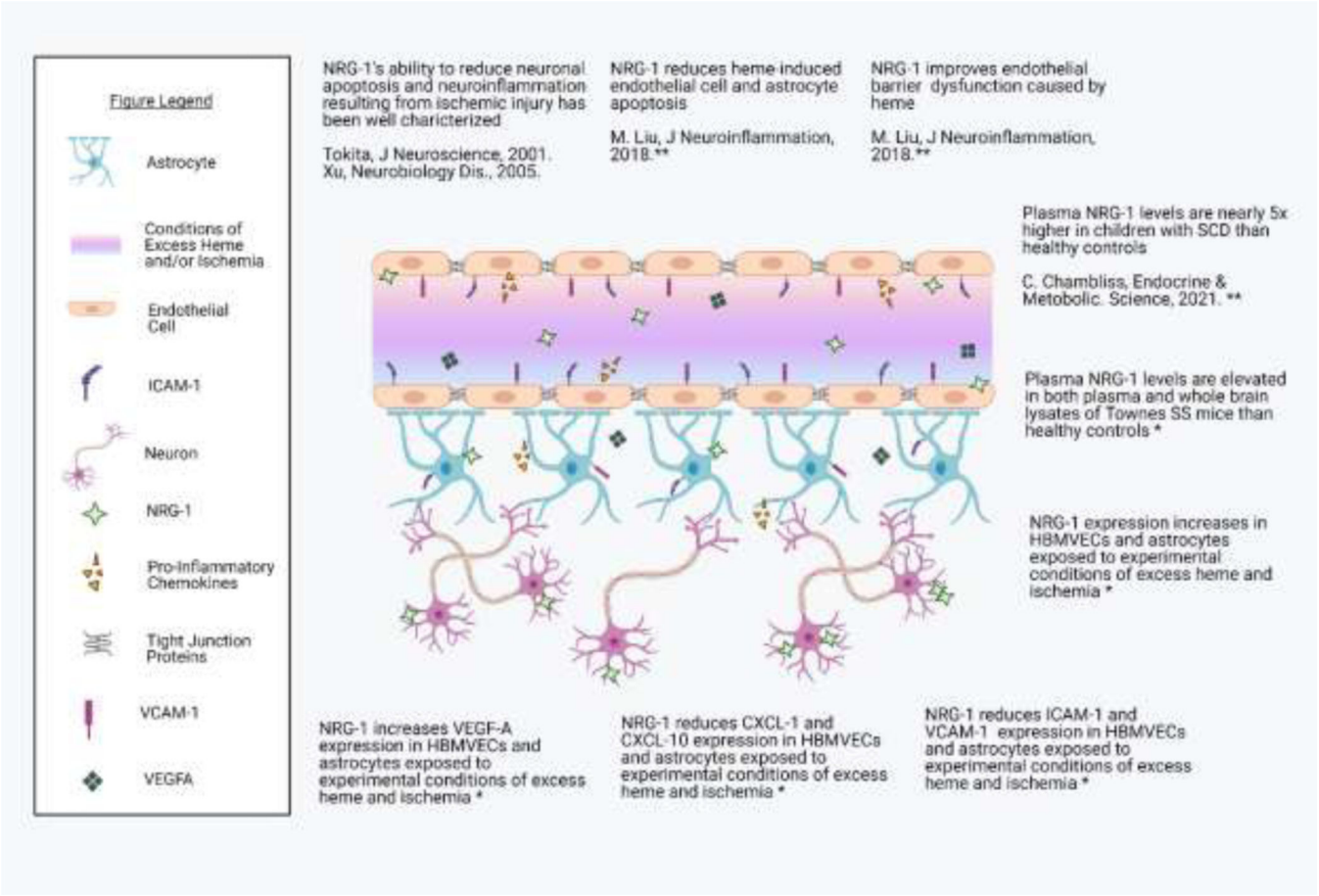

Figure 16. Summary: Identified effects of NRG-1 on cerebral and cerebral vascular dysfunction in conditions of excess heme and ischemia.

Summary of the findings related to NRG-1 and cerebral/cerebrovascular health as contributed by other labs, our labs (**), and this study (*). Neuregulin-1β's ability to aid in recovery from ischemic-induced neuronal injury is well documented [23, 26, 30, 32, 49]. We continue to contribute to expanding the body of knowledge as to NRG-1's ability to combat heme-induced cerebrovascular injury and dysfunction. We plan to characterize NRG-1 in SCD, a largely hemolytic and ischemic disease, while demonstrating NRG-1's ability to reduce cerebrovascular injury in SCD. Conditions of excess heme and ischemia cause disruptions to blood brain barrier integrity and endothelial dysfunction including increased pro-inflammatory chemokine and adhesion molecule production; endothelial, astrocyte, and neuronal apoptosis; and increased NRG-1 production (believed to be a protective response). Further increases in NRG-1 through administration of exogenous NRG-1 have shown to ameliorate cellular responses to injury. Continued investigation of NRG-1's effect on heme- and ischemic- induced cerebral and cerebrovascular injury may prove beneficial to disease models such as SCD.