

Coactivation of NMDA receptors by glutamate and D-serine induces dilation of isolated middle cerebral arteries

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N-methyl-D-aspartate (NMDA) receptors are glutamate-gated cation channels that mediate excitatory neurotransmission in the central nervous system. In addition to glutamate, NMDA receptors are also activated by coagonist binding of the gliotransmitter, D-serine. Neuronal NMDA receptors mediate activity-dependent blood flow regulation in the brain. Our objective was to determine whether NMDA receptors expressed by brain endothelial cells can induce vasodilation of isolated brain arteries. Adult mouse middle cerebral arteries (MCAs) were isolated, pressurized, and precontracted with norepinephrine. N-methyl-D-aspartate receptor agonists, glutamate and NMDA, significantly dilated MCAs in a concentration-dependent manner in the presence of D-serine but not alone. Dilation was significantly inhibited by NMDA receptor antagonists, D-2-amino-5-phosphopentanoate and 5,7-dichlorokynurenic acid, indicating a response dependent on NMDA receptor glutamate and D-serine binding sites, respectively. Vasodilation was inhibited by denuding the endothelium and by selective inhibition or genetic knockout of endothelial nitric oxide synthase (eNOS). We also found evidence for expression of the pan-NMDA receptor subunit, NR1, in mouse primary brain endothelial cells, and for the NMDA receptor subunit NR2C in cortical arteries *in situ*. Overall, we conclude that NMDA receptor coactivation by glutamate and D-serine increases lumen diameter in pressurized MCA in an endothelial and eNOS-dependent mechanism.

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Introduction

Glutamate is a central nervous system excitatory neurotransmitter that can activate both G-protein-coupled (metabotropic) receptors and ligand-gated ion channels, which include AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and N-methyl-D-aspartate (NMDA) receptors. N-methyl-D-aspartate receptors are heterotetramers consisting of seven subunits (NR1, NR2A–D, NR3A,B), and are

critical regulators of neuronal excitability, synaptic plasticity (Collingridge and Singer, 1990), excitotoxicity (Choi *et al*, 1988), and cerebral blood flow (Busija *et al*, 2007). Neuronal NMDA receptors most often consist of two NR1 and two NR2A or NR2B subunits. N-methyl-D-aspartate receptors consisting of two NR1 and combinations of NR2C, NR2D, NR3A, or NR3B have also been identified and differ functionally from NR2A/B configurations. Most notably, they are resistant to baseline voltage-dependent channel blockade by Mg^{2+} that is characteristic of NMDA receptors expressing NR2A/B (Chatterton *et al*, 2002; Monyer *et al*, 1994). Nonneuronal NMDA receptors with such configurations have been identified in cell types including astrocytes, oligodendrocytes, vascular endothelial cells, lymphocytes, and cardiomyocytes, and mediate diverse functions such as myelination, immune response, and blood–brain barrier permeability (Schipke *et al*, 2001; Boldyrev *et al*, 2005; Karadottir *et al*, 2005; Lalo *et al*, 2006;

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Reijerkerk *et al*, 2010). *N*-methyl-D-aspartate receptor activation requires glutamate, which binds NR2 subunits, and binding of a coagonist, glycine or D-serine. While glycine and D-serine are both NMDA receptor coagonists, D-serine binds with greater affinity and dominates as the main endogenous agonist in most of the brain regions (Mothet *et al*, 2000). D-Serine is produced and stored in astrocytes (Schell *et al*, 1995). These stores are released by a Ca^{2+} and SNARE protein-dependent mechanism (Martineau *et al*, 2008; Mothet *et al*, 2005) and, in turn, influence neuronal excitability and plasticity (Mothet *et al*, 2000; Yang *et al*, 2003).

Functional hyperemia is an endogenous regulatory pathway in the brain coupling elevated neuronal activity with vasodilation and increased local cerebral blood flow (Iadecola, 2004). Application of glutamate directly to the brain surface *in vivo* consistently triggers vasodilation and increased blood flow in several animal models (Busija and Leffler, 1989; Faraci and Breese, 1993; Faraci and Brian, 1995; Meng *et al*, 1995; Philip and Armstead, 2004). There is broad literature consensus that glutamate can induce vasodilation in two ways. First, neuronal NMDA receptors can be activated, stimulating Ca^{2+} influx, and nitric oxide (NO) production by neuronal nitric oxide synthase (nNOS) (Bari *et al*, 1996b; Chi *et al*, 2003; Faraci and Brian, 1995; Fergus and Lee, 1997; Meng *et al*, 1995). Second, glutamate can stimulate astrocytic release of vasodilators produced from arachidonic acid, such as epoxyeicosatrienoic acids and prostaglandins (Metea and Newman, 2006; Takano *et al*, 2006). A third possibility with little support to date is that glutamate mediates vasodilation by acting directly at the microvasculature level. Intriguingly, brain endothelial cells reportedly express NMDA receptors (Krizbai *et al*, 1998; Reijerkerk *et al*, 2010; Scott *et al*, 2007; Sharp *et al*, 2003), and glutamate has been shown to stimulate endothelial cell production of vasodilators, such as CO (Parfenova *et al*, 2003) and NO (Scott *et al*, 2007). There is also at least one report that glutamate dilates isolated cerebral arterioles by an endothelium-dependent mechanism (Fiumana *et al*, 2003). On the other hand, several groups have argued against such a mechanism based on limited direct effects of glutamate on lumen diameter in isolated artery preparations using wire or pressure myography (Faraci and Breese, 1993; Hardbo *et al*, 1989; Simandle *et al*, 2005; Wendling *et al*, 1996). There are also reports that specifically refute claims that brain endothelial cells express NMDA receptor subunits (Domoki *et al*, 2008; Morley *et al*, 1998).

We postulated that the demonstrated failure of isolated, pressurized brain arteries to dilate in response to glutamate may in part be explained by an absence of NMDA receptor coagonist in these experiments. The objectives of the current study were to determine whether coactivation of endothelial NMDA receptors by D-serine and glutamate is

sufficient to cause dilation of isolated brain arteries, and to determine whether the pan-NMDA receptor subunit NR1 is expressed in the brain endothelial cells. We report that glutamate or NMDA applied to isolated middle cerebral arteries (MCAs) with D-serine, but not alone, causes vasodilation dependent on endothelial function and endothelial nitric oxide synthase (eNOS) activity. NR1 expression was also detected in primary brain endothelial cell cultures.

Materials and methods

Pressure Myography

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise noted. Animal protocols were approved by the Animal Care Committee at the University of Manitoba. Male CD1 mice (15 weeks old) were anesthetized with 70% CO_2 /30% O_2 and decapitated. The brains were removed and placed in ice-cold Krebs buffer (118 mmol/L NaCl, 4.65 mmol/L KCl, 1.18 mmol/L MgSO_4 , 1.18 mmol/L KHPO_3 , 25 mmol/L NaHCO_3 , 2.5 mmol/L CaCl_2 , 5.5 mmol/L glucose, 0.026 mmol/L EDTA). A MCA was dissected from the brain, mounted between two glass pipettes in a pressure myograph chamber, and adherent tissue was removed. The vessels were secured in place with suture ties and pressurized with a Servo-controlled peristaltic pump to an intraluminal pressure of 30 mmHg. Branches from the main artery were closed by electrocauterization or suture ties. The arteries were bathed in Krebs aerated with 5% CO_2 , 20% O_2 , and 75% N_2 at 37°C throughout the experiments.

Changes in arterial diameter during the drug treatments were measured with a video dimension analyzer (Living Systems Instrumentation, Burlington, VT, USA) attached to a Nikon Eclipse TS100 microscope (Melville, NY, USA). Vessel viability was confirmed before the start of the experiments by $\geq 50\%$ constriction to 125 mmol/L KCl. The vessels were equilibrated for 1 hour between treatments. Arteries were precontracted with norepinephrine (1 $\mu\text{mol/L}$), and vascular responses were measured in response to incremental doses of glutamate or NMDA with or without D-serine.

To assess the role of the endothelium, the endothelial cell layer was removed by pushing a bolus of air through the vessel. Complete denudation of the endothelium was confirmed by failure of the vessel to relax in the presence of acetylcholine (ACh; 10 nmol/L to 10 $\mu\text{mol/L}$). Viability of the smooth muscle layer was verified by treatment with sodium nitroprusside (SNP; 100 nmol/L to 100 $\mu\text{mol/L}$), a NO donor.

Phenolic Denervation and Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase Staining

Residual postdissection nerve terminals on MCA smooth muscle surfaces were chemically destroyed by incubating the arteries in 0.15% phenol and 1.35% ethanol for 3 minutes, which was adapted from previous denervation

methods (Wang and Bukoski, 1999). Vessel viability was confirmed by dilation to ACh (10 nmol/L to 10 μ mol/L) and SNP (100 nmol/L to 100 μ mol/L). To verify denervation, arteries were fixed with 4% paraformaldehyde (pH 7.6) for 10 minutes. Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase solution, containing nitro blue tetrazolium (5 μ mol/L), the reduced form of β -nicotinamide adenine dinucleotide phosphate (10 μ mol/L), and 0.2% Triton X-100 in TBS (Tris-buffered saline; 0.05 mol/L; pH 8.0), was applied for 30 minutes at 37°C to detect nNOS-positive neurons.

Brain Slice Preparation and Two-Photon Imaging

The brains from 14- to 19-day-old CD1 mice were cut on a vibrating blade microtome (Vibratome, Bannockburn, IL, USA) into 350- μ m thick sections. Slices were loaded with Rhod-2 AM (Invitrogen, Burlington, ON, USA), a calcium indicator preferentially taken up by astrocytes, and isolectin B₄ from *Griffonia simplicifolia* tagged with Alexa Fluor 488 (Invitrogen) to label the brain slice vasculature. Slices were incubated in artificial cerebrospinal fluid aerated with 20% O₂, 5% CO₂, and 75% N₂ while imaged on an upright microscope with scanhead for two-photon imaging (Prairie Technologies, Middleton, WI, USA) attached to a pulsed Ti-sapphire laser (Coherent Inc., Santa Clara, CA, USA) at 800 nm. Glutamate (10 μ mol/L) and D-serine (10 μ mol/L) were bath applied to the slices and the change in arteriolar diameter was recorded over time.

Primary Endothelial Cell Culture

Endothelial cells were isolated from 15-week-old male CD1 mouse brains, as described previously (Jung and Levy, 2005). Briefly, the brains were cut into small pieces with a scalpel and digested with 0.05% collagenase. Digested material was resuspended in 17% Dextran solution and centrifuged at 10,000 *g* for 30 minutes at 4°C, which resulted in an endothelial cell pellet. Isolated endothelial cells were collected on glass beads and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 1% penicillin-streptomycin, and 150 μ g/mL endothelial cell growth supplement. Cells were passaged twice before reaching confluence.

Polymerase Chain Reaction

Total RNA was isolated from primary endothelial cell cultures, unpurified brain cultures, or mouse brain using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON, USA), and DNase digested according to the Turbo DNA-free protocol (Applied Biosystems, Carlsbad, CA, USA). Turbo DNA-free RNA was reverse transcribed to cDNA according to the SuperScript III RNase H⁻ Reverse Transcriptase protocol (Invitrogen), and complementary mRNA was digested using RNase H. cDNA was purified according to the PCR Purification Kit (Qiagen).

Mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase; internal control) and NR1 genes were amplified using the following primers: GAPDH forward = CACGGCA

AATTCAACGGCACAGT, GAPDH reverse = TGGGGGCATC GGCAGAAGG; NR1 forward = TGTGCGGGACAACAAGCT CCA, NR1 reverse = ATGCCGATGCCAAAGCCGGA. All gene targets were PCR amplified according to the DreamTaq protocol (Fermentas, Burlington, ON, USA) using 10 ng of cDNA as template. There was an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C (GAPDH), 63°C (NR1), or 65°C (von Willebrand's factor) for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. These PCR products were visualized on a 1% agarose gel.

Immunofluorescence

Primary endothelial cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 hour. Cells were permeabilized for 5 minutes with 0.2% Triton X-100 in PBS and blocked at room temperature with 1% bovine serum albumin in PBS. Goat anti-NR1 polyclonal primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was applied overnight at 4°C. The secondary antibody Alexa Fluor 568 donkey anti-goat IgG (H + L) and endothelial cell marker, Isolectin B₄ tagged with Alexa Fluor 488 (Invitrogen), were applied in 1% bovine serum albumin in PBS for 1 hour at room temperature. Staining was visualized with a Zeiss LSM510 laser-scanning microscope (Oberkochen, Germany).

For brain slice immunofluorescence, mouse brains (15 week old) were rinsed with PBS (pH 7.2) and placed in 4% paraformaldehyde for 24 hours. Frozen sagittal sections (50 μ m) were cut and free-floating sections were exposed to an antigen retrieval step with 10 mmol/L citrate (pH 8.5) at 80°C for 30 minutes. Sections were rinsed in 0.1 mol/L TBS (pH 7.6), treated with a mouse-on-mouse blocking kit (Vector Laboratories, Burlingame, CA, USA) and incubated with rabbit anti-NR2C (Santa Cruz Biotechnology) with either mouse anti-GFAP (glial fibrillary acidic protein) (Sigma) or anti-mouse p-glycoprotein (Mdr-1; Santa Cruz Biotechnology) overnight at 4°C. After washing, the sections were incubated with secondary antibodies for 1 hour at room temperature. Goat anti-rabbit IgG (Alexa Fluor 568) was used to detect NR2C signal, while goat anti-mouse IgG (Alexa Fluor 488) was used to detect GFAP and p-glycoprotein. Stained sections were visualized with a Zeiss LSM510 laser-scanning microscope.

Western Blot

Cultured endothelial cells were scraped in nondenaturing lysis buffer (10% glycerol and 1% Triton X-100 in 20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 2 mmol/L EDTA with protease inhibitors; pH 8) and protein concentrations were determined using a BCA assay. Crude protein was separated on 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Membranes were blocked for 60 minutes with 5% skim milk powder in TBS with 20% Tween 20 (TBS-T) and incubated with rabbit polyclonal anti-NR1 (1:300) (Cell Signaling, Danvers, MA, USA) overnight at 4°C. A goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:1,000)

(Cell Signaling) was added to the membrane for 2 hours (room temperature), and an ECL Plus chemiluminescence kit (GE Life Sciences, Piscataway, NJ, USA) was used to visualize the protein bands with a Fluo-STM MultiImager (Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

For dose–response pressure myography experiments, two-way ANOVA (analysis of variance) for repeated measures were conducted with Bonferroni *post-hoc* tests. For experiments where arteries were treated with a single concentration of drug, data were analyzed by one-way ANOVA test with Newman–Keuls *post-hoc* test.

Results

N-Methyl-D-Aspartate Receptor Agonists Directly Dilate Isolated Middle Cerebral Arteries and Penetrating Cortical Arterioles

Bath application of increasing concentrations (0.1 $\mu\text{mol/L}$ to 10 mmol/L) of NMDA receptor agonists, glutamate and NMDA, to precontracted MCAs had no significant effect on lumen diameter

(Figure 1A). In contrast, with D-serine present (100 $\mu\text{mol/L}$), incremental concentrations of glutamate (Figure 1B), or NMDA (Figure 1C) produced significant concentration-dependent relaxation of MCAs, which was first statistically detectable ($P < 0.05$) at 10 $\mu\text{mol/L}$ glutamate (22.9% \pm 5.7%) or NMDA (13.8% \pm 2.3%). D-Serine concentrations 10-fold lower than this (10 $\mu\text{mol/L}$) were sufficient to evoke vasodilation in the presence of 100 $\mu\text{mol/L}$ glutamate (Figure 1D). This D-serine concentration is consistent with unstimulated forebrain D-serine levels analyzed by microdialysis (Hashimoto *et al*, 1995). In an effort to find the lower range of D-serine and glutamate concentrations able to cause MCA dilation, we used both coagonists at 10 $\mu\text{mol/L}$ and found this condition to cause a significant increase in MCA diameter (Figure 2A). D-Serine was unable initiate vasodilation when administered with another known vasodilator, ACh (Supplementary Figure 1), indicating a specific effect observed only when given with an NMDA receptor glutamate site agonist. To explore whether the vasodilatory effect of D-serine can be extended to arterioles or capillaries in the brain parenchyma, we determined whether the combination of D-serine and glutamate dilates cortical arterioles in mouse brain slices maintained

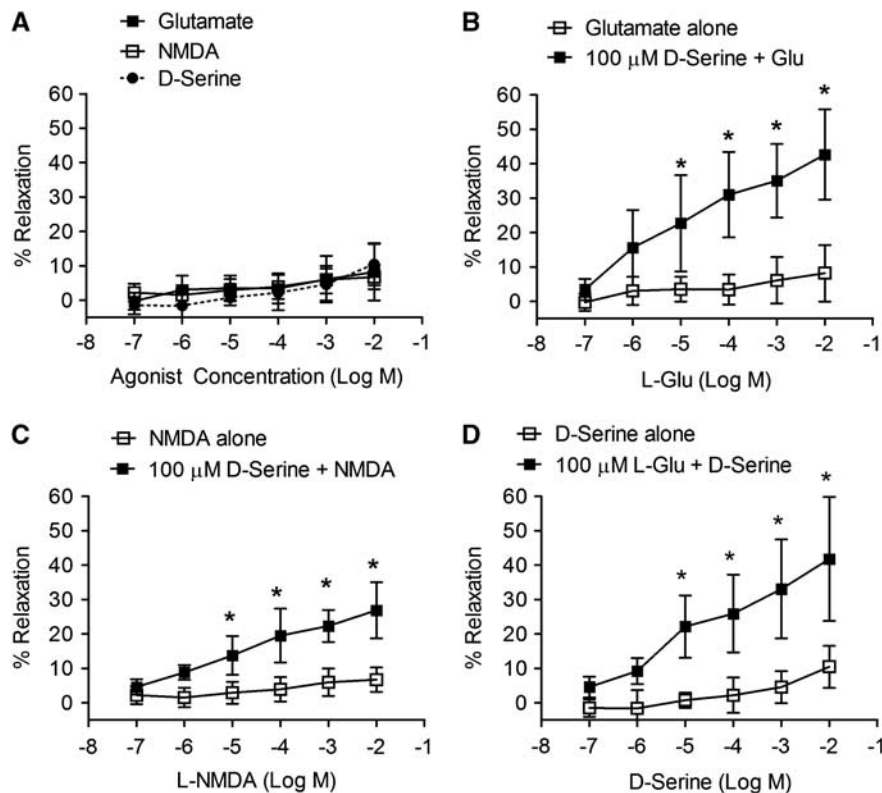


Figure 1 Dilation of isolated middle cerebral arteries (MCAs) by *N*-methyl-D-aspartate (NMDA) and glutamate requires D-serine. (A) Individually, glutamate, NMDA, and D-serine failed to significantly affect vessel diameter ($n = 6$). Glutamate (B) and NMDA (C) caused concentration-dependent vasodilation of MCAs in the presence of fixed (100 $\mu\text{mol/L}$) D-serine ($n = 6$). (D) D-Serine caused concentration-dependent increases in MCA lumen diameter in the presence of fixed glutamate (100 $\mu\text{mol/L}$, $n = 6$). Data are presented as mean \pm s.d.; * $P < 0.05$ compared with other groups using two-way analysis of variance (ANOVA) for repeated measures and Bonferroni *post-hoc* test.

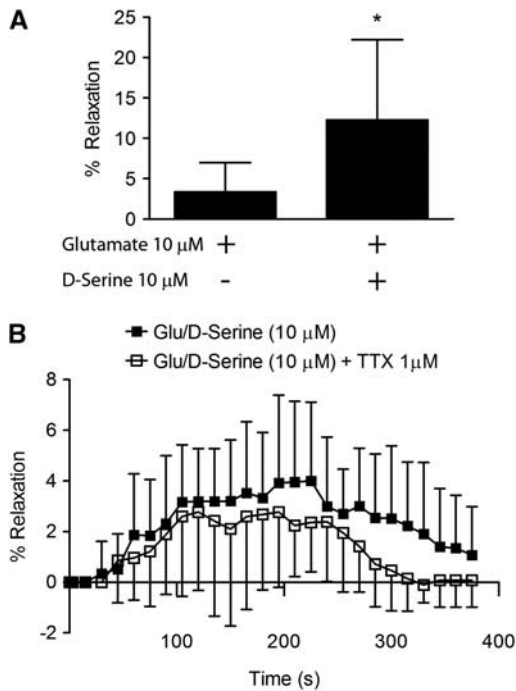


Figure 2 Glutamate and D-serine increase lumen diameter in the brain slice arterioles. **(A)** Combinations of glutamate and D-serine as low as 10 μmol/L each agonist causes significant middle cerebral artery (MCA) dilation. * $P < 0.05$ compared with glutamate only group using a *t*-test. **(B)** The combination of glutamate and D-serine causes significant dilation of penetrating cortical arterioles in cortical slices in the presence and absence of the neuronal excitability blocker, tetrodotoxin (TTX). Data are presented as mean values \pm s.d. of 14 (control) or 18 (TTX group) brain slices and were analyzed by two-way analysis of variance (ANOVA) for repeated measures and Bonferroni *post-hoc* test.

at 20% O₂. Bath application of glutamate and D-serine (each 10 μmol/L) dilated brain slice arterioles (maximum 4.0% \pm 3.1%) over 400 seconds (Figure 2B). Adding tetrodotoxin to block neuronal excitability had no significant effect on dilation (maximum 2.8% \pm 3.0%), thus eliminating the possibility that signaling through neuronal excitation is responsible for this effect of glutamate and D-serine.

Glutamate and D-Serine-Mediated Vasodilation Is N-Methyl-D-Aspartate Receptor Dependent

To determine the glutamate receptor subtype involved in MCA dilation by NMDA receptor coagonists, several antagonists were used, including a competitive glutamate binding site NMDA receptor antagonist, AP5 (D-2-amino-5-phosphonopentanoate), a glycine/D-serine coagonist site NMDA receptor antagonist, DCKA (5,7-dichlorokynurenic acid), and an AMPA/kainate receptor antagonist, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione). D-2-Amino-5-phosphonopentanoate (100 nmol/L) and DCKA

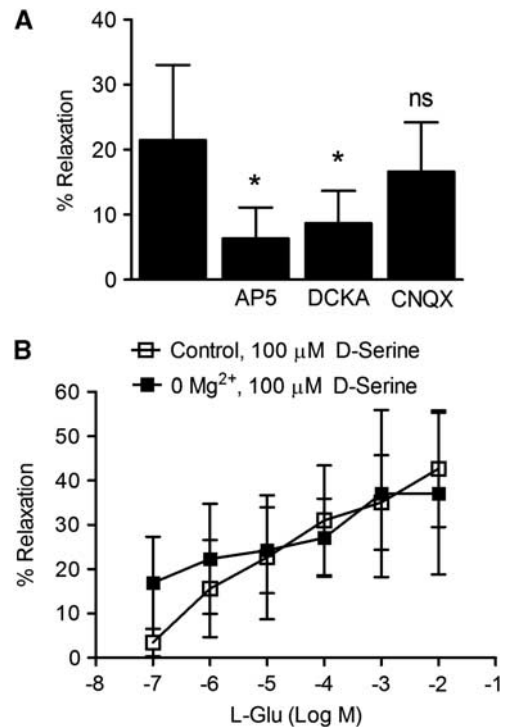


Figure 3 N-methyl-D-aspartate (NMDA) receptors mediate vasodilation in response to glutamate and D-serine. **(A)** Treatment of middle cerebral arteries (MCAs) with glutamate and D-serine together (both 100 μmol/L) caused a significant increase in lumen diameter. NMDA receptor antagonists, AP5 (D-2-amino-5-phosphonopentanoate; 100 nmol/L) and DCKA (5,7-dichlorokynurenic acid; 300 nmol/L) attenuated vasodilation ($n = 6$). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist, did not significantly inhibit vasodilation up to 20 μmol/L ($n = 6$). Data are presented as mean \pm s.d.; * $P < 0.05$ compared with glutamate/D-serine control using one-way analysis of variance (ANOVA) with Newman-Keuls *post-hoc* test. **(B)** No significant differences in vasodilatory response to incremental glutamate in the presence of D-serine (100 μmol/L) were detected between control arteries and arteries incubated in Mg²⁺-free buffer. Data are presented as mean \pm s.d. and were analyzed using two-way ANOVA for repeated measures and Bonferroni *post-hoc* test.

(300 nmol/L) significantly blocked MCA dilation induced by glutamate and D-serine (100 μmol/L each combined) ($P < 0.05$), while CNQX (up to 10 μmol/L) had no significant effect (Figure 3A). This suggests that NMDA receptors, but not AMPA/kainate receptors mediate vasodilation by glutamate and D-serine.

Neuronal NMDA receptors require plasma membrane depolarization to expel channel-bound Mg²⁺, whereas NMDA receptors identified on nonneuronal cell types, such as astrocytes and oligodendrocytes, have little Mg²⁺ sensitivity (Karadottir *et al*, 2005; Lalo *et al*, 2006). Removal of extracellular Mg²⁺ did not significantly change vasodilation induced by glutamate and D-serine (Figure 3B; $P > 0.05$), indicating low or no sensitivity to Mg²⁺ block. This is consistent with other nonneuronal NMDA receptors.

Endothelial-Derived Nitric Oxide Mediates N-Methyl-D-Aspartate Receptor-Mediated Vasodilation

Endothelium was removed from selected MCAs by pushing an air bolus through the lumen. These arteries constricted slightly more strongly than MCAs with intact endothelium (Supplementary Figure 2A), attributable to a loss of baseline NO production, and failed to dilate in response to ACh (10 nmol/L to 10 μ mol/L; Supplementary Figure 2B), confirming endothelial destruction. Denuded arteries dilated normally in response to the NO donor, SNP (100 nmol/L to 100 μ mol/L; Supplementary Figure 2C). Exposure of denuded arteries to glutamate (0.1 μ mol/L to 10 mmol/L) in the presence of D-serine (100 μ mol/L) produced no significant vasodilatory response (Figure 4A), indicating the endothelial layer is required for NMDA receptor-mediated vasodilation.

We next tested whether NO mediates NMDA receptor-induced vasodilation. Treatment of isolated MCAs with the nonselective NOS inhibitor, L-NAME (*N*-nitro-L-arginine methyl ester; 10 μ mol/L), prevented dilation induced by glutamate and D-serine together (Figure 4B). The NOS inhibitor, TRIM (1-(2-(trifluoromethylphenyl))imidazole), at a concentration selective for nNOS (50 μ mol/L) (Handy *et al*, 1995) did not significantly alter vasodilation

by glutamate and D-serine ($P > 0.05$; Figure 4C). In contrast, the NOS inhibitor, L-NIO (N^5 -(1-iminoethyl)-L-ornithine dihydrochloride), significantly blocked relaxation induced by glutamate and D-serine ($P < 0.05$; Figure 4D) at a concentration selective for eNOS (3 μ mol/L) (Chinellato *et al*, 1998; Rees *et al*, 1990). Similarly, isolated MCAs from eNOS knockout mice did not significantly dilate in response to glutamate and D-serine ($P < 0.05$; Figure 4D). This suggests eNOS, but not nNOS, is involved in the mechanism of vasodilation by glutamate and D-serine.

Vascular Nerve Endings do not Contribute to Glutamate/D-Serine-Mediated Vasodilation

To eliminate the possibility that residual vascular nerve endings expressing NOS and/or NMDA receptors contribute to vasodilation, selected MCAs were chemically denervated and tested for responses to glutamate and D-serine. Middle cerebral arteries subjected to nicotinamide adenine dinucleotide phosphate-diaphorase staining displayed NO-positive nerve endings on the smooth muscle surface (Figure 5A). Chemical denervation of arteries in 0.15% phenol and 1.35% ethanol removed the majority of NO-containing terminals (Figure 5B).

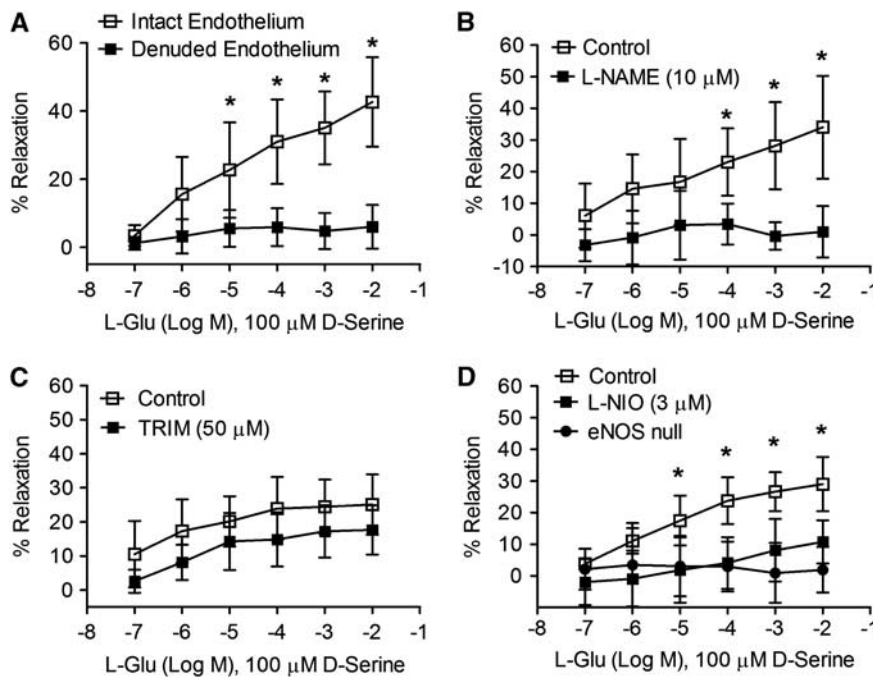


Figure 4 Vasodilation by glutamate and D-serine requires intact endothelium and endothelial nitric oxide synthase (eNOS). **(A)** Removal of the endothelium isolated middle cerebral arteries (MCAs) an air bolus significantly attenuated smooth muscle relaxation caused by incremental glutamate concentrations in the presence of 100 μ mol/L D-serine ($n = 6$). **(B)** *N*-nitro-L-arginine methyl ester (L-NAME; 10 μ mol/L) blocked concentration-dependent vasodilation caused by glutamate and D-serine ($n = 6$). **(C)** Glutamate/D-serine-induced MCA relaxation is not affected by the neuronal NOS inhibitor, 1-(2-(trifluoromethylphenyl))imidazole (TRIM; 50 μ mol/L). **(D)** N^5 -(1-iminoethyl)-L-ornithine (L-NIO; 3 μ mol/L), an eNOS-selective antagonist, and eNOS deletion prevented MCA relaxation by incremental glutamate increases in the presence of 100 μ mol/L D-serine ($n = 6$). Data are presented as mean \pm s.d.; * $P < 0.05$ using two-way analysis of variance (ANOVA) for repeated measures and Bonferroni *post-hoc* test.

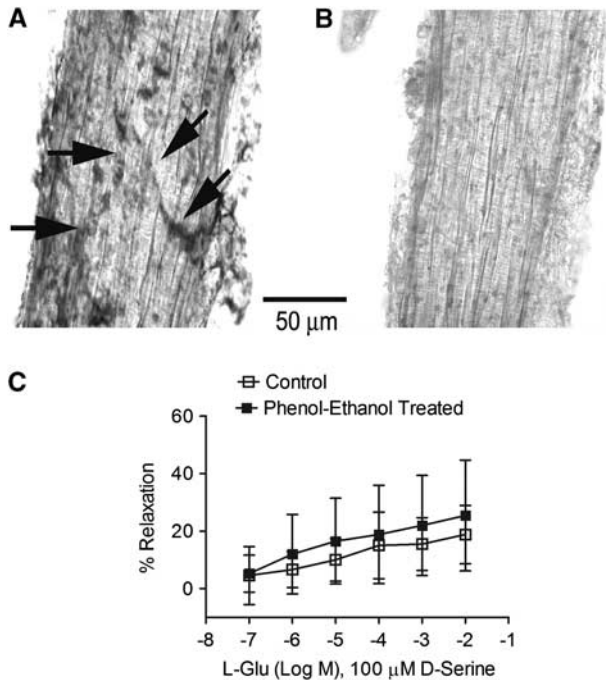


Figure 5 *N*-methyl-D-aspartate (NMDA) receptor-mediated vasodilation persists following chemical denervation of middle cerebral arteries (MCAs). Isolated mouse MCAs were mechanically stripped and treated with vehicle (**A**) or 1.35% ethanol with 0.15% phenol for 3 minutes (**B**) to cause chemical denervation. Vessels were either analyzed for nitric oxide (NO)-containing nerve endings by nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining (**A**, **B**) or analyzed for responses to glutamate/D-serine by pressure myography (**C**). (**A**) Untreated MCA has NADPH-diaphorase-positive nerve fiber tracts (arrows). (**B**) Vessels treated for 3 minutes showed no indication of NADPH-diaphorase staining and therefore neuronal NO. (**C**) Both intact and denervated vessels relaxed significantly in a concentration-dependent manner ($P < 0.05$, $0.1 \mu\text{mol/L}$ versus 10 mmol/L) and there were no significant differences between the groups at any dose. Data are presented as mean values \pm s.d. for six MCAs. Data were compared using two-way analysis of variance (ANOVA) for repeated measures, followed by the Bonferroni test for selected pairs.

Denervated arteries were able to constrict normally in response to norepinephrine and dilate in response to ACh (10 nmol/L to $10 \mu\text{mol/L}$) and SNP (100 nmol/L to $100 \mu\text{mol/L}$; Supplementary Figures 2B and 2C), indicating full functional viability. Chemical denervation did not significantly affect the magnitude of vasodilation caused by glutamate ($0.1 \mu\text{mol/L}$ to 10 mmol/L) in the presence of D-serine ($100 \mu\text{mol/L}$; Figure 5C). This indicates that NOS-containing vascular nerve endings remaining after brain arterial dissections do not contribute to vasodilation by glutamate and D-serine.

Mouse Brain Endothelial Cells Express *N*-Methyl-D-Aspartate Receptor Subunits

To test whether mouse cerebral endothelial cells express NMDA receptors, PCR, immunocytochemis-

try and Western blot approaches were employed using primary brain endothelial cell cultures isolated from 15-week-old mice. mRNA for the pan-NMDA receptor subunit, NR1, and endothelial marker, von Willebrand's factor, were identified, indicating the presence of NR1 transcript in the brain endothelial cells (Figure 6A). An immunoreactive product consistent with NR1 (116 kDa) was detected by Western blot of whole-cell lysates from purified mouse brain primary endothelial cells (Figure 6B). This band was also observed in positive control mouse whole brain homogenates. NR1 immunoreactivity was identified in cultured brain endothelial cells that express isolectin B₄ (Figures 6C–6E), confirming NR1 protein expression in these cells.

We also investigated whether NMDA receptor subunits are expressed by brain slice cortical arteries near the pial surface. Vessels with diameters consistent with those of MCAs used in pressure myography experiments (50 to $80 \mu\text{m}$) were chosen for analysis. Although apparent vascular NR1 immunoreactivity was detected, it was largely obscured by stronger neuronal NR1 signal (not shown). Immunoreactivity for the NMDA receptor subunit, NR2C, which is largely silent in adult cortical neurons, was identified in cortical arteries of the targeted diameter range. NR2C signal was colocalized with the endothelial cell marker, p-glycoprotein (Figures 6F–6H). In contrast, NR2C signal was not colocalized with perivascular astrocytes that express GFAP (Figures 6I–6K). Overall, these results indicate endothelial expression of NR2C immunoreactivity in cortical arteries *in situ*.

Discussion

Here, we present evidence supporting vasodilation of mouse brain arteries by endothelial NMDA receptors and eNOS. *N*-methyl-D-aspartate receptor coagonists glutamate and D-serine stimulated vasodilation of isolated, denervated mouse MCAs in a manner sensitive to NMDA receptor antagonists and eNOS inhibition or deletion. We also found evidence that primary brain endothelial cells and brain arteries *in situ* express NMDA receptor subunits.

Numerous studies covering a range of species have shown that NMDA or glutamate application topically to pial arteries through a cranial window causes vasodilation (Bari *et al*, 1996a; Busija and Leffler, 1989; Faraci and Breese, 1993, 1994; Philip and Armstead, 2004). This effect has been widely attributed to activation of neuronal NMDA receptors and nNOS. In apparent agreement with this, several studies have found no effect of exogenous glutamate or NMDA on lumen diameter in isolated cerebral arteries (Faraci and Breese, 1993; Hardebo *et al*, 1989; Simandle *et al*, 2005; Wendling *et al*, 1996). Our results are consistent with these findings by demonstrating that NMDA receptor agonists alone have little effect on isolated cerebral arterial

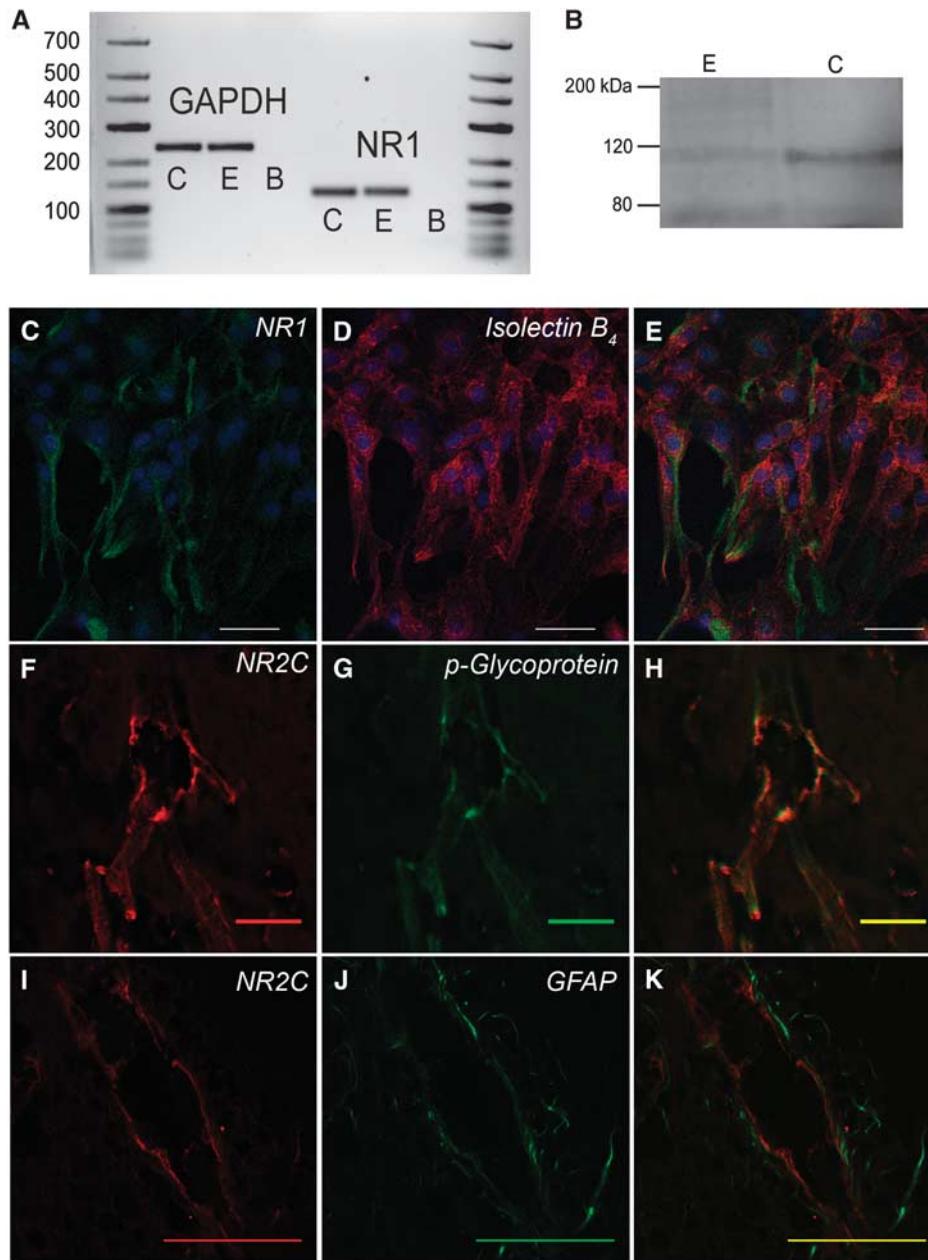


Figure 6 Brain endothelial cells express *N*-methyl-D-aspartate (NMDA) receptor subunits. **(A)** Purified primary adult mouse brain endothelial cultures expressed mRNA for housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and NR1. C denotes cortex; E denotes endothelial culture; B denotes blank control. **(B)** Western blot of protein from cortex (C) and endothelial cultures (E) produced a band for NR1 at 116 kDa. **(C–E)** Immunofluorescence shows colocalization of NR1 (C, green) and the endothelial cell marker, isolectin B₄ (D, red), in cultured endothelial cells. Nuclei were stained with ToPro-3. Scale bars for (C–E) are 100 μ m. **(F–H)** Coimmunofluorescence of NR2C (F, red) and p-glycoprotein (G, green) in cortical arteries near the pial surface. **(I–K)** Immunofluorescence for NR2C (I, red) and astrocyte glial fibrillary acidic protein (GFAP; J, green) does not overlap (K) in cortical arteries near the pial surface. Scale bars for (F–K) are 50 μ m.

diameter. However, for the first time we found significant vasodilation when D-serine was applied with glutamate or NMDA. The question of whether this observation could translate into a physiologically relevant dilatatory mechanism *in vivo* at our experimental glutamate and D-serine concentrations is important. Local extracellular glutamate levels can approach low millimolar levels (Clements *et al*,

1992) and basal D-serine levels *in vivo* have been reported in the 5 to 8 μ mol/L range by microdialysis (Hashimoto *et al*, 1995). We found that glutamate and D-serine concentrations in these ranges (100 μ mol/L glutamate, 10 μ mol/L D-serine) were sufficient to cause a 22.2% dilation of MCAs. According to Poiseuille's law, vasodilation of this magnitude would decrease vascular resistance \sim 2-fold.

We further showed that 10 $\mu\text{mol/L}$ D-serine caused vasodilation in combination with glutamate concentrations as low as 10 $\mu\text{mol/L}$ and that this combination induced dilation of penetrating arterioles in mouse cortical slices. Although further work is required to demonstrate that endogenous D-serine and glutamate are capable of producing cerebral vasodilation, these observations show that locally attainable concentration ranges of D-serine and glutamate dilate isolated brain arteries and, importantly, small arterioles in the more anatomically relevant brain slice model.

Our data support an effect of glutamate and D-serine together at NMDA receptors. The fact that D-serine is required for vasodilation is suggestive of an NMDA receptor-mediated response to begin with, as no other glutamate receptors with a coagonist requirement have been identified. In addition, competitive NMDA receptor antagonists AP5, which blocks the glutamate binding site, and DCKA, which blocks the D-serine/glycine binding site, both significantly inhibited vasodilation induced by combining glutamate and D-serine. Lastly, an AMPA/kainate antagonist, CNQX, did not alter vasodilation. All of these observations support the hypothesis that glutamate and D-serine together influence vessel diameter by an NMDA receptor-mediated mechanism. Neuronal NMDA receptor configurations consist of NR1 and NR2A/B subunits and are sensitive to blockade of the cation channel by Mg^{2+} at resting membrane potential. We tested whether Mg^{2+} -free Krebs buffer could enhance vasodilation to gain insight into the similarity of eNMDA (endothelial cell NMDA) receptor subunit configuration and function in comparison with neuronal NMDA receptors. We found that removing Mg^{2+} had no significant effect on vasodilation induced by glutamate and D-serine, suggesting that eNMDA receptors may contain NR2C, NR2D, or NR3 subunits, which confer much lower Mg^{2+} sensitivity to NMDA receptor function (Chatterton *et al*, 2002). This is consistent with NMDA receptors identified in other nonneuronal subtypes, including astrocytes and oligodendrocytes, which are not subject to Mg^{2+} block and appear to consist of NR2C and NR3 subunits (Karadottir *et al*, 2005; Lalo *et al*, 2006).

Numerous studies in the brain slices and live animals have suggested NMDA receptor activation stimulates NO production via nNOS activity (Bari *et al*, 1996b; Chi *et al*, 2003; Faraci and Breese, 1993; Faraci and Brian, 1995; Fergus and Lee, 1997; Meng *et al*, 1995). In isolated arteries, we have now identified a potential role of eNOS in NMDA receptor-mediated vasodilation. TRIM, at a concentration relatively selective for nNOS (50 $\mu\text{mol/L}$) (Handy *et al*, 1995), did not significantly alter vasodilation by glutamate and D-serine. In contrast, dilation was inhibited by the nonselective NOS inhibitor, L-NAME, by the NOS inhibitor, L-NIO, at a concentration selective for eNOS (3 $\mu\text{mol/L}$) (Chinellato *et al*, 1998; Rees *et al*, 1990), and in

eNOS^{-/-} MCAs. These findings strongly indicate eNOS involvement in a direct vascular effect of NMDA receptors. It is not our position that new observations of a potential role for eNOS in glutamate-mediated vasodilation argue against the established role of nNOS *in vivo*. Rather, our data suggest that eNOS may contribute to glutamate-induced vasodilation alongside or in place of nNOS in certain conditions. Endothelial nitric oxide synthase has a major role in regulation of the brain vascular tone (Iadecola, 1993) and baseline brain blood flow (Ma *et al*, 1996), and while eNOS does not have a significant role in functional hyperemia evoked by whisker stimulation or hypercapnia (Ayata *et al*, 1996; Ma *et al*, 1996), it is a critical mediator of hyperemia resulting from experimental seizures, focal brain trauma (Hlatky *et al*, 2003), and cerebral ischemia (Endres *et al*, 2004). These conditions are associated with large-scale elevations of extracellular glutamate and D-serine levels; thus, it is tempting to speculate that eNOS-induced hyperemia is linked to eNMDA receptors that require high brain glutamate production for activity.

Glutamate and D-serine-mediated dilation was also abolished by removal (denudation) of the endothelium. Thus, our experiments support a link between NMDA receptor activation, endothelial function, and eNOS. Results agree with previous results in a brain endothelial-derived cell line displaying NMDA receptor-dependent eNOS activation (Scott *et al*, 2007), but our study is the first to demonstrate linkage between vascular NMDA receptors and eNOS in intact arteries. We considered the possibility that neuronal NMDA receptors expressed by remnant nerve endings adhering to the vascular smooth muscle surface mediate endothelial-dependent eNOS activation. To address this, we used two approaches. First, we chemically denervated MCA segments and found that this did not impact MCA dose-dependent dilation to glutamate and D-serine. This indicates glutamate and D-serine exert direct effects on vascular NMDA receptors. Second, we presented evidence that NMDA receptor subunits are expressed by endothelial cells. NR1 expression was detected in primary cultured endothelial cells from 15-week-old mice by PCR, Western blot, and immunocytochemistry. This agrees with previous studies reporting the presence of NMDA receptor expression in cultured endothelial cells (Chen *et al*, 2005; Krizbai *et al*, 1998; Scott *et al*, 2007; Sharp *et al*, 2003) and endothelial cells in the brain tissue (Reijerkerk *et al*, 2010). While this is an important result, the preparation used contains microvascular endothelium as well as arterial endothelium. Since the focus of the current study is on brain arteries, we stained fixed cortical slices for NMDA receptor subunits and selected arteries >50 μm in diameter near the pial surface for analysis. Cortical neurons were positive for NR1, as expected. NR1 signal was also apparent in the brain vasculature but was considerably less intense than surrounding neuronal

signal and ambiguous for fine determination of cellular distribution. We surveyed immunoreactivity for other NMDA receptor subunits *in situ* and found a strong signal for NR2C in cortical arteries but not the majority of cortical neurons. NR2C signal overlapped with the endothelial marker, p-glycoprotein, suggesting an endothelial distribution. NR2C was distinctly more luminal than GFAP, further supporting an endothelial distribution. Other groups have specifically argued against endothelial cell NMDA receptor expression (Domoki *et al*, 2008; Morley *et al*, 1998). The reason for disparate results between these two studies and those supporting endothelial NMDA receptor expression are not clear and comprehensive immunochemical analyses of brain endothelium *in situ* are required to address this further.

This study provides new evidence linking D-serine with endothelial NMDA receptor activation and eNOS-mediated dilation in intact brain arteries. Astrocytes mediate activity-dependent changes in the brain blood flow and release both D-serine and glutamate in response to neuronal activity (Mothet *et al*, 2005; Parpura *et al*, 1994). Thus, it is rational to hypothesize that astrocyte D-serine stores contribute to blood flow regulation in hyperamia in certain conditions. The current results lend significant support to this possibility, but further studies in intact brain preparations at the arteriole and capillary level are required to determine the role of D-serine in regulating lumen diameter and blood flow *in vivo*.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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