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N-methyl-D-aspartate receptor Activation, Novel Mechanism of Homocysteine Induced Blood Retinal Barrier Dysfunction

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Abstract

Background: Elevated levels of amino acid homocysteine (Hcy) recognized as hyperhomocysteinemia (HHcy) was reported in several human visual disorders, such as diabetic retinopathy (DR) and age-related macular degeneration (AMD). Breakdown of the blood-retinal barrier (BRB) is concomitant with vision loss in DR and AMD. We previously reported that HHcy alters BRB. Here, we tested the hypothesis that HHcy alters BRB via activation of the N-methyl-D-aspartate receptor (NMDAR).

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

All experiments with animals conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were done following our animal protocol approved by the Institute for Animal Care and Use Committee (IACUC) and Augusta University policies (protocol number AUP 2014-0683).

Consent to participate Not applicable

Availability of data and material Data and material are available in request to corresponding author

Consent for publication not applicable

Methods: Human retinal endothelial cells subjected to high level of Hcy and mouse model of HHcy were used. We injected Hcy intravitreal and used a mouse model of HHcy that lacks cystathionine-β-synthase (cbs). RT-PCR, western blot, and immunofluorescence showed that retinal endothelial cells (RECs) express NMDAR at the gene and protein levels both *in vitro* and *in vivo* and this was increased by HHcy. We assessed BRB function and retinal morphology using fluorescein angiogram (FA) and optical coherence tomography (OCT) under HHcy with and without pharmacological inhibition of NMDAR by (MK801) or in mice lacking endothelial NMDAR ($NMDAR^{E-/-}$ mouse). Additionally, retinal albumin leakage and tight junction proteins ZO-1 and occludin were assessed by western blotting analysis.

Results: Inhibition or elimination of NMDAR was able to improve the altered retinal hyperpermeability and morphology under HHcy as indicated by a significant decrease in retinal albumin leakage and restoration of tight junction proteins ZO-1 and occludin.

Conclusion: Our findings underscore a potential role for endothelial NMDAR in mediating Hcy-induced breakdown of BRB and subsequently as a potential therapeutic target in retinal diseases associated with HHcy such as DR and AMD.

Keywords

N-methyl-D-aspartate receptor; Homocysteine; Blood retinal barrier; cystathionine-β—synthase and mouse

INTRODUCTION

Homocysteine (Hcy), an amino acid which is produced in one-carbon metabolism and metabolized via two enzymatic pathways, re-methylation pathway to form methionine and transsulfuration pathway to cysteine and taurine. The efficacy of Hcy metabolism depends on the availability of vitamin cofactors such as folate, vitamin B12, and vitamin B6 (1). Transsulfuration of Hcy to cysteine, which ultimately produces glutathione, is catalyzed by cystathionine beta-synthase (CBS) enzyme. Elevation of Hcy in plasma or hyperhomocysteinemia (HHcy), is predominant in the human population (5-12%) and several studies have demonstrated an association between HHcy and vascular diseases (2–6). Over the last few years, Hcy has been noticeably reported to be elevated in patients with retinal neurovascular diseases such as diabetic retinopathy (DR) (7–11) and age related macular degeneration (AMD) (12–15). Vasculopathies linked to HHcy include endothelial dysfunction, vessel wall malformations, loss of extracellular matrix collagen, and disruption of the blood-brain barrier (BBB) in rodents and humans (16). There is an association between Hcy and diabetes-induced microangiopathies (diabetic nephropathy, retinopathy, and macular edema) (17–20). Impaired endothelial cell function has been reported in HHcy both *in vitro* and *in vivo* (21). However, the underlying cellular and molecular mechanisms have been not yet clearly defined. Our previous studies on a mouse model of HHcy caused by the deficiency of the CBS ($cbs^{-/-}$ and $cbs^{+/-}$ mice) reported an association between HHcy and retinal vascular dysfunction such as pathological neovascularization, pericyte loss, impaired blood-retinal barrier function, and subsequent retinal hyperpermeability (22–25). Moreover, we did further studies to determine the underlying mechanisms of HHcy-induced

BRB dysfunction. We reported endoplasmic reticulum (ER) stress(23), Oxidative stress(24), epigenetic modification(26) and inflammation(27) as possible mechanisms.

The BRB consists of inner and outer components and an intact BRB is essential for retinal structural and functional integrity as it plays a fundamental role in the maintenance the retinal neuron's microenvironment. It regulates fluids and molecular movement between the ocular vascular and retinal tissues and prevents leakage of macromolecules and other potentially harmful agents into the retina. Vision is adversely affected in clinical conditions associated with BRB breakdown such as DR which is associated with altered inner BRB (28, 29), and AMD which is characterized by alteration in the outer BRB (29–31).

NMDAR, a subtype of ion-gated glutamate channel receptor, consists of 7 subunits (NR1, NR2A-D, NR3A, and B). NMDAR is a receptor for Hcy in neurons (32). However, the expression of NMDAR subunits is not limited to neurons; they are present in astrocytes, osteoblasts and osteoclasts, peripheral neurons (33–35), and endothelial cells (36–39) Activation of NMDAR in cerebral endothelial cells by Hcy results in hyperpermeability and disruption of tight junction proteins (TJs) (16) (40). The NMDAR antagonist memantine prevented blood-brain barrier (BBB) permeability (39, 41) and reversed the attenuating effects of HHcy on TJs in brains of HHcy mice ($cbs^{+/-}$)(16). Thus, NMDAR could affect endothelium by mediating intracellular signal transduction in response to increases in Hcy concentration in the blood. Hcy was reported to induce neurotoxicity mediated via activation of the NMDAR (42), resulting in cytoplasmic calcium influx, activation of the inflammatory pathway, and cellular apoptosis (43–45). Also, Hcy has been reported to interact with NMDAR, initiate oxidative stress, induce apoptosis, trigger mitochondrial dysfunction, and lead to vascular damage(46). The hybridization signals for NR2A and NR2B mRNA were significantly elevated in the dorsal horn of diabetic rats (47). Furthermore, deletion of NMDA-R1 restored cardiac contractility under HHcy(48) and NMDAR pharmacological inhibitor (MK-801) was able to abolish HHcy induced retinal ganglion cell death(42).

The current study was undertaken to investigate whether activation NMDAR in retinal endothelium by elevated Hcy is a possible mechanism of HHcy-induced BRB dysfunction. Accumulating evidence from previous studies and the research data presented in the current study showing activation of endothelial NMDAR in HHcy *in vitro* and *in vivo* models and thus NMDAR is proposed as a therapeutic target for prevention/treatment of ocular diseases associated with HHcy such as DR and AMD.

MATERIALS AND METHODS

Human retinal endothelial cells (HREC)

Human retinal endothelial cells (HRECs) and its complete medium (including supplements, growth and attaching factor) from Cell Systems (Kirkland, WA) was used as an endothelial model system to evaluate the expression of NMDAR and the effect of treatments with different levels of Hcy on NMDAR expression in HRECs and subsequent alteration of its barrier function. The purity of HRECs was confirmed per our previously published research (24) by Immunofluorescence staining using an endothelial cell marker CD31 and pericyte cell marker alpha-smooth muscle actin. HRECs were confirmed for being positive for CD31

but negative for alpha-smooth muscle actin (supplementary figure). Endothelial complete medium containing hydrocortisone was used for growing cells. HRECs were used between 5–7 passages and about 80–90% confluence, the cells were shifted to serum starved-medium (1% serum) overnight before treatment with or without Hcy thiolactone (20, 50, or 100 μM) followed by studies to evaluate the expression of NMDAR and REC barrier function.

Animals

All experiments with animals conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were done in agreement with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Augusta University guidelines. The generation of (*cbs*) mice deficient in cystathionine beta-synthase has been described by Watanabe et al (49). To establish colonies of $cbs^{+/+}$, $cbs^{+/-}$, and $cbs^{-/-}$ mice, breeding pairs of $cbs^{+/-}$ mice (B6.129P2-Cbs^{tm1Un}/J; Jackson Laboratories, Bar Harbor, ME) were used All. NMDAR endothelial conditional knockout mice ($NMDAR^{E-/-}$) were generated in our lab, mice heterozygous or homozygous for mutation of GluN1 subunit of the N-methyl-D-aspartate receptor (B6.129S4-Grin1tm2Stl/J) and age-matched wild type controls (C57BL/6J) are commercially available and were obtained from the Jackson Laboratories (Also Known As: NR1flox, fNR1).These floxed NR1 mice allow deletion of the GluN1 subunit of the N-methyl-D-aspartate receptor in Cre recombinase expressing cells/tissues. These floxed NR1 (Grin1flox) mice possess loxP sites - flanking approximately 12 kbp of sequence of the targeted gene that encodes the entire transmembrane domain and C-terminal region that are homozygous for this allele- are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities (50, 51). Mice were backcrossed with Tie-2 CRE mice (B6.Cg-Tg (Tek-cre) 1Ywa/J to generate endothelial cell conditional knockout mice, useful in studying the NMDAR1 and its downstream signaling molecules/pathways and its involvement in HHcy-induced BRB dysfunction. Animal breading was done in animal facility and animals were maintained in clear plastic cages, subjected to standard 12-hour light/12-hour dark light cycles in the animal room at regulated temperature (22 to 24°C), and allowed to eat and drink *ad libitum.* Light levels at the bottom of cages were controlled at 1.5-foot candles (16.1 lux) to avoid the possibility of light damage to the retina. Mice were genotyped according to the Jackson animal laboratory's protocol. A total of 60 mice were used in this study at ages ranging from 3-24 weeks.

For intravitreal injection, the procedure of injection and the dose of Hcy-thiolactone injection (200 μM) was selected according to our previously published research (25, 26, 52). Briefly, 1 μL L-Homocysteine thiolactone hydrochloride (Sigma-Aldrich, Louis, MO) was used for intravitreal injection to prevent uncontrolled increase of the intraocular pressure (vitreous volume of mouse eye is ~10 μL)(53). L-Homocysteine thiolactone hydrochloride was dissolved in water and a working solution of 10x was acquired by diluting 1 μL of stock solution (200 mM) to 100 μL with PBS, this was followed by intravitreal injection of 1 μL of this working solution to reach a vitreal concentration of 200 μM of Hcy-thiolactone.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-q PCR).

Total RNA was extracted using TRIzolTM Reagent (Invitrogen, Eugene, Oregon, USA). After quantification, 2 μg of RNA was reverse transcribed using iScript™ Synthesis kit (BioRad Laboratories, Hercules, CA). The cDNA was amplified using Absolute QPCR SYBR Green Fluorescein Mix (Thermo Scientific, Surrey, UK), the BioRadiCycler (BioRad, Hercules, CA) and gene specific primers. The primers used were obtained from (Integrated DNA Technologies, IDT). The primers sequences were as following (NMDAR1 human F1: 5′ AAG CTG AGG GTG TGA AAC GG-3′, NMDAR1 human R1: 5′ GAG AGC CTG GAA ACT GGA CC-3′, NMDAR1 human F2: 5′ CCA CAT TTA GGG CTA TCA CCT CC-3′, NMDAR1 human R2: 5′ TCT CCC CCT TAC CGT GTC TTT-3′, NMDAR 1 mouse F: 5′ AAA CCT CGA CCA ACT GTC CT-3′, NMDAR 1 mouse R: 5′ AGC AGA GCC GTC ACA TTC TT-3′). Amplification parameters were; 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Melt curve analysis was used to confirm the purity of the end products. For normalization 18S and GAPDH were used and to obtain fold changes in gene expression, comparative CT method was used.

Protein extraction and Western blot analysis

Washed cultured cells and retinal tissue samples were lysed in modified RIPA buffer supplemented with 1:100 (v/v) of proteinase/phosphatase inhibitor cocktail (Thermo Scientific, Greenville, SC, USA). This was followed by centrifugation at 12,000 xg at 4°C for 30 min to remove insoluble material. Protein was determined by BCA Protein Assay (Thermo Scientific, Greenville, SC, USA) and equal amount of protein was exposed to boiling in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%, Pierce, Rockford, IL), then transferred to nitrocellulose membrane, followed by incubation with specific antibodies. Antibodies for NMDAR1 (Cell signaling, Danvers, MA, USA, Cat# 5704S), NMDAR2A (Cell signaling, Danvers, MA, USA, Cat# 4205s), NMDAR2B (Cell signaling, Danvers, MA, USA, Cat# 4207s), ZO-1 (Abcam, Cambridge, MA, USA, ab59720), occludin (Invitrogen, Waltham, MA, USA, 71-1500), Albumin (Bethyl, TX, USA), GAPDH, (Sigma-Aldrich, St. Louis, MO, USA) and β actin (Cell signaling, Danvers, MA, USA, Cat# 937215) were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (Thermo Scientific, Greenville, SC, USA). Intensity of immunoreactivity was measured by densitometry using Image J software (NIH).

Immunofluorescent assessment of NMDAR expression

NMDR expression was evaluated by Immunofluorescent (IF) staining of HRECs and retinal frozen sections (from two models of HHcy; wild mice injected intravitreal with Hcy, *cbs*$^{+/-}$, and *cbs*$^{-/-}$ mice compared to normal wild type mice). HRECs plated at 1 χ 10^5 in 8-well chamber slides (Sigma-Aldrich Chemical Corp., St. Louis, MO, USA) were treated with or without Hcy thiolactone (20, 50, or 100μM) for 24 hours. Then, cells were fixed with formalin 4% for 10 min, washed with PBS, and blocked for 1 hour with Power Block (BioGenex, Fremont, CA, Cat# BS-1310–25). This was followed by incubation of HRECs overnight at 4 °C with antibodies for NMDAR1 (Invitrogen, Eugene, Oregon, USA, Cat# 32-0500 diluted 1/200), isolectin B4 (Vector Laboratories, Burlingame, Ca, Cat#: B-1205, 7μl/ml) as a marker for endothelial cells and also marker for blood vessels in retinal sections.

Anti CD31 (Novus Biologicals, NB100–2284) and anti α-smooth muscle actin (Abcam, ab5694). CD31 and α-smooth muscle actin markers were used confirm HRECs identity as we did in our previously published work using HRECs (24). After treatment with primary antibody, cells were washed three times with PBS containing 0.3% Triton-X and incubated with appropriate secondary antibodies (Alexafluor and Texas red avidin, Invitrogen). Fluoroshield containing DAPI (Sigma-Aldrich) was used as a counter stain to cover slides. Images were taken by fluorescent microscopy (Carl Zeiss, Göttingen, Germany).

Fluorescein isothiocyanate (FITC)-dextran permeability assay.

HRECs were grown to full confluence on 0.4 μm pores collagen/fibronectin-coated membranes (Transwell; Corning Costar). Then HRECs barrier function was evaluated using FITC-dextran flux permeability assays per our previously described method(24). Briefly, HRECs were seeded on collagen/fibronectin coated membranes with 0.4 μm pores (Transwell; Corning Costar), in normal media. After forming complete confluent layer, the cells were shifted to serum free media overnight before Hcy treatment (50μM) (Sigma-Aldrich) for 24 hours to the upper chambers in the presence or absence of a NMDAR inhibitor MK801 (25μM) followed by addition of FITC-dextran (10μM) to the upper chambers. Aliquots were collected from the upper and lower chambers at different time points of 1, 3, or 6 hours then placed in a 96-well plate for fluorescence intensity measurement with a plate reader. The rate of diffusive flux (Po) FITC-dextran was calculated by the following formula: $Po= [(FA/\Delta t)VA]/(FLA)$(24). Where Po is in centimeters per second; FA is lower chamber fluorescence; FL is upper chamber fluorescence; Δt is change in time; A is the surface area of the filter (in square centimeters); and VA is the volume of the lower chamber (in cubic centimeters).

Optical coherence tomography (OCT) and Fluorescein Angiography (FA)

To evaluate retinal morphology and BRB integrity *in vivo*, OCT, and FA were performed simultaneously in living mice following our previous publication (25, 52). Mice were put to sleep using 2% isoflurane anesthetic, followed by dilation of their pupils using 1% tropicamide eye drop and Genteal gel was applied liberally to keep the eye moist during imaging. Each mouse was evaluated by OCT and FA after being placed on the platform of the Phoenix Micron III retinal imaging microscope supplemented with an OCT imaging device (Phoenix Research Laboratories, Pleasanton, CA). Mice were injected intraperitoneal with fluorescein sodium 10 to 20μL 10% (Apollo Ophthalmics, Newport Beach, CA), followed by rapid acquisition of fluorescent images ensued for ~5 minutes. Leakage of fluorescein manifests as unclear vascular borders increasing progressively to diffusing hazy fluorescence.

RESULTS

Homocysteine Activates NMDAR in Retinal Endothelium

NMDAR consists of 7 subunits (NR1, NR2A–D, NR3A, and B); however, NR1 subunit is necessary for the formation of functional NMDA receptors (38) and serves as the main subunit in the regulation of BBB (37). First, the expression of NMDAR subunit NR1 in HREC was confirmed at the gene level by RT-qPCR. Human neuroblastoma cells (ATCC

CRL-2266) was used as a positive control (Fig.1A). This was followed by confirming the protein expression of NMDA receptor subunits NR1 by immunofluorescence (IF). IF revealed expression of NMDAR1 (green) in HREs stained (red) with a specific marker for endothelial cells, Isolectin B-4 (Fig.1B). Moreover, the activation of NMDAR1 by Hcy was evaluated by WB analysis of protein extracts of HRECs treated with/without homocysteine thiolactone (50μM) which showed significant increases in NMDAR1 (120 kD) expression by Hcy treatment (Fig.1C).

Assessment of NMDAR1 expression in different models of hyperhomocysteinemia (HHcy)

Three models of HHcy were used to assess activation of NMDAR1 by Hcy, in *vitro* HRECs treated with different concentration of Hcy-thiolactone (20, 50 and 100μM, representing mild, moderate and severe HHcy respectively) and in *vivo* in which wild type, C57BL6 mice received intravitreal injection of Hcy or using mouse model of HHcy ($cbs^{+/-}$ *and* $cbs^{-/-}$ that represent mild/moderate and severe HHcy respectively). Immunofluorescence staining of NMDAR (green) and WB analysis showed a dose dependent increase in NMDAR expression in HRECs treated with Hcy (20, 50 and 100μM of Hcy) (Fig.2A and Fig.2B). Additionally, NMDAR expression was increased in the blood vessels stained with Isolectin-B4 (red), in retinal frozen sections of wild type mice injected intravitreal with Hcy (Fig.2C) compared to wild type-non injected and $cbs^{+/-}$ *and* $cbs^{-/-}$ compared to wild type mice ($cbs^{+/+}$) (Fig.2E). WB analysis of Hcy-injected wild type, $cbs^{+/-}$ *and* $cbs^{-/-}$ mice retina showed significant elevation of NMDAR compared to controls (Fig.2D and Fig.2F).

Inhibition or deletion of NMDAR attenuates the HHcy-induced BRB induced disruption

1- Pharmacological Inhibition of NMDAR(MK801)—After confirming expression of NMDAR in retinal endothelium and its activation by Hcy both *in vivo* and *in vitro.* We wanted to examine whether blocking NMDAR will rescue the retina from HHcy-induced BRB. MK801 is a highly selective NMDAR antagonist and was reported to block hypoxia-induced brain endothelial cells barrier disruption (54).

We performed functional assays and investigated whether MK801 protects BRB against elevated Hcy-induced disruption of barrier function in HRECs monolayer by measuring the FITC-dextran flux through the confluent monolayer. HRECs treated with Hcy thiolactone (50μM) were significantly more permeable to FITC-dextran compared to controls as indicated by increased diffusive flux (Po) for FITC-dextran, while MK801 treatment was able to bring HRECs permeability back to normal level (Fig.3A). Barrier function was further evaluated *in vivo*, by evaluating tight junction proteins TJPs (ZO-1 and occludin) using western blotting analysis. ZO-1 and occludin were evaluated in mice retina after intravitreal injection of Hcy (200μM) with/without intraperitoneal injection of MK801 (IP, 25μM) prior to Hcy injection. Hcy injection significantly reduced ZO-1 and occludin in mice retina, while blocking NMADR by MK801 restored ZO-1 and occludin back to normal levels (Fig.3B, 3C and 3D).

2- Genetic deletion of endothelial of NMDAR ($NMDAR^{E-/-}$) mouse—We generated mouse deficient in the endothelial NMDAR ($NMDAR^{E-/-}$) by backcrossing of B6.129S4-Grin1tm2Stl/J: (otherwise known as: NR1flox, fNR1) with Tie-2 CRE mice

(B6.Cg-Tg (Tek-cre) 1Ywa/J to investigate the involvement of NMDAR in HHcy-induced BRB dysfunction. Knocking down of NMDAR in endothelial cells in $NMDAR^{E-/-}$ was confirmed by genotyping using PCR analysis (Fig.4A). Reduction of NMDAR level in endothelial cells was confirmed by western blotting analysis of NMDAR in blood vessels isolated from brains of $NMDAR^{E-/-}$ mice. Because of the defaults to isolate mice retinal endothelial cells or retinal vasculature for WB analysis and attributed to the fact that retina is a part of the brain and knocking down of NMDAR is global in endothelial cells in $NMDAR^{E-/-}$ mice, we isolated cerebral blood vessels, which were subjected to WB analysis for evaluation of NMDAR expression, which was significantly reduced in $NMDAR^{E-/-}$ compared to wild type control mice (Fig.4B). Furthermore, NMDAR knocking out in endothelial cells was confirmed in the vasculature of the $NMDAR^{E-/-}$ mouse by imunofluorescence staining for NMDAR (green) which was colocalized to vasculature, stained with isolectin B-4 (red) in retinal frozen sections, showing marked reduction of NMDAR (yellow arrows) in retinal blood vessels of $NMDAR^{E-/-}$ compared to wild type mice. Meanwhile, there was normal expression of NMDAR in ganglion cells (white arrows) which confirm the specific deletion of NMDAR in endothelial cells (Fig.4C)

Effect of pharmacological and genetic intervention of NMDAR on retinal morphology and vascular function

To test whether blocking NMDAR, through pharmacological (MK801) or genetic ($NMDAR^{E-/-}$ mouse) approach is able to rescue HHcy-induced disruption of BRB, we performed a functional study in which 4 groups of mice were used at age ~6-8 weeks old (WT, WT-Hcy intravitreal injected, WT-Hcy intravitreal injected+MK801(IP, injected) and $NMDAR^{E-/-}$+ Hcy intravitreal injected). Mice were subjected to OCT and FA examination 72 hours after Hcy injection, followed by perfusion with PBS solution and retinas collection for WB analysis as previously described in our publications(25, 26, 52) to assess albumin leakage and NMDAR expression (Fig.5.). **A)** FA, is showing normal well-formed vessels in WT mouse; however, angiograms for Hcy injected mouse retinas exhibit sever vascular leakage appears as diffused hyperfluorescence (white arrows), which was reduced to normal level after blocking of NMDAR with MK801or knocking it out in endothelial cells ($NMDAR^{E-/-}$). **B)** OCT examination is showing normal appearance of retina in the WT mice, while, marked disruption of retinal morphology with focal hyper reflective spots, hyporeflective subretinal lucency and choroidal neovascularization (yellow arrows) in Hcy-injected mice, Blocking NMDAR was able to reduce the retinal disruption and choroidal neovascularization after Hcy injection in both MK801-injected WT mice and $NMDAR^{E-/-}$ mice (yellow arrows). **C)** Vascular leakage was confirmed by measuring the albumin leakage in the retinas by western blotting, which was significantly increased in the Hcy-injected mice and was reduced back to normal level after blocking of NMDAR in both MK801-injected WT mice and $NMDAR^{E-/-}$ mice. **D)** Western blotting assessment of NMDAR expression mice retina showed significant increase in NMDAR by Hcy injected mice, which was significantly reduced after blocking of NMDAR in both MK801-injected WT mice and $NMDAR^{E-/-}$ mice.

In summary, our presented data is proposing activation endothelial NMDAR as a target mechanism for HHcy-induced BRB dysfunction and neovascularization. Our data showed

the expression of NMDAR in retinal endothelial cells at gene and protein levels. Hcy was able to activate endothelial NMDAR in both *in vivo* and *in vitro* models of HHcy. Furthermore, inhibition of NMDAR (pharmacological or genetic) was able to rescue HHcy-induced BRB disruption. Therefore, NMDAR inhibition could be a potential therapeutic target for retinal diseases associated with HHcy such as DR and AMD.

Discussion

The current study presents evidence that suggests activation of NMDAR as a possible mechanism of HHcy-induced BRB dysfunction, the presented data showed 1) Activation of NMDAR by HHcy *in vitro* and *in vivo*, 2) We tested blocking of NMDAR by pharmacological inhibition using MK801 and molecular inhibition using endothelial NMDAR conditional knockout mouse that was created in our lab ($NMDAR^{E-/-}$), 3) Inhibition of NMDAR attenuates HHcy-induced retinal hyperpermeability *in vitro* and *in vivo*.

Hyperhomocysteinemia is an imbalance of methionine/Hcy metabolism that ultimately causes excessive Hcy build-up in the blood. HHcy-induced disruption of BRB concomitant with distorted retinal homeostasis has been reported in various ocular diseases including retinal vein occlusion(55) and DR(56). Furthermore, our research over the last few years studying the effect of HHcy on retinal vasculature and BRB reported the devastating effect of HHcy on retinal vasculature(22, 23) such as increased angiogenic potential and disruption of both inner and outer BRB(23–25). BRB dysfunction plays a major role in the development and progression of common retinal diseases associated with vision loss worldwide such as DR and AMD. Our research is aiming to highlight the importance of HHcy in retinal diseases, especially in aging and diabetic population. We are seeking to find the underlying mechanisms of HHcy-induced retinal changes and also we are endorsing the former recommendations for screening or management of hyperhomocysteinemia. If elevated Hcy levels are discovered, vitamin deficiency should be ruled out to allow specific treatment and prevention of complications. Furthermore, we encourage adherence to the previously suggested daily allowance of dietary sources of folate and vitamins B_{12} as a preventive tool against HHcy-induced complications (57).

We proposed different mechanisms of HHcy induced BRB dysfunction, including ER stress(23), oxidative stress(24), epigenetic modification(26), and inflammation(27). However, the precise receptor mechanism for such deleterious effects is still a missing link. Hcy was reported to induce oxidative/nitorosative stress, inflammation, and apoptosis in cerebral as well as peripheral vascular endothelial cells via NMDAR activation(58). Additionally, NMDAR activation was reported to disturb intracellular Ca^{2+} homeostasis triggering neuronal apoptosis by enhancing ER stress(59). The key findings of the current study are proposing activation of endothelial NMDAR as a mediator of HHcy-induced BRB dysfunction. Therefore, therapeutic interventions that target Hcy-NMDAR interaction could be of significant visual benefits. Especially some of the pharmacological NMDAR blockers are FDA approved and have been already used in the treatment of Alzheimer's and Parkinson's diseases (60)'(61, 62).

Various pharmacological studies demonstrated that Hcy is an agonist operating via NMDA receptor and that Hcy-induced neuronal toxicity is NMDAR dependent. In this context, it was reported that Hcy-induced blood brain barrier disruption, excitotoxicity and synaptic dysfunction is accompanied by increased expression of NMDA-R (NR1) in mice model of Alzheimer's disease(63) and that Hcy treatment triggered neuronal cell death via NMDAR activation. Indeed, Hcy treatment stimulated neuronal NMDAR with subsequent phosphorylation and nuclear translocation of ERK2 leading ultimately to neuronal cell death. This effect was abolished by MK801(64).Moreover, Deep et al, demonstrated that pharmacological inhibition of GluN2A subunit of NMDR using a selective inhibitor NVP-AAM077 attenuated Hcy-induced neurotoxicity via Ca^{2+}-dependent ERK MAPK signaling pathway(65). Similarly, Rajagopal et al reported that NVP-AAM077 attenuated Hcy-induced pro-inflammatory response in neurons and Jinda et al found that it markedly reduced ischemic brain damage in hyperhomocysteinemic rats(66). Of note, the GluN2A subunit of NMDARs represents the preferential target that mediates Hcy-induced toxicity in cortical neurons(67).

Accumulating evidence from our and other previous studies reported that Hcy-induced retinal ganglion cell death via activation of NMDA receptor (42) and that Hcy-stimulated NMDAR in retinal ganglion cells was simultaneous with apoptotic neuronal death (68). In addition to nervous tissue, Hcy seems to activate NMDAR in the cardiovascular system. Indeed, Hcy was reported to up-regulate NMDAR expression in rat vascular smooth muscle cells with the promotion of a pro-inflammatory response (69) and increase the expression of NMDAR in brain endothelial cells along with mitochondrial toxicity and endothelial dysfunction (63). Respectively, our results indicated a similar effect of Hcy on HRECs, whereas Hcy treatment increased expression of NMDA in HRECs at gene and protein levels both in vivo and in vitro, suggesting the implication of NMDA activation in Hcy-related retinal vascular injury.

Endothelial cells constitute the inner BRB where they selectively permit the passage of oxygen and nutrients while restricting the entrance of other molecules and thus maintaining retinal homeostasis. Therefore, disrupting endothelial cells leads to BRB dysfunction and retinal hyperpermeability, exposing retinal neurons to potential bloodborne toxicity(70). This selective permeability is conferred by well-developed tight junctions that consist of more than 40 proteins. Tight junction proteins not only orchestrate paracellular diffusion across the BRB but also regulate cellular signaling and proliferation. Therefore, loss of barrier proteins can lead to a plethora of retinal pathological events (71). Among tight junction proteins, we previously reported that downregulation of ZO-1, occludin, and claudin-5 are linked to HHcy-induced BRB disruption (22, 23). Accordingly, to further prove the involvement of NMDR in HHcy-induced BRB dysfunction, we used two approaches of NMDR inhibition; in the first approach we treated a monolayer of HRECs with Hcy with or without MK801, a known pharmacological inhibitor of NMDAR followed by an assessment of FITC-dextran leakage as an indication of HRECs integrity and intact barrier function (*in-vitro*). Besides, the effect of co-administration of MK801 with Hcy (*in-vivo*) on protein expression of ZO-1 and occludin was evaluated. In the second approach, we tested the impact of pharmacological (MK801) or genetic intervention of NMDAR (deletion of endothelial NMDAR, $NMDAR^{E-/-}$ mice) on retinal permeability after intravitreal

injection of Hcy in mice eye followed by an assessment of retinal barrier function *in vivo* using FA, OCT and albumin measurement by WB. Consistent data were obtained from both approaches, whereas pharmacological and genetic inhibition of NMDAR resulted in the preservation of BRB from HHcy-induced dysfunction. This was evident by suppressed FITC-dextran leakage *in vitro* and albumin hyperpermeability *in vivo.* Moreover, results of FA and OCT indicated preserved vascular integrity and BRB (both inner and outer BRB, as indicated reduction of vascular leakage, preserved TJPs and ameliorated retinal disruption and choroidal neovascularization) in both approaches compared to mice injected intravitreal with Hcy only. Noteworthy, inhibition of NMDAR by MK801 has been reported to attenuate Hcy-induced vascular dysfunction (72) and to mitigate Hcy-induced blood-brain barrier disruption and synapse dysfunction (63). Concurrently, knocking down of NMDA-R1 diminished Hcy-induced mitochondrial toxicity in murine brain endothelial cells (63).

Collectively, the findings of the present study provide new mechanistic insight into Hcy-induced retinal endothelial cell dysfunction in which activation of the NMDAR signaling pathway may play an important role. Thus, NMDAR blockade is a potential novel therapeutic approach to prevent retinal vascular dysfunction in retinal diseases associated with HHcy such as DR and AMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

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- Elevated levels of homocysteine (Hcy) is defined as hyperhomocysteinemia (HHcy).
- HHcy is implicated in diabetic retinopathy and age related macular degeneration.
- HHcy alters BRB via activation of N-methyl-D-aspartate receptor.

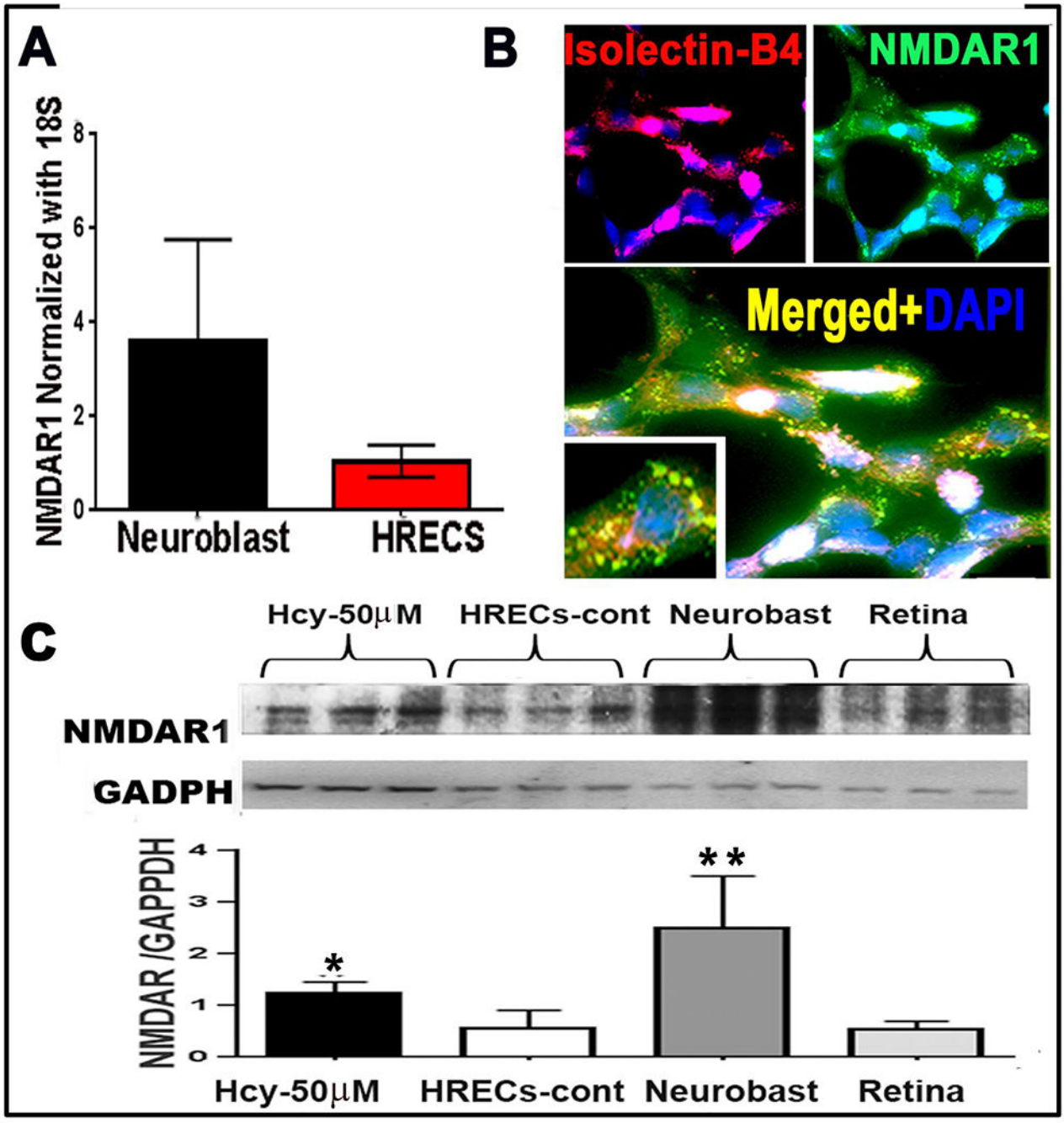

Fig. 1. NMDAR expression in retinal endothelial Cells. RT-PCR.

A) RT-qPCR analysis showing the expression of NMDAR subunit NR1 in HREC cell line compared to human neuroblastoma cells (ATCC CRL-2266) as a positive control. **B)** Immunofluorescence expression of NMDAR1 (green) in HRECS stained with specific marker for endothelial cells, Isolectin B-4 (red), merged image (yellow). Calibration bar is 50 μm. **C)** Western blotting (WB) analysis showing expression of NMDA receptor subunits NR1 (120 kD) in HRECs which was significantly increased after Hcy treatment (50μM). Human neuroblastoma cells was used as a positive control. *$p<0.05$.

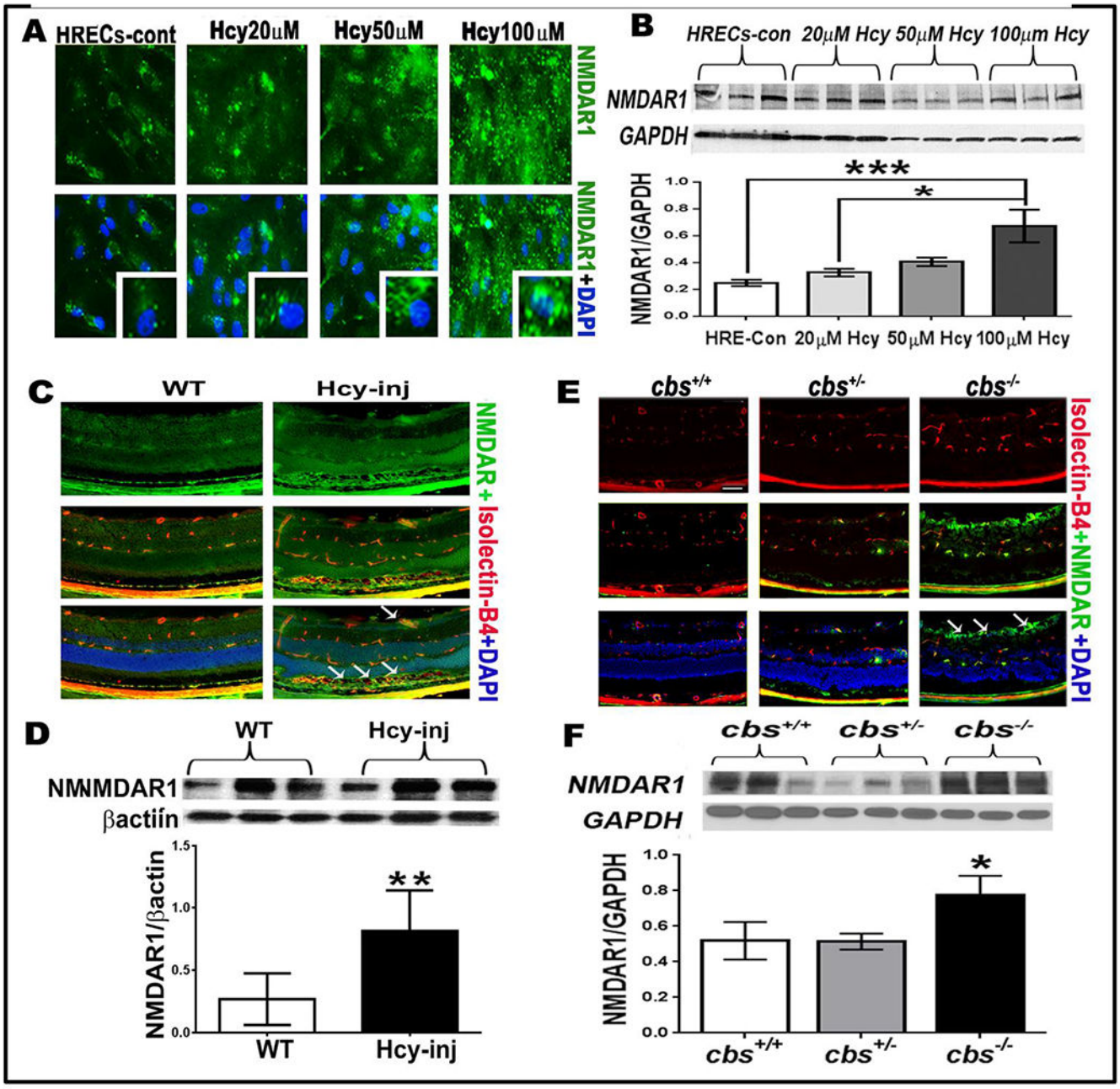

Fig. 2. Evaluation of endothelial NMDAR expression in both *in vivo* and *in vitro* models of HHcy.

A) Immunofluorescence expression of NMDAR (green) in HRECS treated with different concentrations of Hcy (20, 50 and 100μM Hcy-thiolactone). Calibration bar is 50 μm. **B)** Western blot analysis of NMDAR1 in HRECS treated with different concentrations of Hcy (20, 50 and 100μM Hcy-thiolactone), GAPDH was used as the loading control. **C).** Immunofluorescence expression of NMDAR (green) in retinal sections of wild type mice injected intravitreal with Hcy in retinal blood vessels stained with Isolectin-B4 (red), showing higher expression in the areas of CNV induced by Hcy-injection (arrows). **D)** Western blot analysis of NMDAR1 in retinas of wild type mice injected intravitreal with

Hcy. β–actin was used as a loading control. **E)** Immunofluorescence expression of NMDAR (green) in retinal blood vessels stained with Isolectin-B4 (red) of retinal frozen sections from $cbs^{+/+}$, $cbs^{+/-}$ *and* $cbs^{-/-}$ mice showing increased expression in blood vessels and ganglion cells, arrows). **F)** Western blot analysis of NMDAR1 in retinas of $cbs^{+/+}$, $cbs^{+/-}$ *and* $cbs^{-/-}$ mice. , GAPDH was used as the loading control. Calibration bar; 50 μm and *$p<0.05$, **$p<0.01$ and ** *$p<0.001$.

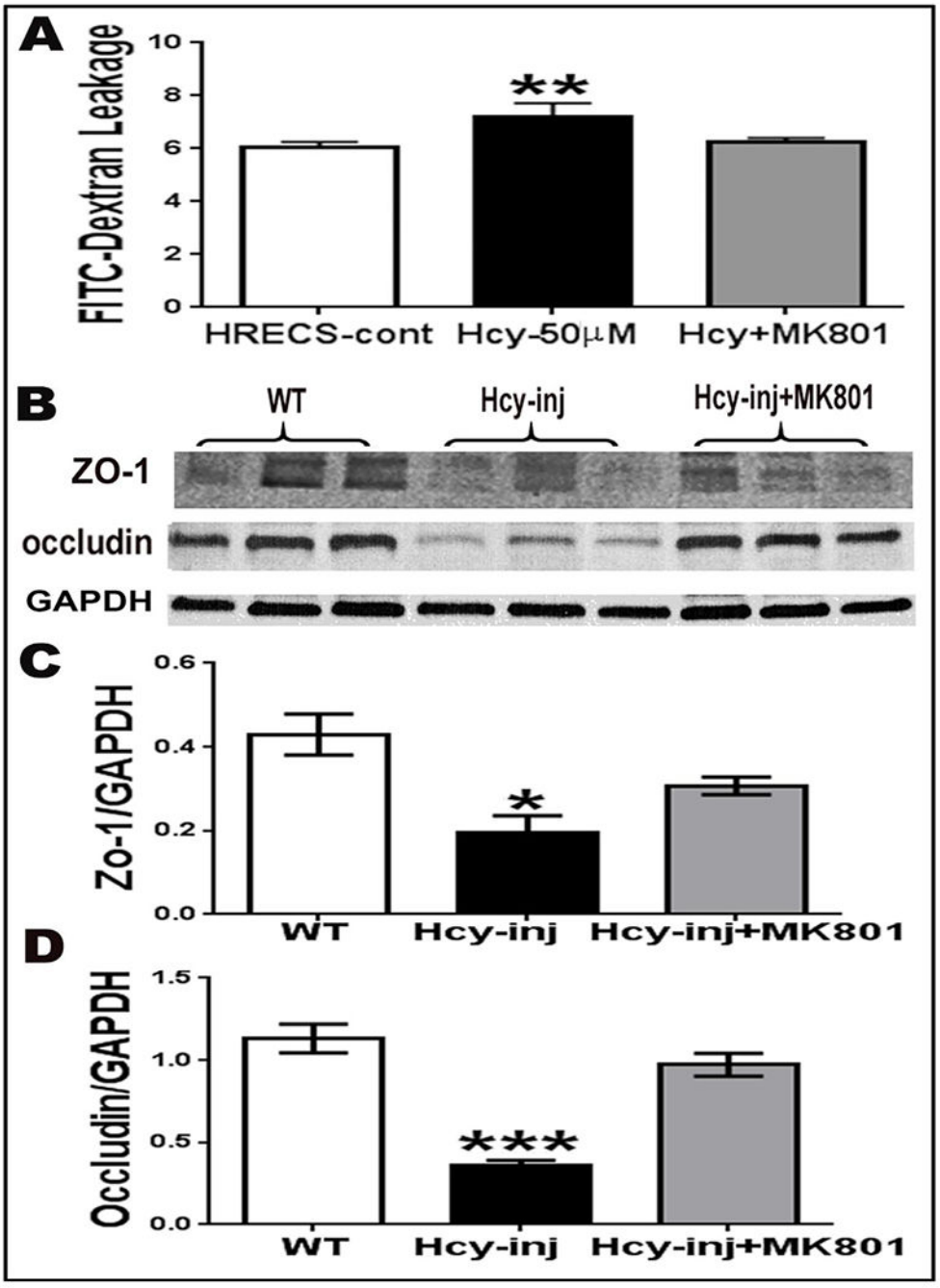

Fig. 3. Effect of NMDAR pharmacological inhibition (MK801) on FITC dextran flux assessment of HRECS and TJPr expression in Hcy-injected wild type mice.

A) FITC dextran flux through the confluent monolayer, which revealed significant increase in FITC dextran leakage in the HREC cells treated with Hcy-thiolactone (50μM), which was significantly reduced by blocking the NMDAR using MK801. *p<0.05 and **p<0.01. **B)** Western blot analysis of ZO-1 (198 kD) and occludin (59 kD) in retina of wild type mice injected intravitreal with Hcy. GAPDH was used as the loading control. **C)** Quantification of densitometric scans of protein bands showing significant decrease in ZO-1 and occludin

expression in which was restored by blocking the NMDAR using MK801 (IP. injection before Hcy intravitreal injection). ***$p<0.001$.

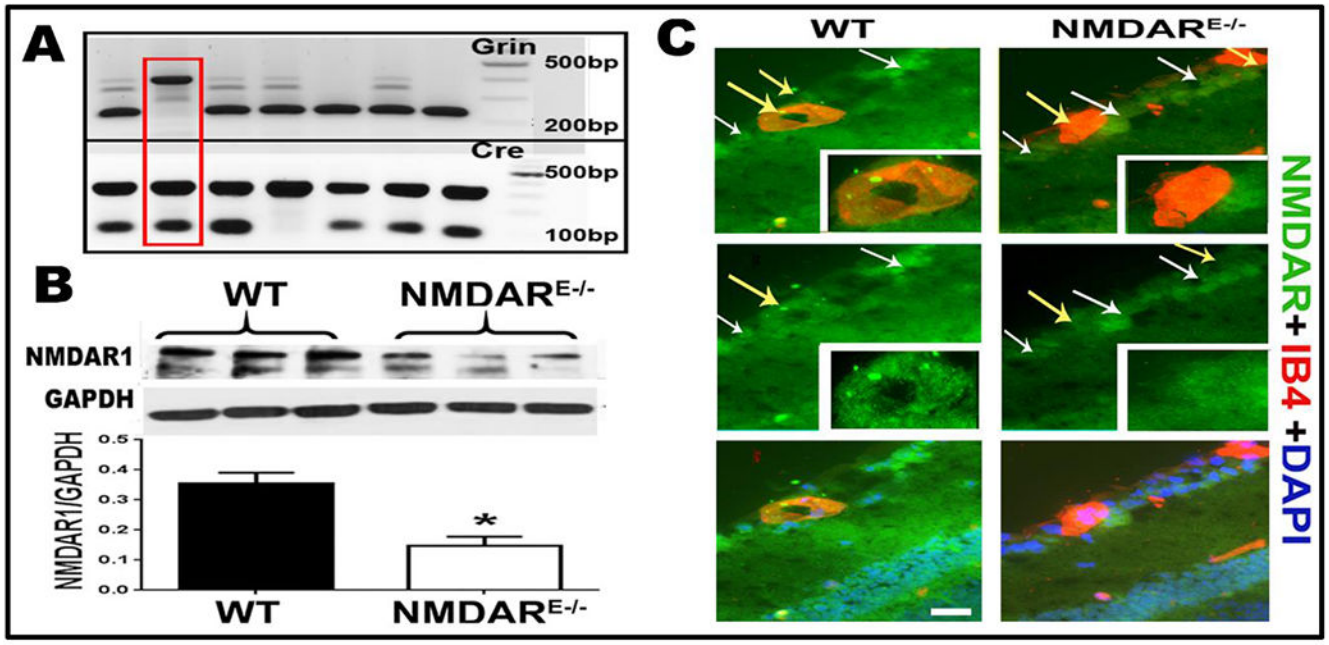

Fig. 4. Mouse with genetic deletion of NMDAR (*NMDAR*$^{E-/-}$).

Mice deficient in NMDAR in the endothelial cells (*NMDAR*$^{E-/-}$) was generated in our lab by backcrossing of B6.129S4-Grin1tm2Stl/J: (otherwise known as: NR1flox, fNR1) with Tie-2 CRE mice (B6.Cg-Tg (Tek-cre). **A)** PCR, genotyping analysis. Grin genotyping results. (*Grin*$^{+/+}$) has only one band ~ 400bp. (*Grin*$^{+/-}$) has two bands ~ 400bp and 232bp. (*Grin*$^{-/-}$), wildtype has a band at ~232bp. Cre genotyping results. Cre+ has band at 100bp plus internal positive control at 400bp, while Cre- has only the 400bp band. The red labeled (*NMDAR*$^{E-/-}$) = *Grin*$^{+/+}$*Cre*$^{+}$. **B)** Western blot analysis to confirm reduced expression of NMDAR in endothelial cells by assessing NMDAR level in blood brain vessels of *NMDARE*$^{-/-}$ which showed marked reduction in comparison to normal WT mice. GAPDH was used as a loading control. *p<0.05 **C)** Immunofluorescence expression of NMDAR (green) in retinal frozen sections of *NMDAR*$^{E-/-}$ which showed marked reduction of NMDAR (arrows), which was colocalized with retinal vasculature indicated by isolectin-B4 (red), specific marker for endothelial cells. Calibration bar; 50 μm.

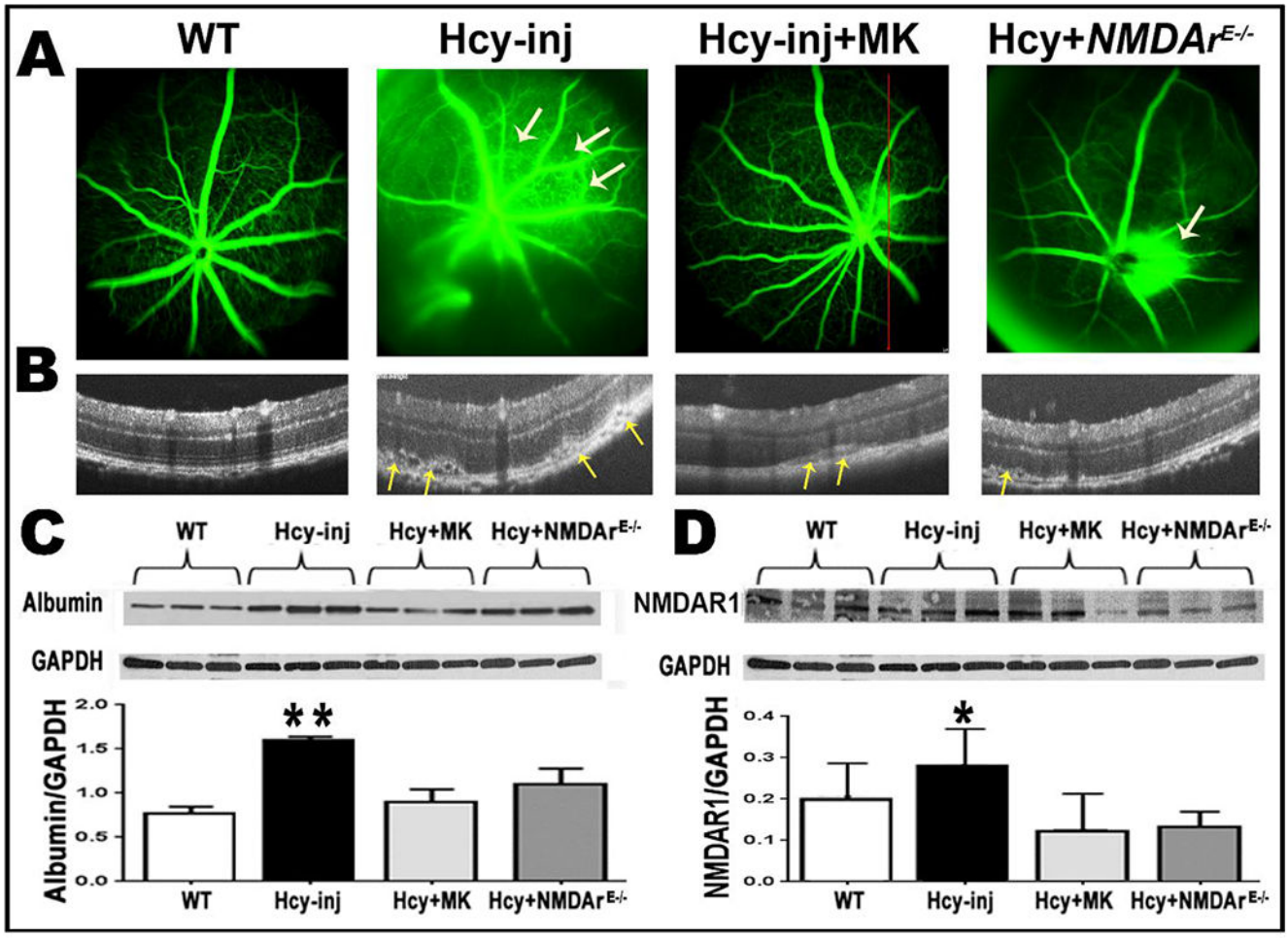

Fig. 5. Retinal Fluorescein angiogram (FA), Optical Coherence Tomography (OCT) and Western Blotting assessment of Albumin leakage and NMDAR expression.

C57BL6 mice with/without Hcy-thiolactone intravitreal injection in the presence/absence of pharmacological (MK801) or genetic inhibition of NMDAR (*NMDAR*$^{E-/-}$) were evaluated by. **A)** FA evaluation showing normal with well-formed vessels in WT mouse; however, angiograms for Hcy injected mouse retinas exhibit sever vascular leakage appears as diffused hyperfluorescence (white arrows), which was reduced to normal level after blocking of NMDAR with MK801 or knocking down endothelial NMDAR in *NMDAR*$^{E-/-}$ mouse. **B**) OCT examination showing normal appearance of retina in the WT mice, marked disruption of retinal morphology with focal hyper reflective spots, hyporeflective subretinal lucency and choroidal neovascularization (yellow arrows). Blocking NMDAR was able to reduce the retinal disruption and choroidal neovascularization after Hcy injection in both MK801 injected and *NMDAR*$^{E-/-}$ mice (yellow arrows), (n = 5 mice). **C)** Vascular leakage was confirmed by measuring the albumin leakage in the retinas by western blotting, which was significantly increased in the Hcy-injected mice eye, which was reduced to normal level after blocking of NMDAR in both MK801 injected and *NMDAR*$^{E-/-}$ mice **p<0.01. **D)** NMDAR expression was assessed by western blotting, showing significant increase in

NMDAR by Hcy injection, which was confirmed to be reduced after blocking of NMDAR in both MK801 injected and *NMDAR*$^{E-/-}$ mice *p<0.05.