Regulation of Brain Blood Flow by Astrocyte D-serine and N-Methyl-D-Aspartate Receptors

By

Jillian LeMaistre

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Therapeutics

Faculty of Medicine

University of Manitoba

ABSTRACT

Functional hyperemia is an endogenous regulatory process coupling synaptic activity and elevated neuronal energy demand with increased local blood flow. This involves signalling between neurons, astrocytes and blood vessels, comprising the neurovascular unit. Astrocyte processes ensheath both synapses and blood vessels, permitting multi-modal responses to synaptic activity, where astrocyte cytoplasmic Ca^{2+} is elevated, triggering endfeet processes to release vasoactive molecules, such as arachidonic acid (AA) metabolites and gliotransmitters, such as D-serine. D-Serine is a co-agonist of the glycine regulatory site at N-methyl-D-aspartate (NMDA)-type glutamate receptors, and NMDA receptors play a role in functional hyperemia *in vivo*. Thus, our aim was to examine the role of astrocyte D-serine in NMDA receptor-mediated vasodilation. Using isolated pressurized mouse middle cerebral arteries (MCAs), we determined that co-application of glutamate and D-serine induced dose-dependent dilation which was mediated by NMDA receptors and endothelial nitric oxide synthase (eNOS) in an endothelial-dependent mechanism. This is the first evidence of direct vascular effects of D-serine and glutamate and suggests a possible role for endothelial NMDA receptor activation. Several studies indicate vascular endothelial cells express NMDA receptor subunits. However, expression in mouse endothelial cells has not been well characterized, so we identified NR1 and NR2C/2D subunit expression in primary brain endothelial cultures by PCR and immunocytochemistry, and further confirmed endothelial NR2C/2D expression *in situ* by immunohistochemistry. To further investigate astrocyte D-serine release and NMDA receptor-mediated functional hyperemia within the neurovascular unit, we used an acute cortical brain slice model

where stimulation of astrocyte cytoplasmic Ca^{2+} induced vasodilation of nearby arterioles. Pharmacologically, D-serine release and NMDA receptor activation were implicated in this vasodilation. Endothelial-derived nitric oxide was also determined to induce dilation by inhibiting the production of an AA metabolite, 20-hydroxyeicostetranoic acid (20-HETE), a vasoconstrictor. This suggests an interaction between astrocyte vasoactive molecules, nitric oxide and D-serine, which warrants further investigation. Overall, our results provide evidence of modulation of NMDA receptor-mediated neurovascular coupling by astrocytic D-serine.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to:

My graduate supervisors, Drs. Chris and Hope Anderson, for their encouragement, patience, and outstanding mentorship. Thank you for keeping me motivated and providing every opportunity to learn and grow as a scientist, teacher, and academic.

My committee members, Dr. Paul Fernyhough, Dr. Don Smyth, and Dr. Jeff Wigle, for their thoughtful suggestions on this project and invaluable guidance.

The Chris Anderson lab group (past and present), including Dr. June Shao, Dr. Lingling Lu, Sam Sanders, and Dr. Catherine Gliddon. Thank you for being incredibly helpful and making the lab a fun place to work. Much of this work would not have been possible without your assistance.

My parents, Mark and Karen, for always encouraging me to try my best and supporting me when times get tough. You have always said I could do anything I put my mind to and this thesis is a prime example. Thanks for being fabulous parents.

My brother, Scott, for his comic-relief.

My best friend, Lilianne Combiadakis, who is always willing to listen and encourage me to take a shopping break.

My fiancé, Dr. Michael Stobart, for always providing excellent suggestions to overcome hurdles, both in science and in life. You are my rock and I look forward to our future collaborations and years of discussing science together.

I have also been fortunate to receive financial support over the course of my studies, and I would like to thank:

- Alzheimer's Society of Manitoba
- Canadian Institutes of Health Research
- Canadian Association for Neuroscience
- Manitoba Health Research Council
- Province of Manitoba
- Society for Neuroscience
- St. Boniface Hospital Research Foundation
- University of Manitoba, including the
  - Faculty of Graduate Studies
  - Faculty of Medicine
  - Department of Pharmacology & Therapeutics
- University of Manitoba Students Union
- University of Manitoba Alumni Association

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATONS

$[K^+]_o$	extracellular potassium concentration
20-HETE	20-hydroxyeicosatetraenoic acid
Aβ	amyloid β-peptide
A_{2A}	adenosine A_{2A} receptor
A_{2B}	adenosine A_{2B} receptor
AA	arachidonic acid
ACh	acetylcholine
AD	Alzheimer's disease
Ado	adenosine
AMPA	α-amino-3-hydroxy-5-methylisoxazole-propionate
ANLSH	astrocyte-neuron lactate shuttle hypothesis
AP5	2-amino-5-phosphonopentanoate, NMDA receptor antagonist
BBB	blood-brain barrier
BK_{Ca}	large conductance Ca^{2+} activated K^+ channels
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione, AMPA antagonist
CO	carbon monoxide
COX	cyclooxygenase

DAAO	D-amino acid oxidase
DCKA	5,7-dichlorokynurenic acid, NMDA receptor antagonist
EAAT	excitatory amino acid transporter
EET	epoxyeicosatrienoic acid
eNMDA	endothelial cell NMDA receptor
eNOS	endothelium nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein, astrocyte marker
HET	HET0016, inhibitor of 20-HETE synthesis
HO	heme oxygenase
INM	indomethacin, COX inhibitor
iNOS	inducible nitric oxide synthase
K_{ATP}	ATP-sensitive potassium channels
K_{ir}	inward rectifying potassium channel
K_mO_2	Michaelis constant for oxygen
LDH	lactate dehydrogenase
L-NAME	N^{ω}-nitro-L-arginine, NOS antagonist
L-NIO	N^5-(1-iminoethyl)-L-ornithine, eNOS-selective antagonist
LTP	long term potentiation

LTD	long term depression
MCA	middle cerebral artery
MCT	monocarboxylate transporter
mGluR	metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
P-gp	P-glycoprotein, endothelial marker
pO_2	partial pressure of oxygen
PSD-95	post-synaptic density protein 95
ROS	reactive oxygen species
sEH	soluble epoxide hydrolase
SNARE	soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor
SNP	sodium nitroprusside
*t*ACPD	(±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid , mGluR agonist
TBS	Tris buffered saline
TRIM	1-(2-(trifluoromethylphenyl))imidazole, nNOS-selective antagonist

TTX	tetrodotoxin, voltage gated sodium channel blocker
VaD	vascular dementia
VGCC	voltage-gated Ca^{2+} channels
vWF	von Willebrand factor

CHAPTER 1: Introduction

1. Astrocytes influence synaptic environments and metabolism

Historically, glia were considered to be the "glue" of the brain, but recent evidence suggests they are critical for normal brain function. There are three major types of glia: oligodendrocytes, which produce myelin, microglia, which act as resident brain macrophages, and astrocytes. Astrocytes are further classified into two main subtypes: fibrous and protoplasmic. Fibrous astrocytes are located in white matter and have several long, straight, fiber-like processes, which overlap with processes from neighbouring astrocytes (Butt et al. 1994; Oberheim et al. 2006). This thesis will deal with protoplasmic astrocytes, which are found in grey matter and have numerous branching processes that dictate a uniform shape. Protoplasmic astrocytes are highly organized into domains, where each cell occupies a relatively homogeneous three dimensional space (Oberheim et al. 2006), and there is limited regional overlap between glia- only 4.6% of the total cell volume in mouse hippocampus (Ogata and Kosaka 2002). Processes within each domain ensheath nearby synapses (Oberheim et al. 2006; Ventura and Harris 1999), permitting astrocytes to sense and modulate neuronal activity. Domain organizations appear to be important for astrocyte function, since domains are most distinct in areas of high synaptic density, such as the cortex and hippocampus. This translates to prominent astrocyte ensheathment of these synapses, like in the stratum radiatum where 80% of synapses are surrounded by astrocytic processes (Ventura and Harris 1999).

Astrocyte processes also extend and completely envelop blood vessels with end-feet (Oberheim et al. 2006), which are in continuous contact with the basal lamina

(Simard et al. 2003), and express surface proteins, such as glucose transporters, for uptake of energy substrates from the endothelium (Kacem et al. 1998). Therefore, a single cortical astrocyte is in contact with different blood vessels, neuronal cell bodies and up to 140,000 synapses in rodent brain, as estimated in the hippocampus (Bushong et al. 2002). This provides specific spatial orientation for monitoring changes in neuronal activity and altering blood flow as required by neurons (Iadecola 2004). This arrangement of neurons, astrocytes, and blood vessels is known as the "neurovascular unit".

Protoplasmic astrocytes form a functional syncytium, where distal processes are connected by connexin gap junctions permitting diffusion of ions and metabolites between neighbouring astrocytes (Giaume and McCarthy 1996; Scemes et al. 1998; Tabernero et al. 2006). This creates a conduit for intercellular communication, but also allows intracellular communication through autocellular junctions between processes of the same cell (Rouach et al. 2002; Wolff et al. 1998). Connexin proteins also form hemichannels, which do not connect to another cell, but allow release of small molecules from the cytoplasm into the extracellular space (Contreras et al. 2002; Rouach et al. 2002; Ye et al. 2003). This network of gap junctions is central to astrocyte function, facilitating communication, information processing and coordinating activity within and around astrocyte domains.

Astrocyte size and complexity increases with evolved cognitive abilities (Oberheim et al. 2009; Oberheim et al. 2006). For example, rodent cortical astrocytes typically have three or four main processes and non-symmetrical domains compared to

human protoplasmic astrocytes which have a larger volume (27-fold greater), up to 40 main processes and symmetrical domains (Oberheim et al. 2009; Oberheim et al. 2006) This suggests that increased astrocyte size and complexity may facilitate greater information processing and cognitive function.

Based on spatial orientation, gap junction connections, and complexity, astrocytes are well situated to influence synaptic environments and function as "gatekeepers" of neuronal metabolism and blood flow coupling. Astrocytes modulate the synaptic space by a complex multi-modal response. **First**, they express numerous neurotransmitter receptors, which activate a wide array of signalling cascades and intracellular second messenger pathways that process information in parallel to neurons (Hamilton and Attwell 2010). **Second,** astrocytes contain machinery for uptake and recycling neurotransmitters, such as glutamate (Anderson and Swanson 2000). **Third,** astrocytes can also release gliotransmitters, such as glutamate (Bezzi et al. 1998; Parpura et al. 1994), ATP (Newman 2001; Pryazhnikov and Khiroug 2008), and D-serine (Martineau et al. 2008; Mothet et al. 2005), thereby modulating neuronal excitability and synaptic plasticity. **Fourth,** astrocytes maintain synaptic homeostasis through buffering of extracellular ions. **Fifth,** astrocytes sustain cerebral bioenergetics by directly supplying neurons with substrates for oxidative phosphorylation (Pellerin et al. 1998). **Finally,** astrocytes also couple neuronal energy demand with local arteriole blood flow (Gordon et al. 2008; Metea and Newman 2006; Mulligan and MacVicar 2004; Takano et al. 2006; Zonta et al. 2003).

1.1 Astrocytes recycle synaptic glutamate

Glutamate is an abundant central nervous system (CNS) neurotransmitter (Fonnum 1984). High synaptic concentrations of glutamate can cause extreme neuronal excitation and ultimately, cell damage, which contributes to neuronal death in multiple diseases (Dong et al. 2009). Therefore, regulation of synaptic glutamate levels is crucial for neuronal survival. Under normal conditions, glutamate balance in the neuropil is tightly controlled by astrocytes. Astrocytic processes enveloping glutamatergic synapses express various amino acid transporters, which are the main route of extracellular glutamate removal (Danbolt 2001; Rothstein et al. 1994). The primary glutamate transporters are Na^+/glutamate co-transporters of the SLC gene family, termed excitatory amino acid transporter 1 and 2 in human tissue (Shashidharan et al. 1994) or glutamate transporter-1 and L-glutamate/L-aspartate transporter in rodents (Pines et al. 1992; Storck et al. 1992). These proteins co-transport glutamate and three Na^+ ions using the Na^+ electrochemical gradient, maintained by Na^+/K^+ ATPase activity, to drive the removal of glutamate from extracellular spaces (Fig. 1). Therefore, glutamate uptake is energetically expensive as it is coupled to ATP consumption by Na^+/K^+ ATPases. Another Na^+-independent, glutamate/cysteine antiporter, is expressed by astrocytes but these conduct cysteine more than glutamate and are considered a secondary mechanism of glutamate uptake (Cho and Bannai 1990). Neurons may also take up glutamate through excitatory amino acid transporter 3, although this transporter may be predominately used for cysteine uptake (Chen and Swanson 2003).

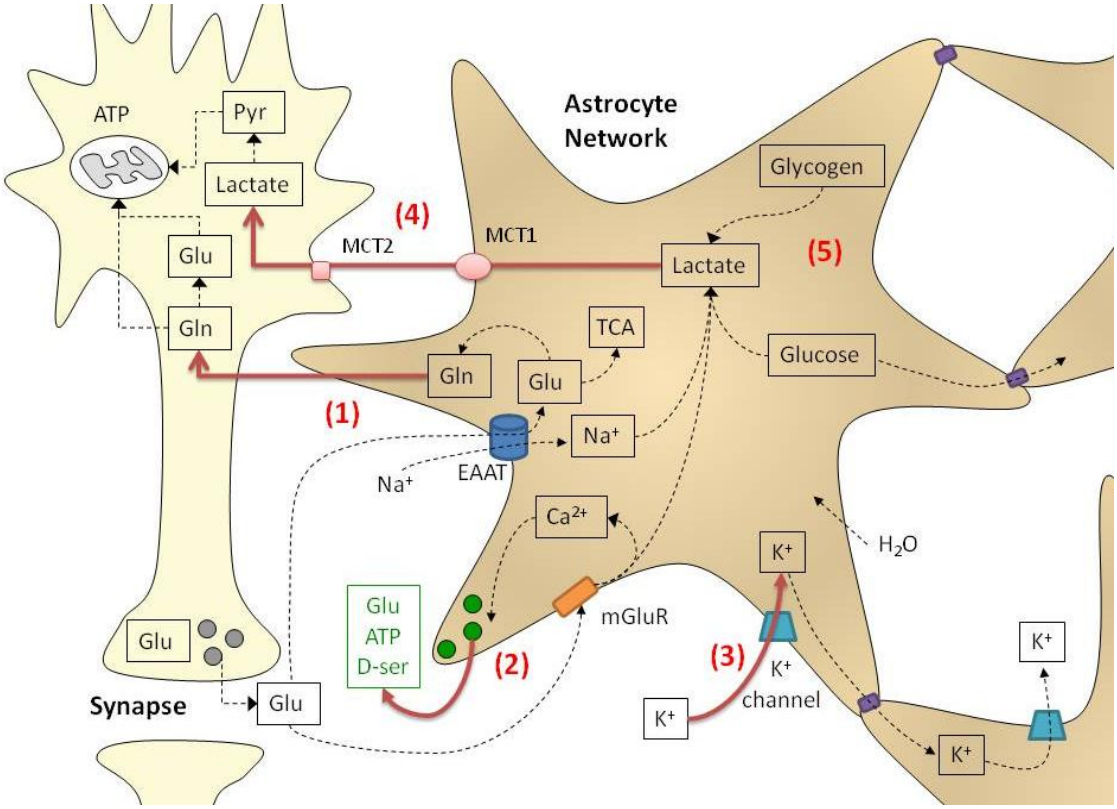

Figure 1: Astrocytes modulate synaptic environments and neuronal metabolism.

(1) Astrocytes take up synaptic glutamate (Glu) through excitatory amino acid transporters (EAATs), which co-transport glutamate and Na^+. Glutamate is metabolized into tricarboxylic acid (TCA) cycle intermediates or glutamine (Gln) to be shuttled to neurons for conversion back to glutamate. **(2)** When intracellular Ca^{2+} increases in response to mGluR activation, astrocytes release vesicles of gliotransmitters, such as glutamate, ATP, and D-serine (D-ser), which can modulate synaptic activity. **(3)** Astrocytes take up K^+ through K^+ channels, which can cause cell swelling by osmosis. K^+ is siphoned through astrocytes (possibly through connexin gap junctions) from high concentrations to regions of low concentrations. **(4)** Astrocytes produce lactate in response to glutamate uptake and increased intracellular Na^+ and Ca^{2+}. Lactate is shuttled to neurons through MCTs for oxidative phosphorylation. **(5)** Energy is stored long-term in astrocytes as glycogen, which can be converted to lactate for neuronal consumption.

Once synaptic glutamate enters astrocytes, one-third is used as a substrate for oxidative metabolism (Hertz et al. 2007; Hertz and Zielke 2004). Astrocytes convert glutamate to α-ketoglutarate by glutamate dehydrogenase or aspartate aminotransferase to replenish components of the tricarboxylic acid cycle (Faff-Michalak and Albrecht 1993; McKenna et al. 2006). A portion of salvaged glutamate is recycled for neurotransmission through a process known as the glutamate-glutamine shuttle (Fig. 1), involving conversion of glutamate to glutamine by glutamine synthase within astrocytes (McKenna 2007). Glutamine is transferred to neurons were it is converted back to glutamate by phosphate-activated glutaminase (McKenna 2007). Glutamate is repackaged into vesicles for synaptic release upon further stimulation. This shuttle process is vital because neurons do not express enzymes for *de novo* synthesis of glutamate, so neuronal glutamate is entirely derived from astrocyte glutamine or α-ketoglutarate (Shank et al. 1985; Yu et al. 1983).

Astrocytes play a critical role in synaptic management, taking up glutamate and shuttling it back to neurons as glutamine. However, extracellular glutamate also acts as a messenger that stimulates astrocyte metabolism (Charles 2005) and release of signalling molecules, which can activate neighbouring astrocytes (Scemes and Giaume 2006), alter neurotransmission (Bezzi et al. 1998), or influence vascular tone of blood vessels (Gordon et al. 2008; Metea and Newman 2006; Mulligan and MacVicar 2004; Takano et al. 2006; Zonta et al. 2003). This multi-modal astrocytic response to glutamate is central to astrocyte information processing and will be discussed in more detail below.

1.2 Astrocyte Ca^{2+} and release of gliotransmitters

Astrocytes express neurotransmitter receptors that facilitate changes in membrane potential and/or increases in cytosolic Ca^{2+} in response to synaptic transmission (Bezzi et al. 1998; Guthrie et al. 1999). Glutamate and ATP stimulate increases in astrocytic Ca^{2+} mainly through metabotropic glutamate receptors (mGluRs) and purinergic receptors, $P2Y_1$ and $P2Y_2$, respectively (Cornell-Bell et al. 1990; Guthrie et al. 1999). These receptor types are G-protein coupled receptors, which activate phospholipase C, stimulating inositol-3-phosphate production and release of Ca^{2+} into the cytoplasm from the endoplasmic reticulum (Idestrup and Salter 1998; Sheppard et al. 1997). Through mGluR and P2Y receptors, astrocytes can sense synaptic transmission as elevated cytosolic Ca^{2+}, which is essential to astrocyte communication and processing. Part of this communication involves propagation of astrocyte Ca^{2+} waves to other neighbouring astrocytes through connexin gap junctions (Blomstrand et al. 1999; Scemes et al. 1998), as fast, long-distance intercellular communication between glia (Scemes and Giaume 2006).

Increased intracellular Ca^{2+} also triggers astrocytes to release gliotransmitters, which act on nearby neurons to affect synaptic activity (Fossat et al. 2011; Nedergaard 1994; Parpura et al. 1994), contribute to Ca^{2+} wave propagation in other astrocytes (Guthrie et al. 1999; Hassinger et al. 1996), have an autocrine effect to amplify Ca^{2+} signals (Suadicani et al. 2006), or influence blood vessel diameter (Simard et al. 2003; Zonta et al. 2003). Many gliotransmitters, including glutamate, ATP, and D-serine, are believed to be packaged in vesicles and stored in astrocyte endfeet. Small vesicles similar to those at neuronal synapses have been detected in hippocampal astrocytes

(Bezzi et al. 2004; Jourdain et al. 2007) and contain vesicular glutamate transporter 1 and 2 for accumulating glutamate (Bezzi et al. 2004). Inhibition of vacuolar (H^+) ATPase, which generates a pH gradient across vesicle membranes for driving gliotransmitter accumulation, reduces the release of glutamate (Araque et al. 2000), ATP (Coco et al. 2003), and D-serine (Mothet et al. 2005) suggesting these gliotransmitters are collected within astrocyte vesicles. Vesicles are released by Ca^{2+} and soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor (SNARE) dependent exocytosis. Cultured astrocytes express SNARE proteins including synaptobrevin II, cellubrevin, syntaxin, and either synaptosomal-associated protein 23 or 25 (Hepp et al. 1999; Maienschein et al. 1999; Parpura et al. 1995). Synaptobrevin II or cellubrevin have been shown to associate with D-serine, glutamate or ATP vesicles in astrocyte culture and *in situ* (Bezzi et al. 2004; Maienschein et al. 1999; Martineau et al. 2008). As indicated by Figure 1, Ca^{2+}-dependent gliotransmitter release is key for propagating extracellular signals within the glia network, but also for modulating neurotransmission (Fossat et al. 2011; Guthrie et al. 1999).

Astrocyte Ca^{2+} signals vary in strength, duration, and location (soma vs. processes) (Gordon et al. 2009; Shigetomi et al. 2010), which correlates with synaptic transmission (Di Castro et al. 2011). Recent characterization of Ca^{2+} signals in astrocyte processes suggest there are two types of signals stimulated by neurotransmission. Expanded Ca^{2+} signals are long-lasting and may spread to the soma in response to neuronal action potentials (Di Castro et al. 2011), while focal microdomain responses (Shigetomi et al. 2010) are short, frequent Ca^{2+} transients in astrocyte processes, which correlate with spontaneous synaptic activity (Di Castro et al. 2011). Expanded Ca^{2+}

events trigger astrocyte activity, gliotransmission, etc., which help potentiate synaptic transmission, but the physiologic role of focal Ca^{2+} transients remains unclear (Di Castro et al. 2011). Astrocyte Ca^{2+} signalling is key for astrocyte neuromodulation via release of gliotransmitters, signal propagation to other glia, and neurovascular coupling, however, more work is required to understand the heterogeneity of Ca^{2+} signals and how they affect astrocyte processing.

1.3 Astrocytes buffer the extracellular ionic environment

Astrocytes regulate the synaptic environment to optimize neurotransmission. During action potentials, K^+ ions enter the extracellular space, where they can alter membrane potential, and astrocytes buffer $[K^+]_o$ (extracellular K^+) through uptake mechanisms (Kofuji and Newman 2004). K^+ uptake involves influx of K^+ through transporters or channels, which requires Cl^- influx or Na^+ efflux to maintain membrane potential, and results in swelling due to osmotic forces (Amedee et al. 1997). Astrocytes are capable of "siphoning" K^+ from regions of high concentration to low concentration. The driving force for this spatial buffering is astrocyte membrane potential, as astrocytes are highly permeable to K^+ and hyperpolarized. Passive K^+ uptake from the synapse (regions of high $[K^+]_o$) depolarizes astrocytes, which is propagated throughout the glial network and triggers a net driving force for K^+ to leave glia (Fig. 1) in regions of lower $[K^+]_o$ (Amedee et al. 1997; Kofuji and Newman 2004). Spatial K^+ buffering is likely mediated by astrocyte inward rectifying K^+ (K_{ir}) channels, which are open at resting membrane potential and maintain hyperpolarization (Butt and Kalsi 2006; Sontheimer 1994). K_{ir} channels co-localize with astrocyte water channel, aquaporin 4 (Nielsen et al. 1997), and K^+ transport is coupled to water transfer and swelling. Therefore, through the uptake of

K^+ and other ions, astrocytes optimize the synaptic environment including extracellular volume for proper neuronal function. Spatial buffering of K^+ may also play a role in neurovascular coupling, as discussed below.

1.4 Astrocytes mediate neuronal metabolism

The human brain receives 10% of cardiac output and utilizes 20% of total blood glucose and oxygen during cerebral activity, even though it only makes up 2% of total body mass (Attwell et al. 2010). Maintenance of adequate energy substrates and oxygen supply is essential for restoring ion gradients after action potential conduction (Magistretti 2006; Magistretti and Pellerin 1999). Perisynaptic astrocytes that extend process endfeet to local arterioles create a conduit for flow of energy substrates from blood to neurons. These energy metabolites also spread long distances through the glial network (Giaume and McCarthy 1996), which may greatly influence brain metabolism (Rouach et al. 2008; Tsacopoulos and Magistretti 1996). Astrocytes also store energy as glycogen, and supply neurons with lactate for oxidative phosphorylation (Brown and Ransom 2007; Pellerin et al. 1998).

1.4.1 Astrocyte lactate fuels neuronal activity

Astrocytes play an important role in brain metabolism by converting glucose to lactate in an activity-dependent and glutamate-mediated manner for delivery to neurons to satiate their energy requirements (Magistretti et al. 1999; Pellerin and Magistretti 1994; Pellerin et al. 1998). This is known as the astrocyte-neuron lactate shuttle hypothesis (ANLSH) and suggests lactate is an oxidative substrate for neurons, not a potentially damaging final metabolite of anaerobic glycolysis (Kasischke 2008). In

rodent brain slices, inhibition of lactate transport and glycolysis during exposure to glutamate causes a permanent loss of neuronal function (Schurr et al. 1999), while addition of lactate maintains synaptic activity in the absence of glucose (Fowler 1993; Izumi et al. 1997; Schurr et al. 1988), preventing neurotoxicity (Cater et al. 2001; Maus et al. 1999; Schurr et al. 1997).

Lactate metabolism is mediated by lactate dehydrogenase (LDH), which reversibly converts pyruvate to lactate with oxidation of NADH to NAD^+ (Tsacopoulos and Magistretti 1996). Neuronal glutamatergic transmission stimulates glucose utilization, glycolysis (Cholet et al. 2001; Pellerin and Magistretti 1994), and lactate production (Caesar et al. 2008; Schurr et al. 1999) by astrocytes (Cholet et al. 2001; Pellerin and Magistretti 1994; Voutsinos-Porche et al. 2003). This increase in astrocyte metabolism occurs due to the energetic expense of glutamate uptake and balancing Na^+ and Ca^{2+} gradients, as intracellular Na^+ is elevated through Na^+ co-transport with glutamate and glutamate receptor stimulation increases intracellular Ca^{2+}. This also triggers intercellular Na^+ and Ca^{2+} waves, elevating glucose uptake and metabolism in neighbouring astrocytes (Bernardinelli et al. 2004; Charles 2005). Lactate is transported between the intracellular and extracellular spaces by monocarboxylate transporters (MCT). MCT are symporters; co-transporting lactate anions with H^+, suggesting lactate transport is mediated by pH (Barros and Deitmer 2009; Schneider et al. 1993). Lactate is taken up rapidly by neurons through high affinity MCT2, which associates with post-synaptic density proteins in dendritic spines (Bergersen et al. 2001). Based on the ANSLH as outlined in Figure 1, lactate is transported out of astrocytes (through MCT1,

2, or 4), taken up by MCT2 into neurons, and converted to pyruvate for use in oxidative metabolism (Magistretti and Pellerin 1999).

Extracellular lactate dynamics change during constant brain activity. At rest, the extracellular space around neurons and astrocytes has a homogenous concentration of lactate and glucose (Barros and Deitmer 2009; Barros and Martinez 2007; Simpson et al. 2007). Brain lactate is produced locally, as blood lactate does not pass the blood-brain barrier as easily as glucose (Chih and Roberts Jr 2003). Extracellular *in vivo* lactate decreases slightly during short periods of brain activation *in vivo* (Hu and Wilson 1997; Mangia et al. 2003), possibly because neurons are utilizing lactate for oxidative metabolism (Kasischke et al. 2004). However, extracellular lactate rapidly rises as neuronal stimulation continues for longer periods (Mangia et al. 2007; Prichard et al. 1991), and astrocyte glycolysis is elevated as indicated by increased cytoplasmic NADH (Kasischke et al. 2004). Thus, astrocytes may replenish extracellular lactate pools for shuttling to neurons during prolonged activation (Bouzier-Sore et al. 2002; Magistretti and Pellerin 1999; Magistretti et al. 1999; Pellerin et al. 1998).

Astrocyte lactate production may influence neuronal metabolism, but it also acts as a signalling molecule in other brain processes, including blood glucose sensing in the hypothalamus (Lam et al. 2007; Lam et al. 2005), and sodium sensing in the subfornical organ (Shimizu et al. 2007). During blood glucose sensing and control, elevated blood glucose leads to increased glial lactate production in the hypothalamus (Lam et al. 2005). Lactate conversion to pyruvate in hypothalamic neurons is required to activate ATP-sensitive K^+ channels (K_{ATP}), which provide negative feedback to the liver, decreasing

liver gluconeogensis (Lam et al. 2005) and secretion of hepatic very-low density lipoprotein (Lam et al. 2007). Astrocyte negative feedback on liver release of glucose and lipids is important to characterize, since metabolic syndromes, such as obesity and hepatic insulin resistance, could be related to dysfunction of this mechanism (Lam et al. 2007). Similarly, in the subfornical organ, lactate influences salt intake behaviour and blood Na^+ sensing (Shimizu et al. 2007). Glial cells of the subfornical organ express atypical sodium channels, which trigger anaerobic glucose metabolism and lactate production upon Na^+ influx (Shimizu et al. 2007). Release of astrocyte lactate stimulates inhibitory neurons in the subfornical organ by a MCT-dependent mechanism, reducing salt-intake behaviour (Shimizu et al. 2007). These studies of the role of lactate in glucose and sodium sensing indicate an exciting new role for lactate as a signalling molecule. Lactate is also involved in brain blood flow regulation discussed below (Gordon et al. 2008).

1.4.2 Astrocyte glycogen as energy source

Glycogen is the main mammalian storage depot of glucose, produced when glucose exceeds immediate energy requirements, and is metabolized when glucose levels cannot meet energy demands (Brown and Ransom 2007). Glycogen is primarily produced in liver and muscle. In the brain, astrocytes are the main glycogen repository. Astrocytes express both glycogen synthase (for glycogen formation) and glycogen phosphorylase (for glycogen degradation) (Pellegri et al. 1996). Glycogen stores are primarily located in regions of high synaptic density, such as grey matter, (Phelps 1972; Sagar et al. 1987), and during hypoglycemia, astrocyte glycogen is critical for maintaining neuronal survival and synaptic activity *in vitro* (Swanson and Choi 1993),

and *in vivo* in cortex, hippocampus (Suh et al. 2007), and optic nerve (Wender et al. 2000). Glycogenolysis does not result in free glucose, since astrocytes do not express glucose-6-phosphatase, the enzyme necessary for free glucose production (Dringen and Hamprecht 1993). Instead, glucose-6-phosphate from glycogenolysis is metabolized to lactate, as shown in cultured astrocytes (Dringen et al. 1993) and mouse optic nerves (Tekkok et al. 2005), linking glycogen utilization and the ANLSH. Also, the astrocytic glycogen reservoir is influenced by glutamatergic transmission and uptake (Fig. 1). When astrocyte calcium rises due to glutamate release, glycogen phosphorylase is up-regulated, triggering glycogenolysis (Shulman et al. 2001). Concurrently, energy from glycogenolysis sustains the glutamate-glutamine cycle, since astrocyte glycogen breakdown is linked to glutamate uptake (Sickmann et al. 2009; Walls et al. 2009) and *de novo* synthesis of glutamate and glutamine (Gibbs et al. 2006; Gibbs et al. 2007). While glycogen degradation is important for glutamate recycling, extracellular glutamate can elevate glycogen synthesis in astrocytes *in vitro* by encouraging glucose uptake and phosphorylation (Hamai et al. 1999). This suggests a tight coupling exists between glutamate neurotransmission, glutamate uptake, glycogen reservoir dynamics, and the ANLSH.

2. Control of Cerebral Blood Flow

Cerebral blood supply stems from the circle of Willis at the base of the brain, which branches several times into smaller pial arteries on the cerebral surface (Iadecola and Nedergaard 2007). Pial arteries are separated from brain cells by the Virchow-Robin space, but branch into arteries that penetrate the cortex and are in direct contact with astrocyte endfeet. These arteries split into smaller cortical and subcortical arterioles and

capillaries (a single layer of endothelial cells) which are also in contact with astrocyte endfeet and small contractile cells known as pericytes (Peppiatt et al. 2006; Simard et al. 2003). Cerebral blood flow is regulated by two mechanisms: autoregulation and functional hyperemia. Autoregulation maintains a constant flow of blood to the brain when systemic blood pressure is altered (Lassen 1959). Functional hyperemia is rapid increases in local blood flow upon elevated neuronal activity, enhancing energy and oxygen supplies to meet brain energy demands (Iadecola 2004; Roy and Sherrington 1890). Neurons and glia consume large amounts of ATP restoring ion gradients after synaptic transmission, so energy balance is critical for neuronal function and survival (Attwell and Laughlin 2001).

Functional hyperemia is a complex, multi-modal response involving numerous signalling pathways, which results in dilation of penetrating arterioles upstream of areas of activity and constriction in regions with abundant substrate supply and lower activity (Devor et al. 2007). Historically, increased blood flow was believed to be linked to elevated neuronal metabolic by-products (Roy and Sherrington 1890), such as CO_2, H^+, lactate, K^+, and adenosine. It was expected that increased CO_2 (by bicarbonate buffering) and H^+ would acidify the extracellular environment, triggering dilation of neighbouring blood vessels (Tian et al. 1995). In contrast, recent studies have shown that the extracellular space becomes alkaline shortly after neurotransmission, due to action of the Ca^{2+}/H^+ ATPase to restore neuronal intracellular Ca^{2+} levels. This indicates CO_2 and H^+ may not act as metabolic dilators (Astrup et al 1978, Makani Chesler 2010). Lactate is produced via increased glycolysis during neuronal activity, and can elevate local blood flow (Ido et al. 2001), likely through modulation of prostaglandin levels, discussed in the

next section (Gordon et al. 2008).

Extracellular K^+ ($[K^+]_{o)}$, released during metabolism, neuronal depolarization and astrocyte activity can alter diameter of neighbouring arterioles during functional hyperemia (Edwards et al. 1988; Kuschinsky et al. 1972; McCarron and Halpern 1990). The type of vascular response is determined by K^+ concentration- $[K^+]_o$ less than 20 mM causes vasodilation and $[K^+]_o$ greater than 20 mM triggers vasoconstriction (Girouard et al. 2010; Knot et al. 1996). K^+ controls vascular tone through vascular smooth muscle voltage-gated Ca^{2+} channels (VGCC), which are very sensitive to changes in membrane potential (Nelson et al. 1990; Rubart et al. 1996). Polarity in smooth muscle responses to extracelluar K^+ exists because at 3-20 mM $[K^+]_o$, conductance of smooth muscle inward rectifying $K_{ir}2.1$ K^+ channels (Bradley et al. 1999) is increased causing hyperpolarization, reduced VGCC activity, and less smooth muscle contraction (Girouard et al. 2010). At larger $[K^+]_o$ concentrations, smooth muscle cells depolarize, increasing conductance of VGCC and causing constriction (Knot and Nelson 1998; Knot et al. 1996). Na^+/K^+ ATPase and other K^+ transporters likely terminate the extracellular signal by taking up perivascular K^+ (Girouard et al. 2010). Smooth muscle also expresses ATP- sensitive K^+ (K_{ATP}) channels, which can dilate arterioles when activated (Sun and Hu 2010), however the role of these channels in functional hyperemia is uncertain.

Adenosine is also considered a neuro-metabolic signal, as it is the end-product of ATP degradation, produced during neuronal energy consumption and synaptic release (Ko, Ngai, Winn 1990). Adenosine is an important inhibitory neuromodulator, but it is also involved in neurovascular coupling (Haglund et al. 2008; Sciotti et al. 1993).

Several studies have shown adenosine triggers vasodilation of arterioles in brain slices (Gordon et al. 2008), somatosensory cortical arterioles *in vivo* after whisker stimulation (Dirnagl et al. 1994; Shi et al. 2007) and somatosensory pial arteries *in vivo* after sciatic nerve stimulation (Ko et al. 1990; Meno et al. 2005). Adenosine binds vascular smooth muscle A_2 adenosine receptors (Miekisiak et al. 2008), triggering vasodilation by increasing cAMP and phosphorylation of K^+ channels, which hyperpolarizes the cell and decreases Ca^{2+} entry through VGCC (Pelligrino et al. 2010). There are two types of adenosine A_2 receptors (A_{2A} and A_{2B}) and A_{2A} has a greater affinity for adenosine (Hinschen et al. 2003). Debate exists over the vascular receptor subtype involved in dilation, as an A_{2A} antagonist inhibits pial arteriole dilation to sciatic nerve stimulation *in vivo* (Meno et al. 2001), while A_{2B} inhibition reduces somatosensory functional hyperemia *in vivo* (Shi et al. 2007). Also, A_{2A} receptors appear to mediate hyperemia during hypoxia (Miekisiak et al. 2008). Adenosine is released from neurons, but it can also be produced from ecto-nucleotidase metabolism of astrocyte ATP during gliotransmission (Pelligrino et al. 2011).

Lactate, potassium, and adenosine are metabolites produced by neurons involved in functional hyperemia. However, blood flow increases regardless of the time course of metabolite accumulation, suggesting other mechanisms exist (Pelligrino et al. 2011; Sandor 1999). Recent studies show functional hyperemia involves specific neuronal signalling to blood vessels within the neurovascular unit, either through direct innervation and release of vasoactive neuromodulators (such as ATP, norepinephrine, acetylcholine, or nitric oxide (discussed in Section 3.4)) or metabolites (lactate, adenosine, K^+), or

indirectly by stimulating astrocytes to release vasoactive molecules near blood vessels.

2.1 Astrocytes modulate blood vessel diameter

As previously mentioned, astrocyte spatial orientation permits relay of signals from neurons to blood vessels, since astrocyte processes contact both synapses and the vasculature. As part of the multi-faceted response of astrocytes to increased neuronal activity, synaptic glutamate and/or ATP triggers elevated intracellular astrocyte Ca^{2+} through $mGluR_5$ (Zonta et al. 2003) and P2Y receptors (Simard et al. 2003). Astrocyte cytosolic Ca^{2+} elevations (Filosa et al. 2004; Simard et al. 2003; Zonta et al. 2003), and inositol-3-phosphate signalling (Straub et al. 2006) are central to neurovascular coupling, stimulating release of vasoactive compounds (Fig. 2) that dilate or constrict neighbouring arterioles (Gordon et al. 2008; Metea and Newman 2006; Mulligan and MacVicar 2004; Takano et al. 2006; Zonta et al. 2003). Multiple parallel pathways occur simultaneously to regulate blood flow. Several of these mechanisms will be discussed below.

2.1.1 Arachidonic Acid Metabolites

ATP or glutamate increases intracellular Ca^{2+} in astrocytes, stimulating activity of phospholipase A_2 (PLA_2), which hydrolyzes phospholipids to produce arachidonic acid (AA) (Byrnes et al. 2009; Mulligan and MacVicar 2004; Sun et al. 2005). AA metabolism by several enzymes produces different molecules with variable vascular effects (Fig. 2). In brain slices and hyperemia *in vivo*, AA can be metabolized by astrocyte cyclooxygenase (COX) (Takano et al. 2006; Zonta et al. 2003) to prostaglandin E_2 (PGE_2). Treatment with a non-selective COX or COX-1 inhibitor in slices or *in vivo*

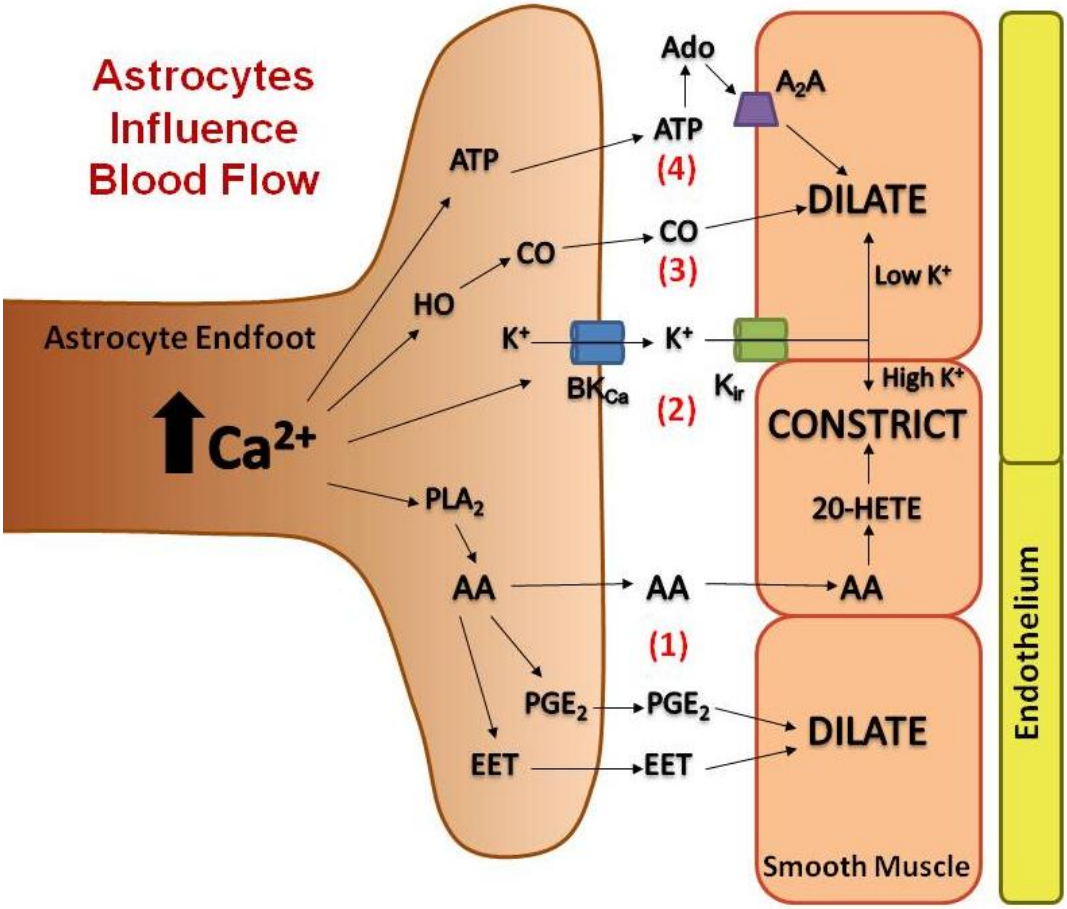

Figure 2: Astrocyte intracellular Ca^{2+} elevations trigger release of vasoactive molecules.

(1) PLA_2 is activated by Ca^{2+} and produces AA. AA is metabolized in astrocyte endfeet to PGE_2 or EET which dilate arterioles, or AA can diffuse to smooth muscle where it forms 20-HETE and causes constriction. **(2)** K^+ is released from astrocyte endfeet through BK_{Ca}, and the amount of K^+ released is directly proportional to astrocyte Ca^{2+} level. K^+ is taken up into smooth muscle through K_{ir} and causes dilation at low concentrations and constriction at high concentrations. **(3)** HO is activated by Ca^{2+} and produces CO, which diffuses to smooth muscle and triggers dilation. **(4)** ATP released from astrocytes is metabolized to adenosine (Ado). Ado binds to smooth muscle A_2 adenosine receptors causing arteriolar dilation.

blocks arteriolar vasodilation after astrocyte Ca^{2+} stimulations, suggesting it is PGE_2-mediated (Gordon et al. 2008; Takano et al. 2006; Zonta et al. 2003). Also, in astrocytes and retinal glial cells, AA is metabolized by cytochrome P450 epoxygenase to epoxyeicosatrienoic acids (EETs), which cause vasodilation (Liu et al. 2011; Metea and Newman 2006; Peng et al. 2002). Both PGE_2 and EETs generate vasodilation by opening arteriole smooth muscle large conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels, triggering hyperpolarization and decreased VGCC activity (Gebremedhin et al. 1992; Higashimori et al. 2010; Miura and Gutterman 1998). EETs also indirectly stimulate BK_{Ca} channels by increasing Ca^{2+} sparks (Earley et al. 2005). Conversely, AA can also diffuse to smooth muscle cells where it is rapidly metabolized by ω-hydroxylase (another cytochrome P450 enzyme) to produce the vasoconstrictor, 20-hydroxyeicosatetraenoic acid (20-HETE) (Mulligan and MacVicar 2004). 20-HETE causes smooth muscle contraction and vasoconstriction by inhibiting vascular BK_{Ca} K^+ channels, leading to depolarization and increased Ca^{2+} entry through VGCC. 20-HETE is also implicated in vasoconstriction of the retina in response to light stimulation (Metea and Newman 2006).

At first, these opposing effects of astrocyte AA metabolism on vaso-reactivity represented a confusing dichotomy in the field. However, recent reports have begun to bring clarity by showing that the directional control of AA metabolism is finely controlled by metabolic need and nitric oxide. In brain slices and retinal preparations aerated with 95-100% oxygen (common oxygen concentrations used in electrophysiological recordings), elevated astrocyte Ca^{2+} leads to vasoconstriction mediated by 20-HETE production (Gordon et al. 2008; Mishra et al. 2011; Mulligan and MacVicar 2004). However, in brain slices and retinal preparations treated with

20% oxygen, astrocyte Ca^{2+} elevations cause vasodilation induced by PGE_2 produced by COX-1 (Gordon et al. 2008; Mishra et al. 2011), similar to *in vivo* studies (Takano et al. 2006). At 20% oxygen, astrocytes display heightened glycolysis and lactate production (Gordon et al. 2008), similar to studies of the ANLSH (Magistretti and Pellerin 1999). Also, astrocyte endfeet express a prostaglandin-lactate transporter, known to exchange intracellular lactate for extracellular PGE_2 (Chan et al. 2002). Thus, at 20% oxygen, as represented in Figure 3, increased extracellular lactate from astrocyte glycolysis inhibits the prostaglandin-lactate transporter, resulting in elevated extracellular PGE_2 and vasodilation (Gordon et al. 2008). Current consensus suggests astrocytes maintain vascular tone equilibrium (between vasodilation and vasoconstriction) under physiological conditions. When synaptic activity is low and oxygen is not being consumed, vasoconstriction by 20-HETE is favoured because PGE_2 is taken up rapidly through prostaglandin-lactate transporters. During periods of greater brain activity, oxygen is depleted and lactate is released from astrocytes following glycolysis. This leads to inhibition of the prostaglandin-lactate transporter, more extracellular PGE_2 and vasodilation. This mechanism couples cerebral blood flow regulation and the ANLSH, since astrocyte lactate production may act as a neuronal energy source and signalling molecule to increase blood flow. It is also the first direct mechanism linking brain tissue energy deficits and increased blood flow (Sandor 1999). While astrocytes may "sense" the oxygen content in their local environment through the production of lactate (Gordon et al. 2008), and trigger either vasodilation or vasoconstriction, the nature of how this switch occurs is still unclear. Recent studies *in vivo* suggest oxygen levels may not influence neurovascular coupling compared to *in vitro* preparations

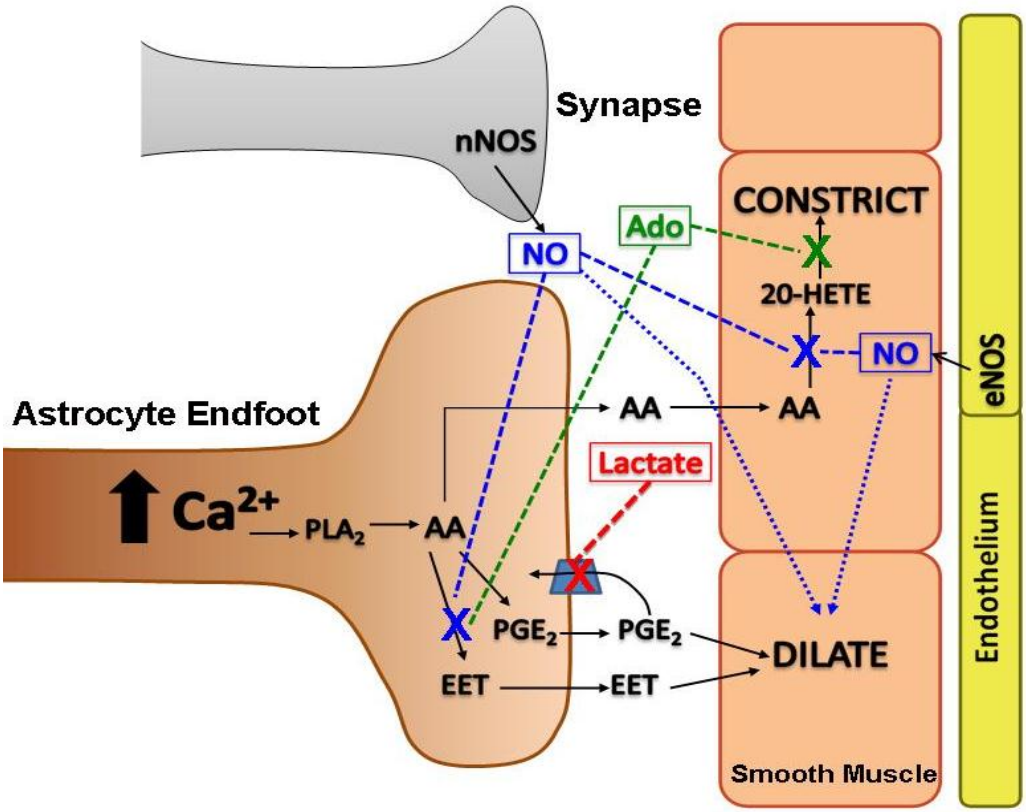

Figure 3: Lactate, NO, and adenosine can affect AA metabolite signalling.

At low O_2 concentrations (20%), lactate and adenosine (Ado) enhance arteriole vasodilation. Lactate production is increased due to elevated glycolysis, leading to export through MCTs. Extracellular lactate inhibits the prostaglandin/lactate transporter, reducing lactate release and increasing perivascular PGE_2 (Gordon et al. 2008). Ado, from increased metabolism and ATP release, blocks 20-HETE vasoconstriction, possibly through vascular A_{2A} receptors (Gordon et al. 2008). Also, Ado can inhibit EET production or release through astrocyte A_{2B} receptors (Shi et al. 2007), but how this impacts neurovascular coupling is unknown. Nitric oxide (NO), from nNOS and eNOS, can inhibit EET and 20-HETE production (Metea and Newman 2006) and dilate smooth muscle directly.

(Lindauer et al. 2010; Mishra et al. 2011). In *ex vivo* retinal preparations, incubation with 100% oxygen drastically increases tissue partial pressure of oxygen (pO_2) 16-fold, while administering 100% oxygen to anesthetised rats only modestly elevates retinal pO_2 (Mishra et al. 2011). Consequently, retinal neurovascular coupling favours vasodilation under normoxic and hyperoxic conditions *in vivo*, compared to vasoconstrictions *in vitro* under high pO_2. Physiologic cerebral oxygen levels are between 12-38 mmHg (Jamieson and Vandenbrenk 1963; Metzger et al. 1971; O'Hara et al. 2005), suggesting 20-HETE synthesis, which is dependent on binding of molecular oxygen as a cofactor and has a K_mO_2 (Michaelis constant for oxygen) of 60-70 mmHg (Harder et al. 1996), is reduced under normoxic conditions. Conversely, production of prostaglandins and EETs, both with $K_mO_2 \leq 10$ mmHg (Harder et al. 1996; Juranek et al. 1999), would be favoured at physiologic oxygen levels. Therefore, the role of cerebral oxygen tension in switching between dilation and constriction might be less significant *in vivo* and further studies are required to fully test this hypothesis.

The role of nitric oxide (NO) in functional hyperemia further complicates neurovascular signalling, as NO modulates AA metabolite activity (Metea and Newman 2006). Nitric oxide is produced by three different enzymes: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). nNOS influences neurotransmission (Karlsson et al. 2007), and eNOS activity regulates vascular tone (Ignarro et al. 1999). Both of these enzymes also play a role in cerebral vasodilation, which will be discussed in more detail below. iNOS is expressed by numerous brain cell types including neurons, astrocytes and microglia, and is activated during inflammation and trauma (Block et al. 2007; Heneka and

Feinstein 2001; Hewett et al. 1994). Traditionally, NO acts directly as a vasodilator, stimulating vascular smooth muscle guanylyl cyclase leading to activation of K^+ channels and hyperpolarization (Ignarro et al. 1999). However, NO can also inhibit cytochrome P450 enzymes (Fig. 3), such as ω-hydroxylase, decreasing 20-HETE production (Alonso-Galicia et al. 1999; Alonso-Galicia et al. 1998), or cytochrome P450 epoxygenase, reducing EET production (Udosen et al. 2003). Also, NO weakly activates COX-1, while suppressing COX-2 (Fujimoto et al. 2004), which may affect prostaglandin levels. Thus, cerebral vasodilation by NO likely involves both direct smooth muscle effects and indirect inhibition of 20-HETE, there by favouring dilation by prostaglandins and EETs (Attwell et al. 2010; Sun et al. 2000). In retinal preparations aerated with 95% O_2, NO enhances constriction after neuronal and glial cell activation, possibly by reducing EET production and favouring the 20-HETE pathway (Metea and Newman 2006). *In vivo* NO inhibits 20-HETE production, since blood flow reduction by a NOS antagonist is reversed by 20-HETE inhibition (Liu et al. 2008). High oxygen tension stimulates nNOS activity in brain slices (Abu-Soud et al. 1996; Elayan et al. 2000). Therefore, Attwell et al. (2010) suggest high O_2 levels favour vasoconstriction by stimulating NO production which reduces EET concentrations. Lower O_2 levels limit 20-HETE synthesis, favouring vasodilation. However, further studies are required to determine the validity of this hypothesis and the effects of oxygen on NO and AA metabolites. NO modulation could be important for neurovascular coupling during pathological situations, since inhibition of iNOS, which is elevated in retinal glia of diabetic animal models, rescues functional hyperemia (Mishra and Newman 2010). NO inhibition of EET production is suggested to mediate this response, but has not been tested (Mishra and Newman 2010).

Adenosine derived from astrocytes or neuronal metabolism may also interact with AA metabolites (Fig. 3). First, adenosine activity is influenced by tissue oxygen tension and may influence dilation pathways, since A_{2A}-mediated dilation is capable of blocking 20-HETE-induced vasoconstriction in brain slices treated with high oxygen (95%) (Gordon et al. 2008). Also, by adding adenosine with a prostaglandin transporter blocker, which increased the extracellular PGE_2 concentration, constrictions could be converted to dilations at high oxygen (Gordon et al. 2008). However, these brain slice experiments were conducted at room temperature, which may greatly reduce adenosine release from brain slices (Masino and Dunwiddie 1999), making the role of adenosine in this model difficult to interpret. Second, astrocyte adenosine A_{2B} receptors appear to regulate EET production during somatosensory stimulation *in vivo* (Shi et al. 2007), but the extent of interaction between endogenous adenosine and AA metabolites, along with the role of both vascular A_{2A} and A_{2B} receptors in neurovascular coupling is unclear. Further work is required to dissect out these complex pathways.

Basal blood vessel tone can also dictate polarity of vascular responses to AA metabolites. In brain slices aerated with 95% O_2, astrocyte stimulation triggers dilation of intracortical arterioles pre-constricted greater than 50%, but causes constriction of arterioles pre-constricted moderately or not at all (Blanco et al. 2008). This suggests there is a "set-point" of blood vessel tone that is optimal for functional hyperemia, but the details are uncertain. While astrocytic production of AA metabolites controls vascular tone, inhibition of these pathways does not completely block vasodilation, suggesting other signalling pathways, such as those discussed below, are involved.

2.1.2 Potassium

Astrocytes express machinery, such as inwardly rectifying $K_{ir}4.1$ K^+ channels or large conductance Ca^{2+} sensitive K^+ channels (BK_{Ca}), to "siphon" K^+ by taking it up from synaptic spaces (high concentrations) and releasing it into the perivascular space (low concentrations) (Kofuji and Newman 2004). As mentioned above, smooth muscle cells respond to extracellular K^+, which is an important pathway of neurovascular coupling, since inhibition of BK_{Ca} or K_{ir} channels reduces cortical functional hyperemia *in vivo* (Gerrits et al. 2002; Leithner et al. 2010). In retina, it was suggested vasodilation can be triggered via K^+ efflux through glial endfoot $K_{ir}4.1$ channels in response to neurotransmission (Newman et al. 1984; Paulson and Newman 1987), but studies of $K_{ir}4.1$ knockout mice show no difference in K^+-induced dilation compared to controls (Metea et al. 2007). Recently, another mechanism was proposed involving astrocyte BK_{Ca} channels, which are Ca^{2+}-dependent (Filosa et al. 2006), and mediate constriction or dilation (Fig. 2) (Girouard et al. 2010). Cytosolic Ca^{2+} signal intensity dictates astrocyte BK_{Ca} channel activation and K^+ efflux. For example, moderate astrocyte Ca^{2+} increases trigger dilation of neighbouring arterioles, likely due to modest K^+ release (less that 20 mM) from BK_{Ca} channels. Larger astrocyte Ca^{2+} signals convert dilations to constrictions, via greater BK_{Ca} channel opening and higher astrocyte K^+ release (greater than 20 mM) (Girouard et al. 2010). Therefore, through astrocyte BK_{Ca} channels, synaptic transmission-induced astrocyte Ca^{2+} elevations are coupled with K^+-mediated alterations of blood flow, and conceivably, astrocytes could select for dilation or constriction based on magnitude of K^+ release (Dunn and Nelson 2010).

AA metabolite and K^+ signalling occur in parallel to regulate cerebral blood flow

(Filosa et al. 2006), and may interact since AA metabolites also affect smooth muscle ion conductance. For example, in renal arteries, PGE_2 can induce smooth muscle BK_{Ca} channel- mediated dilation through EP2 or EP4 prostanoid receptors (Zhang et al. 2005), but this mechanism has not been tested in cerebral arteries. Also, astrocyte BK_{Ca} channel activity is increased by EETs (Higashimori et al. 2010), suggesting AA metabolites can modulate K^+ release into the perivascular space, but the extent of interaction between these two pathways requires further investigation.

2.1.3 Carbon Monoxide

Carbon monoxide (CO) is produced from heme oxygenase (HO) and can dilate blood vessels by increasing the coupling of smooth muscle BK_{Ca} channels with local Ca^{2+} transients or "sparks" which elevate BK_{Ca} channel activity (Jaggar et al. 2002; Wu et al. 2002; Xi et al. 2010). As mentioned above, BK_{Ca} channels can cause smooth muscle hyperpolarization, reduce VGCC activity and dilate blood vessels. Glutamate-induced vasodilation of piglet cerebral arteries involves CO, since inhibitors of HO block vasorelaxation of isolated arteries (Fiumana et al. 2003) and pial arteries *in vivo* (Leffler et al. 1999; Robinson et al. 2002). Glutamate stimulates CO production from endothelial and smooth muscle cells in microvessels, triggering endothelium-dependent dilation (Fiumana et al. 2003; Leffler et al. 2003). However, glutamate also induces CO production from astrocyte endfeet (Leffler et al. 2006) by intracellular Ca^{2+} and calmodulin-dependent activation of HO (Fig. 2) (Xi et al. 2011) This CO increase can mediate vasodilation *in vivo* (Li et al. 2008). Thus, CO is another diffusible, vasoactive molecule, released when neurotransmission activates astrocytes. Similar to AA metabolites, NO (possibly derived from the endothelium (Barkoudah et al. 2004))

increases CO production *in vivo* (Leffler et al. 2005a; Leffler et al. 2005b), however the extent of this modulation is unclear. Furthermore, all CO studies have involved newborn piglets, which show a greater dilation to CO than juvenile or adult pigs (Holt et al. 2007). While CO is possibly an important mediator of functional hyperemia, further investigation of other ages and species would benefit the characterization of this mechanism.

2.2 Capillary Blood Flow Regulation

Recent evidence suggest pericytes can regulate blood flow at the capillary level (Peppiatt et al. 2006), since they express contractile machinery and directly contact capillary endothelial cells even though they do not form a continuous layer like smooth muscle cells (Hamilton et al. 2010). Several signalling molecules may mediate pericyte tone, since cultured pericytes and isolated retinal capillaries contract or relax to neurotransmitters and possibly arachidonic acid derivatives (Puro 2007; Shepro and Morel 1993). Pericytes in rat brain slices dilate to glutamate and contract to norepinephrine and ATP (Peppiatt et al. 2006). Also, relaxation or constriction signals are propagated between pericytes (Peppiatt et al. 2006; Puro 2007), but it is unclear if signals spread between pericytes directly (possibly through gap junctions) or pass through endothelial cells via gap junctions and feed back to neighbouring pericytes (Attwell et al. 2010). While pericytes have the necessary machinery to regulate blood flow, there is still debate over their role in neurovascular coupling. Most studies have been conducted *in vitro* under high oxygen concentrations, and it remains unknown if pericytes are sensitive to tissue oxygen tension, as in arteriole pathways where oxygen modulates constriction/relaxation pathways (Hamilton et al. 2010). Also, a recent *in vivo*

study determined blood flow increases in response to neuronal activity occurred by arteriolar dilation and not changes at the capillary level (Fernandez-Klett et al. 2010). However, pericytes can contract capillaries *in vivo* (Fernandez-Klett et al. 2010) and this could be important under pathological conditions such as ischemia (Yemisci et al. 2009). Further studies of pericytes will help to better elucidate their role in neurovascular coupling.

3. NMDA Receptors and Blood Flow

Glutamate is the primary neurotransmitter of the central nervous system and binds to two families of receptors: metabotropic and ionotropic receptors (Bonvento et al. 2002). There are three groups of metabotropic glutamate receptors (mGluRs): Group I, which activate excitatory second messenger pathways, and Group II and III, which stimulate inhibitory signalling (Niswender and Conn 2010; Ohashi et al. 2002). There are also two types of ionotropic glutamate receptors: α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors (Garthwaite 1991; Lerma et al. 1997; McDonald and Johnston 1990). AMPA receptors conduct Na^+ and K^+, while NMDA receptors permit mainly Ca^{2+} influx, bind glutamate with a higher affinity and have a slower and longer channel conductance than AMPA receptors (Dingledine et al. 1999). At rest, NMDA receptors are blocked by Mg^{2+}, which is removed by AMPA-induced depolarization (Dingledine et al. 1999). As a result, AMPA and NMDA receptors often co-localize on post-synaptic neurons, and together these receptors are critical for proper neuronal function (Bliss and Collingridge 1993).

Due to high Ca^{2+} permeability, NMDA receptors are involved in neuronal signalling

pathways, such as synaptic plasticity, but can also contribute to cell death when over-stimulated, which is known as excitotoxicity. Synaptic plasticity is represented experimentally by long-term potentiation (LTP) and long-term depression (LTD), which are believed to facilitate signal transduction within a neural circuit, and mediate information storage and induction of long-term memory formation (Malenka 1994). LTP is prolonged synaptic strength, as a result of brief, high-frequency electrical stimulation. Repetitive activation of NMDA receptors results in sufficient intracellular Ca^{2+} concentrations to trigger second messenger pathways, including kinase activation (like Ca^{2+}-calmodulin kinase II (Malinow et al. 1989)), which induce LTP (Bliss and Collingridge 1993; Malenka 1994), Conversely, LTD is prolonged synaptic weakness, as a result of longer, low-frequency electrical stimulation. LTD is also dependent on NMDA receptor Ca^{2+} conductance, however, the amplitude of intracellular Ca^{2+} is lower and more prolonged than LTP (Yang et al. 1999), which activates signalling pathways, such as phosphatases (Mulkey et al. 1994). Through Ca^{2+} conductance, NMDA receptors can dictate synaptic plasticity and mediate both LTP and LTD to increase flexibility of information processing. Prolonged NMDA receptor activation can also lead to massive Ca^{2+} influx and cell death known as excitotoxicity, which is prevalent in numerous neurodegenerative disorders where excessive glutamate is released (Choi 1987; Choi et al. 1988). This dysfunctional Ca^{2+} homeostasis causes cell swelling, mitochondrial damage, oxidative stress and free radical production, and activation of calcium-dependent proteases (Lau and Tymianski 2010). All of these factors cause cell injury and possibly death, and provide a mechanism by which NMDA receptors mediate disease situations.

3.1 Properties of NMDA receptors

NMDA receptor activation requires binding of glutamate and a co-agonist, glycine or D-serine, and these receptors conduct ions, including Ca^{2+}, when open. NMDA receptors are complex heterotetrameric structures made up of seven different subunits- NR1, NR2A-D, NR3A and 3B, of which there are numerous splice variants (Blahos and Wenthold 1996; Dingledine et al. 1999). For example, human NR1 has eight different isoforms due to various combinations from three alternative splice sites (Cull-Candy and Leszkiewicz 2004). Each of the seven subunits has an extracellular N-terminus and intracellular C-terminus, with three transmembrane domains and re-entrant membrane loop (Dingledine et al. 1999). These proteins also have extensive glycosylation sites, which ensure proper channel function (Dingledine et al. 1999). Glutamate binds to sites on NR2 subunits, and NR1 and NR3 subunits contain the glycine/D-serine binding site (Chatterton et al. 2002; Dingledine et al. 1999). Neuronal NMDA receptors typically consist of NR1/NR2 subunits (Behe et al. 1995; Dingledine et al. 1999), most commonly NR2A and/or NR2B in adult brain (Blahos and Wenthold 1996), but there is also evidence for NMDA receptors composed of NR1/NR2/NR3 (Salter and Fern 2005) or NR1/NR3 (Chatterton et al. 2002). At rest, NMDA receptors are blocked by an extracellular Mg^{2+} ion, which binds the channel pore (Fig. 4). Membrane depolarization, typically by AMPA receptor activation, repels Mg^{2+}, facilitating NMDA receptor activation (Dingledine et al. 1999). Mg^{2+} sensitivity is dictated by the NR2 subunit composition, as the re-entrant loop of NR2A or NR2B contains an asparagine residue (Cull-Candy and Leszkiewicz 2004), which permits high affinity Mg^{2+} binding compared to NR2C or NR2D (Monyer et al. 1994) or NR3A or NR3B (Chatterton et al. 2002).

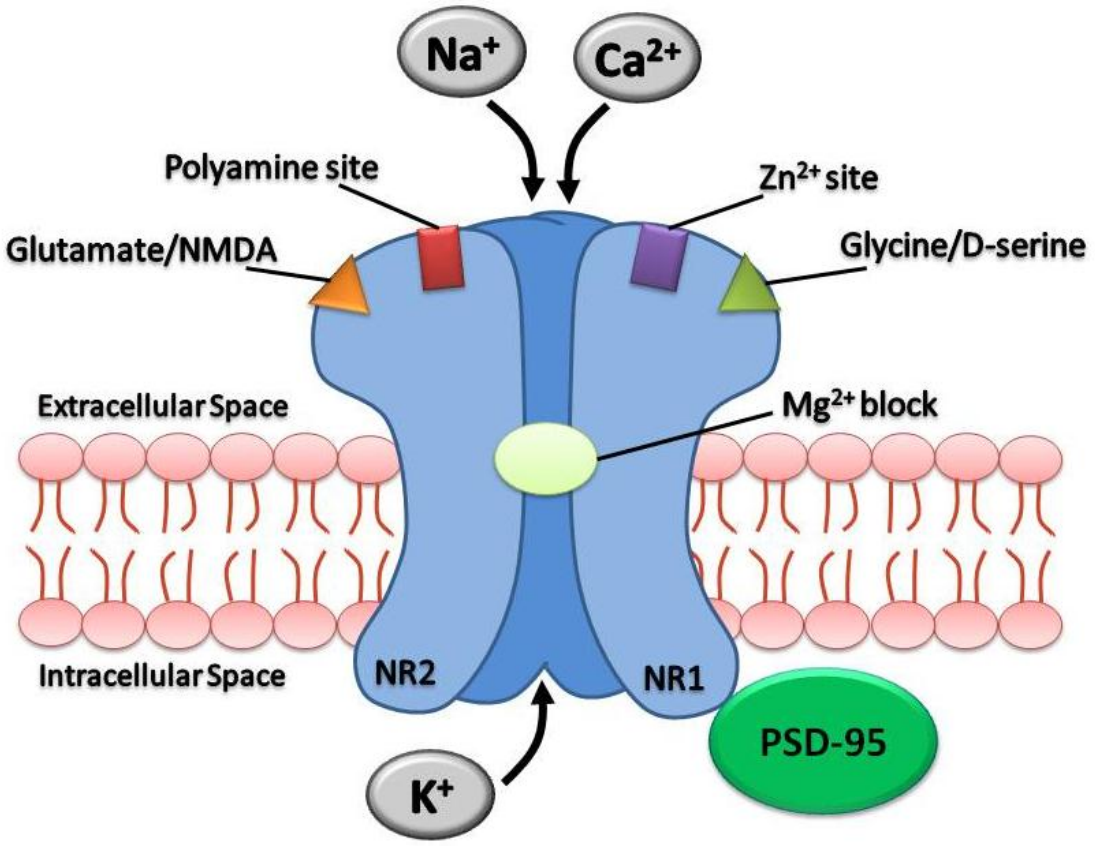

Figure 4: NMDA receptor structure and binding sites.

NMDA receptors are tetramers typically made up of NR1 and NR2 subunits. They are activated by glutamate or NMDA (which binds NR2 subunits) and glycine or D-serine (which binds NR1 subunits), and conduct mainly Ca^{2+} ions when open. At resting membrane potential, an Mg^{2+} ion blocks the pore, which is removed by membrane depolarization. NMDA receptors also have several sites where allosteric modulators, such as Zn^{2+} and polyamines, can alter channel activation and conductance. The C-terminus of NR1 associates with PSD-95, which facilitates localization to synaptic membranes and association with other proteins such as K^+ channels and nNOS.

NMDA receptor population diversity is mainly dictated by the subunit composition, as this determines channel properties. For example, the presence of NR2A decreases binding affinity of glycine on NR1, compared to other NR2 subunits (Buller et al. 1994; Kutsuwada et al. 1992), indicating subunit composition influences receptor potentiation. Subunit expression also changes with development, as the proportion of subunits in sensory pathways shift from NR2B to NR2A. Expression of NR2B is critical for synaptic plasticity and initiation of LTP during early stages (Cull-Candy and Leszkiewicz 2004), but NR2A is responsible for LTP at mature synapses (Massey et al. 2004). In mature neurons, post-synaptic membranes contain NR2A subunits for direct synaptic transmission, while NR2B is found on extra-synaptic membranes and could be required for LTD induction (Massey et al. 2004) and sensing large glutamate signals during excitotoxicity (Scimemi et al. 2004). NR2B is also expressed in presynaptic autoreceptors, which modulate glutamate release (Scimemi et al. 2004). NR3 subunits further complicate receptor properties. NR3A expression with NR1/NR2 reduces Ca^{2+} influx and channel conductance, as measured by single channel and whole cell recordings (Ciabarra et al. 1995; Das et al. 1998). Also, NR1/NR3 receptors are unusual excitatory receptors because they require only glycine for potentiation, they are insensitive to Mg^{2+} and impermeable to Ca^{2+} (Chatterton et al. 2002). Evidence also suggests glycine binding to NR3 alone is sufficient to activate these receptors and NR1 binding acts as a modulatory site to decrease channel conductance (Awobuluyi et al. 2007). Therefore, the subunit composition, NR2A vs. 2B and NR3A or 3B, dictates channel properties and these receptors have diverse distributions throughout the CNS.

NMDA receptor function is also influenced by allosteric modulation of Zn^{2+}, H^{+},

polyamines, and oxidizing/reducing agents (Fig. 4). Blockage of NMDA receptors by Zn^{2+} is voltage-dependent, involving some of the same residues required for Mg^{2+} binding. It is also voltage-independent, which depends on the subunit composition (Peters et al. 1987; Trombley and Shepherd 1996), as NR2A-containing receptors bind Zn^{2+} with high affinity (Paoletti et al. 1997). Extracellular pH also affects NMDA receptor function, since extracellular acidity diminishes the frequency of channel opening, possibly by altering movement of the channel gate (Traynelis et al. 1998). Some subunits are more sensitive to H^{+}, such that physiologic pH (7.4) may inhibit part of the NMDA receptor population (Dingledine et al. 1999; Traynelis et al. 1998). Since synaptic pH changes during prolonged neurotransmission, it is possible NMDA receptor inhibition is elevated during excitotoxicity, which may have neuroprotective effects (Gray et al. 1997; Kaku et al. 1993). Endogenous extracellular polyamines, such as spermine and spermidine, also alter NMDA receptor function by voltage-dependent inhibition, glycine-dependent potentiation, and voltage and glycine-independent potentiation. Voltage-dependent inhibition involves the same mechanism and residues as Mg^{2+} and Zn^{2+} block, but with lower affinity (Benveniste and Mayer 1993; Rock and MacDonald 1992). Polyamines also increase glycine binding affinity, decreasing the glycine concentration required for co-agonist activity (Benveniste and Mayer 1993; Ransom and Deschenes 1990). It is currently unknown if this increased binding affinity and potentiation also applies to D-serine. Polyamines also encourage NMDA receptor potentiation, independent of depolarization or glycine, possibly by altering the pH sensor and reducing H^{+} inhibition (Dingledine et al. 1999; Traynelis et al. 1998). Oxidation/reduction of sulfide groups of two cysteine residues on NR1 and NR2 subunits

also alters NMDA receptor properties. Reduction increases the probability of channel opening, while oxidation decreases channel conduction, possibly by altering Mg^{2+} and Zn^{2+} inhibition (Dingledine et al. 1999; Tang and Aizenman 1993). Most forms of allosteric modulation effect NR1 subunits and different NR1 isoforms have different degrees of modulation. For example, isoforms lacking exon 5 are more sensitive to inhibition by pH changes (Traynelis et al. 1995). This contributes to diversity of NMDA receptor properties and makes allosteric modulation complex, particularly because different modulators have overlapping modulatory sites, which may affect one another.

As well as conducting ions, NMDA receptors associate with various cell signalling intermediates, which influence receptor function and intracellular pathways. For example, C-terminal splice variants of NR1 associate with different proteins, such as α-actinin, postsynaptic density (PSD)-95 (Fig. 4), or calmodulin proteins (Dingledine et al. 1999). Association with α-actinin, an actin binding protein, modulates NMDA receptor localization, while PSD-95 causes clustering of numerous NMDA receptors within the synapse (Wyszynski et al. 1997). Other proteins indirectly complex with NMDA receptors through PSD-95, such as K^+ channels (Kim et al. 1996) and neuronal nitric oxide synthase (nNOS) (Brenman et al. 1996). Intracellular regions of some NMDA receptor subunits can also be phosphorylated at serine/threonine or tyrosine residues, enhancing receptor function and population heterogeneity. Protein kinase A or C phosphorylate NR1 serine/threonines, and while the effects of protein kinase A phosphorylation are not well characterized, protein kinase C phosphorylation reduces blockage by Mg^{2+}, allowing increased channel activation (Chen and Huang 1992). Phosphorylation of C-terminal tyrosine sites of NR2 subunits by Src kinase, also

potentiates NMDA receptor activity, by reducing Zn^{2+} binding (Zheng et al. 1998). Potentiation by tyrosine phosphorylation is greatest with NR2A, since it has a higher affinity for Zn^{2+} than NR2B, NR2C, and NR2D (Dingledine et al. 1999). Tyrosine phosphorylation has been linked to LTP induction in CA1 neurons (Lu et al. 1998), and therefore plays a crucial role in NMDA receptor physiologic function (Dingledine et al. 1999). Increased channel activity and influx of Ca^{2+} through NMDA receptors, activates pathways to decrease NMDA receptor potentiation. For example, phosphatases, such as calcineurin and tyrosine phosphatases, are activated and reverse enhancement of serine/threonine and tyrosine phosphorylation, diminishing channel conductance (Dingledine et al. 1999; Lieberman and Mody 1994). Also, elevated intracellular Ca^{2+} complexes with calmodulin and binds NR1 to decrease the probability of channel opening (Ehlers et al. 1996). Thus, NMDA receptor activity is modulated by kinases and phosphatases, which form a feedback loop controlled by intracellular Ca^{2+} influx.

NMDA receptors are complex glutamate receptors. Receptor kinetics and characteristics are dictated by heterogeneous subunit composition and subunit expression is varied throughout the CNS to regulate plasticity and development. NMDA receptor activity is further complicated by numerous modulation sites, which form feedback loops to greatly influence receptor function.

3.2 D-serine- Endogenous NMDA receptor co-agonist

Glycine and D-serine both bind the glycine modulatory site on NMDA receptors, but D-serine has greater efficacy (Fadda et al. 1988; Hashimoto et al. 1995; Matsui et al. 1995) and 3-4 fold greater potency compared to glycine (Matsui et al. 1995). D-serine is

enriched in brain regions with high NMDA receptor expression, and application of an enzyme to degrade D-serine (D-amino acid oxidase), significantly reduces NMDA receptor activity (Mothet et al. 2000; Panatier et al. 2006; Shleper et al. 2005; Stevens et al. 2003; Yang et al. 2003), suggesting D-serine is the endogenous co-agonist in these regions.

D-Serine immunostaining co-localizes with cultured astrocytes (Martineau et al. 2008) and protoplasmic astrocytes *in situ* near synapses with many NMDA receptors, such as the CA1 region of the hippocampus (Fossat et al. 2011; Hashimoto et al. 1993; Schell et al. 1995) and the prefrontal cortex (Fossat et al. 2011). Also, some populations of neurons in the cortex, forebrain, and brainstem produce D-serine (Fossat et al. 2011; Kartvelishvily et al. 2006; Miya et al. 2008; Rosenberg et al.). D-Serine release in culture is triggered by activation of metabotropic and AMPA glutamate receptors on glial cells (Martineau et al. 2008; Mothet et al. 2005). These receptors elevate intracellular calcium, leading to vesicular release of D-serine by SNARE- dependent exocytosis (Martineau et al. 2008; Mothet et al. 2005). Due to requirement of astrocytic Ca^{2+} activation for release, D-serine is considered to be a gliotransmitter and an important mediator of neuron-glia interactions through NMDA receptor modulation. Basal D-serine levels in the prefrontal cortex and striatum *in vivo* range between 5-7 µM, as measured by microdialysis (Hashimoto et al. 1995), however, peripheral free D-serine levels in blood serum or other organs are near 1-2 µM (Ohnuma et al. 2008).

D-Serine is produced by serine racemase, a pyridoxial-5’ phosphate-containing enzyme which catalyzes the enantiomeric conversion of L-serine to D-serine (Wolosker

et al. 1999b), and is expressed by astrocytes (Wolosker et al. 1999a) and some neurons (Ding et al. 2011). Extracellular D-serine is taken up with low affinity through the Na^+ dependent amino acid transporter, ASCT2 (Ribeiro et al. 2002), which has a higher affinity for L-cysteine, L-alanine, and L-serine and is expressed in cultured astrocytes (Shao et al. 2009; Yamamoto et al. 2003), neurons (Shao et al. 2009), and retinal Müller cells (Dun et al. 2007), as well as neuronal dendrites *in situ* (Gliddon et al. 2009). D-Serine is also taken up with high affinity through the Na^+-independent alanine-serine-cysteine transporter 1, Asc-1, which is reported in presynaptic terminals, dendrites and cell bodies of neurons *in situ* and *in vitro* (Helboe et al. 2003; Shao et al. 2009). Intracellular D-serine is degraded by a peroxisomal flavin adenine dinucleotide-dependent enzyme, D-amino acid oxidase (DAAO), which breaks down D-amino acids to α-keto acids and ammonia through oxidative deamination (Pollegioni et al. 2007). Together, serine racemase, amino acid transporters, and DAAO modulate intracellular and extracellular D-serine concentrations.

D-Serine influences synaptic NMDA receptor activity during neuronal development (Kim et al. 2005) and synaptic plasticity, as well as pathological situations such as models of excitotoxicity (Shleper et al. 2005). Incubation with DAAO reduces NMDA receptor currents in several preparations including co-cultures of astrocytes and hippocampal neurons (Mothet et al. 2000; Yang et al. 2003), acute slices of hippocampus (Mothet et al. 2006; Yang et al. 2003; Zhang et al. 2008b), prefrontal cortex (Fossat et al. 2011), and hypothalamus (Panatier et al. 2006) and retinal preparations (Gustafson et al. 2007; Stevens et al. 2003). Through neuronal NMDA receptors, astrocyte D-serine influences LTP and synaptic plasticity in cultured neurons and hippocampal or

prefrontal cortical slices (Fossat et al. 2011; Henneberger et al. 2010; Mothet et al. 2006; Panatier et al. 2006; Yang et al. 2003), regulating induction of LTP, synaptic plasticity and learning and memory (Braunewell and Manahan-Vaughan 2001). In addition, D-serine is also involved in long-term depression (LTD) and apears to modulate LTD in a bell-shaped concentration-dependent manner, but the precise mechanism of these effects is unknown (Zhang et al. 2008b). With roles in LTP, LTD, and synaptic plasticity, D-serine has emerged as an important gliotransmitter and modulator NMDA receptor activity, which impacts memory formation, retrieval and cognition.

Several studies implicating D-serine as a major modulator of NMDA receptor function have utilized several types of serine racemase knockout mice (Basu et al. 2009; Miya et al. 2008). Brain D-serine concentrations in these mice is reduced 90% compared to wildtype animals. Serine racemase knockout mice are viable without obvious defects, but they display impaired learning and memory (Basu et al. 2009) and schizophrenia-like symptoms including increased anxiety and decreased sociability (Labrie et al. 2009). These traits are attributed to decreased NMDA receptor function and provide protection during excitotoxicity, as described below.

D-Serine also plays a role in neurodegenerative diseases, including stroke, amyotrophic lateral sclerosis, and Alzheimer's disease. Excess D-serine contributes to NMDA receptor-mediated excitotoxicity (Martineau et al. 2006), a common trigger of neuronal death in Alzheimer's disease and other neurodegenerative diseases. Treatment of cultured neurons with amyloid β-peptide (Aβ) enriched with D-serine causes massive neurotoxicity, which is rescued by addition of DAAO (Wu et al. 2004). Serine racemase

knockout mice display neuroprotection during excitotoxicity and forebrain injury by Aβ peptide treatment (Inoue et al. 2008). During oxygen/glucose deprivation and other models of cerebral ischemia, excess glycine or D-serine perpetuates neurotoxicity (Katsuki et al. 2007); however, neuronal death is reduced by treatment with DAAO (Katsuki et al. 2004) or serine racemase deletion (Mustafa et al. 2010). Similarly, progression of amyotrophic lateral sclerosis correlates with increased glial serine racemase expression and D-serine production (Sasabe et al. 2007), but further evidence is required to link motor-neuron excitotoxicity with excess D-serine. While D-serine plays a pathological role in NMDA receptor activation, it has also been linked to diseases involving reduced NMDA receptor signalling pathways, such as schizophrenia. Schizophrenic patients have significantly lower serum levels of D-serine (Hashimoto et al. 2003), generating a hypothesis that schizophrenia pathogenesis is related to altered gene expression of proteins for D-serine metabolism, such as serine racemase and DAAO (Pollegioni and Sacchi 2010). Current clinical research is now targeting these pathways for therapeutic development.

3.3 Non-neuronal NMDA receptors

NMDA receptors have been identified as being expressed by glia of the CNS, such as oligodendrocytes (Karadottir et al. 2005; Salter and Fern 2005), activated microglia (Murugan et al. 2011) and astrocytes (Lalo et al. 2006; Lee et al. 2010). Astrocyte NMDA receptors are likely composed of NR2C and insensitive to block by Mg^{2+} (Palygin et al. 2011). They are activated by synaptic transmission and contribute to elevated intracellular Ca^{2+} (Lee et al. 2010; Palygin et al. 2010). However, the extent of

NMDA receptor involvement in astrocyte Ca^{2+} signalling and neurovascular coupling is not yet clear.

Interestingly, cells outside the CNS, including kidney (Deng et al. 2002; Leung et al. 2002), heart (Gao et al. 2007), pancreas (Inagaki et al. 1995), lung (Said et al. 1996), lymphocytes (Boldyrev et al. 2004), platelets (Franconi et al. 1996), bone cells (osteoblasts and osteoclasts) (Patton et al. 1998), keratinocytes (Genever et al. 1999), and vascular endothelium (Andras et al. 2007; Reijerkerk et al. 2010; Sharp 2003) also express NMDA receptors. In many cases, NMDA receptor function in these tissues is unknown, but recent evidence is helping to elucidate a role of glutamate signalling in other cell types. For example, activation of brain endothelial cell NMDA receptors may increase activity of heme oxygenase (HO) and CO production (Parfenova et al. 2003) and eNOS activity and NO production (Scott et al. 2007), suggesting endothelial cells could be involved in glutamate-mediated vasodilation. However, over-activation of endothelial NMDA receptors during diseases such as ischemia could damage the endothelium and disrupt blood-brain barrier (BBB) integrity (Liu et al. 2010). NMDA receptor activation upregulates P-glycoprotein (an efflux transporter) (Zhu and Liu 2004), alters occludin (a tight junction protein) expression thereby reducing transendothelial electrical resistance (Andras et al. 2007; Neuhaus et al. 2011b), and encourages monocyte migration across the BBB (Koenig et al. 1992; Reijerkerk et al. 2010). Also, BBB disruption by endothelial NMDA receptor activation leads to oxidative stress (Kuhlmann et al. 2008; Sharp 2003), which activates NADPH-oxidase and increases reactive oxygen species (ROS) (Kuhlmann et al. 2009; Sharp et al. 2005). Oxidative stress can feedback to upregulate NMDA receptor expression (Betzen et al. 2009). There is also a link

between endothelial glucose transport and NMDA receptors, since oxygen-glucose deprivation of endothelial cells *in vitro*, upregulates NR1 and glucose transporter 1 and increases glucose uptake, while inhibition of NMDA receptors reduces glucose transporter 1 expression (Neuhaus et al. 2011a). Thus, endothelial NMDA receptors may mediate BBB breakdown and endothelial damage during pathological conditions and could be targeted therapeutically. It is important to note majority of these studies utilized endothelial cell lines or primary cultures, which often differ in their proteomic expression and BBB properties, so further studies are required with other models to better understand endothelial NMDA receptor function. Overall, glutamate signalling and NMDA receptors may serve critical roles in numerous peripheral tissues, but further studies are warranted.

3.4 NMDA receptors can mediate cerebral vasodilation

Glutamate and NMDA activate NMDA receptors to mediate dilation of cerebral arterioles *in vivo*, which appears to occur universally across species, including piglet, rat, and rabbit (Busija and Leffler 1989; Domoki et al. 2002; Faraci and Breese 1993; Iliff et al. 2003; Simandle et al. 2005). Most of these studies monitored the response of pial arterioles to glutamate or NMDA application through a cranial window (Busija et al. 2007; Busija and Leffler 1989; Domoki et al. 2002; Faraci and Breese 1993; Faraci and Breese 1994; Iliff et al. 2003), and found reproducible vasodilation and no evidence of excitotoxicity or vessel damage (Busija and Leffler 1989; Pelligrino et al. 1995). Dilation was inhibited by N^{ω}-nitro-L-arginine (L-NAME), a general NOS inhibitor, (Meng et al. 1995; Pelligrino et al. 1995), 7-nitroindazole, an nNOS selective antagonist (Bari et al. 1996b; Faraci and Brian 1995), and in some cases, tetrodotoxin (TTX), a

voltage-gated sodium channel blocker (Faraci and Breese 1993; Pelligrino et al. 1996), implicating neuronal activity and NO production as part of the dilation mechanism (Chi et al. 2003; Faraci and Breese 1993).

NMDA receptors and nNOS are functionally-linked via Ca^{2+} influx. Nitric oxide synthase enzymes degrade L-arginine to NO and L-citrulline, and require oxygen and several co-factors including, NADPH and calmodulin (Alderton et al. 2001). When NMDA receptors are activated, cytoplasmic Ca^{2+} increases and complexes with calmodulin, which bind nNOS stimulating enzymatic activity (Roman et al. 2000). NMDA receptors and nNOS are also physically linked, since they are both tethered to PSD-95 in the synaptic membrane (Brenman et al. 1996). This allows a tight coupling between NMDA receptor channel conductance and nNOS activation, and suggests NMDA receptor-mediated functional hyperemia involves vascular smooth muscle dilation via NO from neighbouring neurons. However, nNOS and NMDA receptor neuronal co-localization has been difficult to pin-point near cortical blood vessels by immunohistochemistry, and variations are apparent between species, age, and anatomical region (Aoki et al. 1997; Bari et al. 1998c; Gracy and Pickel 1997). Thus, NO may not be the major mediator of NMDA receptor-induced vasodilation in all brain regions, and other compounds such as adenosine and AA metabolites may also be involved (Bhardwaj et al. 2000; Iliff et al. 2003).

Astrocyte D-serine and neuronal NO can influence each other. D-Serine potentiates neuronal NMDA receptors, increasing NO production. This is evident in serine racemase knockout mice, which display lower D-serine levels and decreased NO production and

nitrosylated proteins (Mustafa et al. 2010). Neuronal NO can diffuse to astrocytes and nitrosylate and inhibit serine racemase while activating DAAO, which reduces D-serine production overall (Shoji et al. 2006a; Shoji et al. 2006b). This creates a feedback loop where glutamatergic neurotransmission triggers astrocyte D-serine release, activating NMDA receptors and nNOS, leading to NO diffusion and inhibition of D-serine production. Currently, the role of D-serine in NMDA receptor-mediated functional hyperemia is unknown, but it may help increase NO production.

Using wire or pressure myography, several studies indicate glutamate and NMDA dilate isolated arteries free of neural circuitry (Crespi et al. 2000; Fiumana et al. 2003; Nguyen-Duong 2001; Parfenova et al. 2003), by an endothelium-dependent mechanism (Fiumana et al. 2003). However, several groups have demonstrated that NMDA receptor agonists do not directly dilate isolated arteries (Faraci and Breese 1993; Hardebo et al. 1989; Simandle et al. 2005; Wendling et al. 1996), suggesting innervation is required. Lack of dilation of isolated arteries could be due to absence of glycine site co-agonists, glycine or D-serine, which have not been considered to date. Also, expression of NMDA receptor subunits by brain endothelial cells has been demonstrated (Andras et al. 2007; Krizbai et al. 1998; Reijerkerk et al. 2010; Scott et al. 2007; Sharp 2003), but this has been refuted in other studies (Domoki et al. 2008; Morley et al. 1998). Results from various studies vary greatly and it is unclear if activation of endothelial NMDA receptors can trigger vasodilation in brain. However, glutamate treatment of cultured endothelial cells stimulates HO and eNOS to release CO and NO metabolites (Parfenova et al. 2003; Scott et al. 2007), which could relax vascular smooth muscle. eNOS is a crucial mediator of baseline brain vascular tone (Iadecola 1993) and functional hyperemia resulting

from experimental seizures (Pereira de Vasconcelos et al. 2006), focal brain trauma (Hlatky et al. 2003) and cerebral ischemia (Endres et al. 2004). During these conditions, extracellular glutamate is greatly elevated and may trigger excitotoxicity; therefore, it is possible that hyperemia involving eNOS requires high glutamate concentrations for activation. A recent study by de Labra et al. (2009) demonstrated both eNOS and nNOS activity were important for control of functional hyperemia, but the degree of neuronal activation dictated which enzyme was activated. During low frequency stimulation, vascular NO, likely derived from eNOS, had a greater effect on vascular responses, while nNOS mediated effects were triggered by high frequency stimulation (de Labra et al. 2009). This work helps to determine the relative importance of eNOS vs. nNOS in neurovascular coupling, but further studies would better distinguish the contributions of these enzymes.

4. Astrocyte Dysfunction and Neurovascular Coupling in Disease

Multiple brain diseases are associated with abnormal energy metabolism, dysfunctional glutamate cycling by astrocytes, and altered functional hyperemia. Here, we discuss astrocytes in Alzheimer's disease, vascular dementia, cerebral ischemia, epilepsy, and diabetes.

4.1 Alzheimer's Disease and Vascular Dementia

Alzheimer's disease (AD) is the most common form of dementia, characterized by declining cognitive performance and memory (McKhann et al. 1984). AD pathology is characterized by two types of lesions- amyloid-β (Aβ) plaques, consisting of insoluble, extracellular deposits of Aβ peptide fibrils, and neurofibrillary tangles, composed of

intracellular neuronal deposits of hyper-phosphorylated and crosslinked tau protein. Aβ plaques are linked to synaptic dysfunction, activation of microglia and astrocytes, and oxidative stress (Fuller et al. 2009).

During AD, astrocytes undergo morphological changes, related to proximity of Aβ deposits. Near neuritic and Aβ plaques, astrocytes from AD patients undergo reactive gliosis (Simpson et al. 2010), where glial fibrillary acidic protein (an intermediate filament protein) is upregulated and cytokines (transforming growth factor-β and interleukin- 1β) (Reilly et al. 1998) are released. In dementia patients (Senitz et al. 1995) and AD transgenic mice (Rodriguez et al. 2009) astrocytes distal to Aβ plaques display decreased surface area and volume of processes. This astrocyte dystrophy and reactive astrogliosis may greatly impair astrocytic modulation of synaptic environments and neuronal metabolism, exacerbating AD progression (Fuller et al. 2009). For example, astrocytes from AD patients display decreased glutamate transporter expression (Li et al. 1997; Masliah et al. 2000), diminished glutamine synthetase activity, and reduced glutamine concentration in cerebrospinal fluid (Csernansky et al. 1996; Jimenez-Jimenez et al. 1998), suggesting dysfunctional glutamate uptake and glutamine shuttling to neurons. Also, AD patients exhibit reduced glucose uptake (Alexander et al. 2002), often before Aβ plaques or neurofibrillary tangles are detected (Morley et al. 1998), suggesting energy metabolism is altered early in disease. Lactate can rescue learning consolidation impaired by Aβ peptide administration to day-old chicks (Gibbs et al. 2009), signifying astrocyte lactate production and influence over neuronal metabolism are linked to Aβ memory deficits. Thus, dysfunctional astrocytes could be central to AD pathogenesis.

As AD progresses, vascular dysfunction contributes to vasoconstriction (Niwa et al. 2001), while reducing neurovascular coupling (Iadecola 2004; Niwa et al. 2000) and resting blood flow (Mentis et al. 1996; Warkentin and Passant 1997). Astrocytes from transgenic AD mice *in vivo* exhibit increased frequency of spontaneous, focal intracellular Ca^{2+} responses, which are not coupled with neuronal activity and may impact release of vasoactive molecules and neurovascular coupling (Kuchibhotla et al. 2009; Takano et al. 2007). Also, amyloid β-peptide accumulates in blood vessel walls (Selkoe and Schenk 2003), possibly because it is cleared from cerebral interstitial fluid through a mechanism involving perivascular drainage and transport across the BBB (Preston et al. 2003) Vascular Aβ accumulation causes endothelial cell deformity, smooth muscle deterioration (Farkas and Luiten 2001), and pericyte toxicity (Wilhelmus et al. 2007), which is linked to reduced free NO and vasoconstriction (Niwa et al. 2001; Thomas et al. 1996). Thus, dysfunctional neurovascular coupling during AD could be caused by altered Ca^{2+} signalling in astrocytes and gross vascular abnormalities, which change normal intrinsic vascular tone. Astrocytes are central to AD initiation and progression, and have now become future therapeutic targets (Fuller et al. 2009).

A growing body of evidence suggests defective vascular function acts precipitously to cause chronic generalized subcortical ischemia and associated brain dysfunction, leading to a type of dementia known as vascular dementia (VaD). VaD is associated with cardiovascular risk factors such as hypertension and diabetes (Chui 2001). Cerebrovascular dysfunction, including impaired functional hyperemia, is the major contributor to VaD. Cognitive symptoms and vascular dysfunction in VaD are similar to AD and up to 60% of AD patients also have VaD, making it difficult to differentiate

between these two dementias (Lee 2011). Abnormalities in astrocyte control of blood flow have not been studied in VaD, but may contribute to vascular effects as in AD.

4.2 Ischemia

During cerebral ischemia, blood flow is occluded either by artery blockage (focal ischemia) or cardiac arrest (global ischemia), causing bioenergetic failure (Hertz 2008). Reduced oxygen and glucose levels stimulate a chain reaction where glycolysis produces lactate and proton acidification of the cytoplasm (Silver et al. 1997) and reactive oxygen species (ROS) form due to electron transport chain leakage (Abramov et al. 2007). ATP becomes depleted, inhibiting Na^+/ K^+ ATPase and triggering membrane depolarization (Silver et al. 1997). Extracellular glutamate is elevated because of depolarization and subsequent vesicular release by non-vesicular egress mechanisms. This initiates further membrane depolarization, mitochondrial damage, excitotoxicity, and neuronal death (Brookes et al. 2004; Nicholls 2004; Nicholls et al. 2007; Schild et al. 2003). Neurons are very sensitive to this chain reaction, while astrocytes are more resistant because they can increase their glycolytic rate (Walz and Mukerji 1990) or utilize alternate energy substrates for ATP production (Edmond et al. 1987; Hertz 2003; Hertz and Hertz 2003). Astrocytes also exploit glutathione stores to limit ROS damage (Juurlink 1997).

During ischemia, astrocyte glutamate recycling becomes dysfunctional in an attempt to balance increased extracellular glutamate. Excessive glutamate uptake through transporters elevates astrocyte cytoplasmic Na^+, leading to cell swelling. Astrocytes express volume-regulated anion channels, which efflux glutamate and other amino acids to reduce ischemic swelling, further elevating extracellular glutamate

(Kimelberg et al. 1990). Finally, as ATP levels drop, astrocytes depolarize leading to glutamate transporter reversal and glutamate efflux (Bonde et al. 2003; Phillis et al. 2000). Astrocyte glutamate handling has become a potential ischemic therapeutic target, since upregulating expression or activity of glutamate transporters or inhibiting volume-regulated anion channels may decrease glutamate excitotoxicity (Rossi et al. 2007).

Exhaustion of ATP supplies and progression of neuronal death during ischemia can be prevented by increased availability of energy substrates, such as astrocyte lactate and glycogen (Brown et al. 2005; Schurr et al. 2001; Tekkok et al. 2005). However, as oxygen is depleted, oxidative phosphorylation is inhibited and anaerobic glycolysis (particularly of glial glycogen) increases (Rossi et al. 2007), which causes lactic acidosis and exacerbates brain damage (Li and Siesjo 1997). In early stages of ischemia, increasing glycogen stores (by elevating glycogen synthase activity, for example) may increase protective benefits of glycogen (Rossi et al. 2007). However, in later stages of ischemia, inhibition of lactic acid damage, (such as blocking H^+ sensing ion channels (Xiong et al. 2004)) may be important for neuroprotection (Rossi et al. 2007).

During ischemia, propagation of signals and metabolites through the glial network is reduced at astrocyte gap junctions, but increased through hemichannels (Contreras et al. 2002). Increasing diffusion through gap junctions could be beneficial since they conduct protective molecules to the injury site and remove toxic molecules (Contreras et al. 2002). However, increased hemichannel activity allows Na^+ and Ca^{2+} to diffuse into astrocytes, while glutamate flows out, furthering excitotoxicity (Ye et al. 2003). Also, an important astrocytic antioxidant, glutathione, is lost through astrocyte hemichannels,

limiting ROS protection (Rana and Dringen 2007). While both hemichannels and gap junctions respond to ischemic signals, they are difficult therapeutic targets as both are inhibited by the same antagonists, obscuring potential benefits (Rossi et al. 2007).

Reperfusion after ischemia is characterized by reduced blood flow (Leffler et al. 1989) and functional hyperemia due to disruption of the neurovascular unit via neuronal and vascular ischemic damage (del Zoppo 2010). Reduced neurovascular coupling exacerbates ischemic injury, which may increase infarct size. Blood flow is partly reduced because fibrin, activated platelets and/or leukocytes occlude capillaries and venules (del Zoppo and Mabuchi 2003). However, hypoxia and ischemia reduce dilation of pial arteries to NMDA receptor agonists *in vivo* (Bari et al. 1996a; Bari et al. 1998a; Bari et al. 1998b; Busija et al. 1996) and decrease arteriolar K^+ channel activity (Bari et al. 1996c), which could reduce the dilatory effects of astrocyte K^+ siphoning. Neurovascular dysfunction is also apparent at the capillary level where pericytes cause constriction (Peppiatt et al. 2006; Yemisci et al. 2009), which is likely stimulated by peroxynitrite formation by endothelial cells (Yemisci et al. 2009). Evidence also suggests that AA metabolite (EETs and 20-HETE) signalling is altered during ischemia, which also contributes to decreased blood flow and neurovascular coupling. Recent therapeutic studies have elevated EET levels using inhibitors of soluble epoxide hydrolase (sEH), an enzyme that degrades EETs (Imig and Hammock 2009). sEH inhibitors are beneficial regardless of administration time, since infarct size is decreased in rodents when the drug is given chronically, shortly after the ischemic insult or during reperfusion (Dorrance et al. 2005; Simpkins et al. 2009; Zhang et al. 2007; Zhang et al. 2008a). EETs mediate this protection, since inhibition of CYP epoxygenase (the

EET synthesis enzyme) prevents sEH benefits (Zhang et al. 2007; Zhang et al. 2008a). This protective mechanism increases astrocyte survival (Liu and Alkayed 2005), elevates anti-apoptotic factors (Simpkins et al. 2009) and increases neurovascular coupling (Zhang et al. 2007; Zhang et al. 2008a). Conversely, 20-HETE is elevated during ischemia (Tanaka et al. 2007), and inhibition of 20-HETE production is also neuroprotective in rodent models (Dunn et al. 2008; Miyata et al. 2005; Poloyac et al. 2006; Renic et al. 2009; Tanaka et al. 2007). Reduction of 20-HETE inhibits ROS production (Dunn et al. 2008), limits vasoconstriction and increases blood flow during reperfusion (Dunn et al. 2008; Miyata et al. 2005). Taken together, evidence suggests AA metabolite signalling is dysfunctional during and after cerebral ischemia, where EETs are decreased and 20-HETE is elevated. By inhibiting EET degradation and 20-HETE production, functional hyperemia can be restored, and these pathways make promising therapeutic targets.

Focal cerebral ischemia causes altered glutamate handling and lack of energy substrates, which triggers neuronal excitotoxicity, ATP depletion, and ROS production (Hertz 2008). In early stages of ischemia, astrocytes are less susceptible to damage and may help protect neurons through glutamate uptake, glycogen hydrolysis to lactate for energy, and conduction of protective molecules through gap junctions. However, prolonged ischemia damages the neurovascular unit reducing blood flow and functional hyperemia during reperfusion. Current therapeutic targets are meant to promote astrocyte protection of neurons and help restore proper circulation after stroke, but further study is necessary to better develop these therapies.

4.3 Epilepsy

Epilepsy is characterized by sudden, temporary synchronization of electrical charges in groups of neurons, which may manifest as seizures. The origins of this disorder are not completely understood (McCormick and Contreras 2001; Scharfman 2007), but neuronal hyperexcitability is believed to be caused by disequilibrium between glutamatergic and GABAergic neurotransmission, either by decreased inhibitory (GABA) circuits or excessive glutamatergic release (Dudek et al. 1999; Uhlhaas and Singer 2006). Dysfunctional astrocyte glutamate-glutamine cycling is also involved (Tian et al. 2005), since astrocyte expression of glutamate transporters is diminished in epilepsy patients (Fotheringham et al. 2007; Proper et al. 2002), and knock-down of glutamate transporters (Tanaka et al. 1997); (Watase et al. 1998) in animal models exacerbates neuronal excitability. Also, glutamine synthetase expression is reduced by 40% in astrocytes of epilepsy patients, suggesting that glutamate degradation is greatly diminished (Eid et al. 2004). Therefore, dysfunctional glutamate metabolism in astrocytes could contribute to neuronal synchronization and hyperexcitability.

Astrocytes in epilepsy may also mismanage K^+ buffering, since both K_{ir} currents and aquaporin 4 expression are reduced (D'Ambrosio 2004; Eid et al. 2005) This results in elevated extracellular K^+, decreased water homeostasis, and reduced seizure thresholds (Binder and Steinhauser 2006). Astrocytes also display elevated intracellular Ca^{2+} signals before and during seizure activity in rodents (Gomez-Gonzalo et al. 2011; Gomez-Gonzalo et al. 2010; Tian et al. 2005), which is mediated by mGluR and purinergic receptors, and may further exacerbate neuronal activation by triggering gliotransmission (Gomez-Gonzalo et al. 2010). Common anti-epileptic drugs, such as

valproate and phenytoin, reduce astrocytic Ca^{2+} increases (Tian et al. 2005).

Cerebral bioenergetics is also altered during epilepsy, but the precise changes remain unknown. Epilepsy patients display high levels of glucose uptake and hypermetabolism during seizures (Engel et al. 1983), and low levels of glucose uptake and hypometabolism between seizures (Engel et al. 1982). Glycolytic inhibitors, such as 2-deoxy-D-glucose, have anti-epileptic properties (Garriga-Canut et al. 2006), suggesting glycolysis is necessary for neuronal hyperexcitability and synchronization. In animal models of epilepsy, astrocyte glycogen accumulates before the onset of seizures as a possible neuronal energy substrate (Bernard-Helary et al. 2000). Also, connexin knockout mice experience spontaneous interictal bursts and neuronal hyperexcitability, suggesting inter-astrocyte signalling and redistribution of metabolites within the astrocyte network is altered in epileptic brains (Cloix and Hevor 2009; Wallraff et al. 2006).

Epileptiform activity triggers increased blood flow and deoxygenates haemoglobin (Suh et al. 2006) to meet energy and oxygen demand of active neurons (Kuhl et al. 1980). However, hyperemia may not fully support neurons, since some studies suggest chronic epilepsy may cause ischemic-like tissue damage (Suh et al. 2006). A lag time was identified between astrocyte endfeet Ca^{2+} elevations and the vasodilation of preconstricted arterioles during synchronous bursts in rat brain slices treated with 95% oxygen, indicating astrocyte-independent mechanisms may be more prevalent in epilepsy (Gomez-Gonzalo et al. 2011). For example, experimental seizure models have shown eNOS is crucial for local vasodilation (Pereira de Vasconcelos et al. 2006). Thus, other molecular mechanisms may control cerebral blood flow during seizures, and the role of

known astrocyte signals, such as AA metabolites, is not well characterized (Gomez-Gonzalo et al. 2011).

Astrocytes may play an important role in epilepsy, but it is unclear if they promote neuronal excitability, or merely sustain seizures and epileptogenesis. Several astrocyte functions are altered during epilepsy including glutamate-glutamine shuttle, ion homeostasis and movement of metabolites, but the role of astrocytes in functional hyperemia during seizure activity is unknown. In the future, astrocyte glutamate uptake, blood flow control or metabolism could be targeted to limit neuron excitability.

4.4 Cognitive Decline and Retinopathy in Diabetes

Prolonged, uncontrolled diabetes can lead to complications, including cognitive decline (Awad et al. 2004; Pasquier et al. 2006) and retinopathy (Fletcher et al. 2008). The mechanism of cognitive impairment is poorly understood, but involves vascular changes such as BBB leakage (Mogi and Horiuchi 2011). NMDA receptor-induced vasodilation and functional hyperemia *in vivo* are reduced by hyperglycemia (Mayhan and Patel 1995). In astrocytes, high glucose culture conditions cause oxidative stress and reduces gap-junction trafficking (Gandhi et al. 2010). Thus, astrocyte dysfunction including reduced flow of metabolites and intercellular signalling, may impact neurovascular coupling, decreasing blood flow and contributing to diabetes-induced cognitive decline (Mogi and Horiuchi 2011), but further studies are required to better understand this mechanism.

Diabetic retinopathy is a leading cause of blindness (Fletcher et al. 2007). It involves both neuronal dysfunction (Fletcher et al. 2008) and vascular changes, including

neovascularisation, reduced blood flow and haemorrhaging (Antonetti 2009). Recent evidence suggests neuronal and vascular dysfunction could be partly attributed to dysregulation of normal glial signalling (Fletcher et al. 2007; Fletcher et al. 2005). Retinal glia, such as Müller glia, regulate synaptic environments, release signalling molecules and control blood flow, similar to CNS astrocytes (Rungger-Brandle et al. 2000). During diabetes, Müller cells undergo reactive gliosis, which decreases expression of K^+ channels and reduces K^+ siphoning (Bringmann et al. 2002) increasing the extracellular space. Müller glia in diabetic rats also have altered glutamate handling and display increased glutamate uptake (Ward et al. 2005), which could decrease neuronal expression of glutamate receptors leading to dysfunction (Fletcher et al. 2007). Müller glia also contribute to changes in vascular structure, as reactive gliosis releases several angiogenic factors, such as vascular endothelial growth factor, which contribute to neovascularisation (Aiello et al. 1994). PGE_2, COX, and NO are upregulated in diabetic retina and hyperglycemic cultured Müller cells; however, it is not clear if this influences inflammation and/or glial signalling during neurovascular coupling (do Carmo et al. 1998; Du et al. 2004). Thus, diabetes induces glia alterations which contribute to vascular and neuronal dysfunction, but these mechanisms require further investigation.

5. Hypotheses

Neurovascular coupling is important for maintaining energy supply to neurons. Astrocytes are central to this mechanism since they influence arteriolar tone through several pathways, and multiple signals likely occur in concert to ensure tight coupling

with neuronal activity. While several pathways involving astrocyte signalling molecules (AA metabolites, etc.) have been identified, the role of astrocyte D-serine modulation of NMDA receptor-mediated vasodilation has not been characterized.

In this thesis, my <u>overall hypothesis</u> is that glutamate and D-serine, an astrocyte gliotransmitter, are released during periods of elevated neuronal activity, and act directly on endothelial NMDA receptors to stimulate eNOS-induced vasodilation to meet tissue energy demands. This is outlined in Figure 5 and is a new model for neurovascular coupling. To study this hypothesis, I will examine three main objectives:

<u>Objective 1:</u> Investigate the role of D-serine in NMDA receptor-induced vasodilation. <u>Rationale:</u> Glutamatergic neurotransmission triggers astrocyte Ca^{2+} elevations and release of vasoactive metabolites and gliotransmitters, such as D-serine (Mothet et al. 2005). D-serine is an NMDA receptor co-agonist (Fossat et al. 2011), and NMDA receptor activity can trigger vasodilation *in vivo* (Busija et al. 2007).

Hypothesis 1: D-serine, a gliotransmitter released from astrocytes in response to neurotransmission, potentiates NMDA receptor-mediated vasodilation and contributes to cerebral blood flow control.

This hypothesis will be examined in Chapters 2 and 4 using pressure myography of isolated mouse cerebral arteries and two-photon microscopy of brain slices.

<u>Objective 2:</u> Characterize the distribution of D-serine and NMDA receptors around blood vessels. <u>Rationale:</u> Several studies have shown that endothelial cells express NMDA receptors (Reijerkerk et al. 2010; Scott et al. 2007; Sharp et al. 2005), while others refute

these claims (Domoki et al. 2008). Thus, the existence and subunit composition of endothelial NMDA receptors is unclear. Also, D-serine is localized to astrocytes, and could be available for release near blood vessels (Schell et al. 1995).

Hypothesis 2: D-serine is localized to astrocyte endfeet near blood vessels expressing endothelial NMDA receptors with heterogeneous subunit composition.

This hypothesis will be tested in Chapter 3 and 4, using cultured mouse primary endothelial cells and immunohistochemistry of brain slices to determine NMDA receptor and D-serine localization.

Objective 3: Determine the mechanism of endothelial cell NMDA receptor-induced vasodilation. Rationale: Treatment of cultured endothelial cells with glutamate has been linked to nitric oxide production (Scott et al. 2007). NMDA receptors are Ca^{2+} channels, which activate nNOS in neurons (Roman et al. 2000). eNOS is also activated by Ca^{2+}/calmodulin binding, suggesting endothelial NMDA receptor activation could also stimulate eNOS. NO is a potent vasodilator and modulator of cerebral blood flow, which can regulate AA- metabolite signalling from astrocytes to cerebral arterioles (Attwell et al. 2010; Metea and Newman 2006).

Hypothesis 3: Astrocyte D-serine acts on endothelial NMDA receptors, triggering eNOS activation that interacts with other known pathways of neurovascular coupling, such as AA metabolites.

This hypothesis will be examined in Chapter 2 and 4 using pressure myography of isolated arteries and two-photon microscopy of brain slices. A better understanding of this mechanism will further our knowledge of functional hyperemia and may impact the

treatment of numerous cerebral disorders, such as AD and ischemia, where neurovascular coupling is dysfunctional.

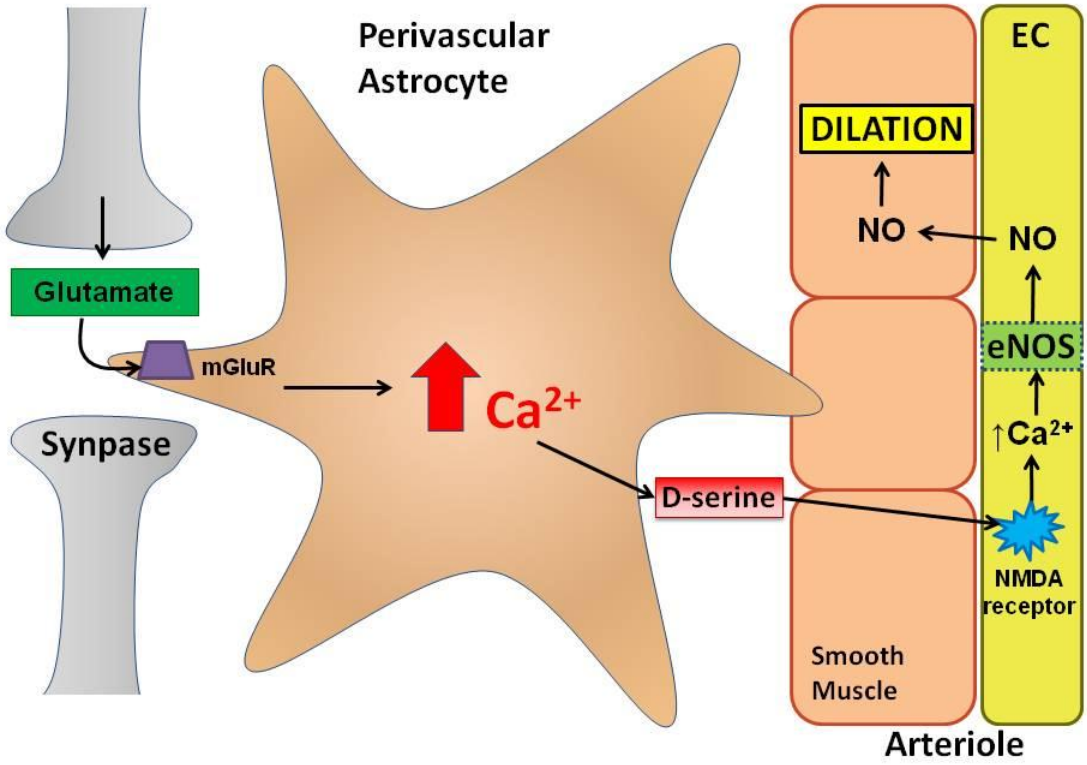

Figure 5: New mechanism of NMDA receptor-mediated dilation involving D-serine. Increased astrocyte Ca^{2+}, from glutamatergic neurotransmission, triggers D-serine exocytosis. D-serine and glutamate potentiates endothelial NMDA receptors, elevating endothelial cytoplasmic Ca^{2+}, which complexes with calmodulin. This stimulates eNOS activity and NO production, which causes smooth muscle vasodilation, possibly by modulating AA metabolite levels.

[1]CHAPTER 2: Co-activation of NMDA receptors by glutamate and D-serine induces dilation of isolated middle cerebral arteries

SUMMARY

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 co-agonist binding of the gliotransmitter, D-serine. Neuronal NMDA receptors mediate activity-dependent blood flow regulation in brain. Our objective was to determine whether NMDA receptors can induce vasodilation of isolated brain arteries. Adult mouse middle cerebral arteries (MCA) were isolated, pressurized and pre-constricted with norepinephrine. NMDA receptor agonists, glutamate and NMDA, significantly dilated MCAs in a concentration-dependent fashion in the presence of D-serine but not alone. Dilation was significantly inhibited by NMDA receptor antagonists, D-2-amino-5-phosphonopentanoate (AP5) and 5,7-dichlorokynurenic acid (DCKA), indicating a response dependent on NMDA receptor glutamate and D-serine binding sites, respectively. Vasodilation was inhibited by denuding the endothelium and by either selective inhibition or genetic knockout of endothelial nitric oxide synthase (eNOS). Overall, we conclude that NMDA receptor co-

[1] LeMaistre JL, Sanders S, Stobart MJ, Lu L, Anderson HD, Anderson CM. (2012). Co-activation of NMDA receptors by glutamate and D-serine induces dilation of isolated middle cerebral arteries. J Cereb Blood Flow Metab 32(3):537-47.

activation by glutamate and D-serine increases lumen diameter in pressurized MCA in an endothelial and eNOS-dependent mechanism.

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 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. NMDA 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. NMDA 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). Non-neuronal 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 (Boldyrev et al. 2005; Karadottir et al. 2005; Reijerkerk et al. 2010). NMDA receptor activation requires glutamate, which binds NR2 subunits, and binding of a co-agonist, glycine or D-serine. While glycine and D-serine are both NMDA receptor co-agonists, D-serine binds with greater affinity and dominates

as the main endogenous co-agonist in most 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 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 (Faraci and Breese 1993; Faraci and Brian 1995; Meng et al. 1995; Philip and Armstead 2004). There is broad 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) (Chi et al. 2003; Faraci and Brian 1995; Fergus and Lee 1997; Meng et al. 1995). Second, glutamate can stimulate astrocytic release of vasodilators such as arachidonic acid metabolites-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.; Scott et al. 2007; Sharp 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 the limited direct effects of glutamate on lumen diameter in isolated artery preparations using wire or pressure myography (Faraci and Breese 1993; Hardebo 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 co-agonist in these experiments. The objectives of the current study were to determine whether co-activation of endothelial NMDA receptors by D-serine and glutamate is sufficient to cause dilation of isolated brain arteries. We report that glutamate or NMDA applied to isolated middle cerebral arteries with D-serine, but not alone, causes vasodilation dependent on both endothelial function and endothelial nitric oxide synthase (eNOS) activity.

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 anesthetised with 70% CO_2/30% O_2 and decapitated. Brains were removed and placed in ice cold Krebs buffer

(118 mM NaCl, 4.65 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM $KHPO_3$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5.5 mM glucose, 0.026 mM EDTA). A middle cerebral artery (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 the stopcock was closed at the distal pipette to permit pressurization with a Servo-controlled peristaltic pump to an intraluminal pressure of 30 mm Hg. 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 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 experiments by ≥ 50% constriction to 125 mM KCl. The vessels were equilibrated for one hour between treatments. Arteries were pre-constricted with norepinephrine (NE; 1 μM), and vascular responses were measured in response to incremental doses of glutamate or N-methyl-D-aspartate (NMDA) with or without D-serine.

Arteries were treated with several antagonists for NMDA and AMPA receptors, including 2-amino-5-phosphonopentanoate (AP5; 100 nM), 5,7-dichlorokynurenic acid (DCKA; 300 nM; Tocris Bioscience), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM). AP5 is a competitive antagonist for the NMDA receptor glutamate binding site, which binds with high selectivity and potency (K_i = 370 nM) (Lodge et al. 1988). DCKA

inhibits the NMDA receptor glycine (D-serine)-site competitively and with high selecitivty (Leeson et al. 1991) and has a K_i = 79 nM (Baron et al. 1990). AMPA/kainate receptor antagonist, CNQX, binds the receptors competitively and with strong potency (IC_{50}= 300 nM) (Honore et al. 1988). This drug may also competitively inhibit the NMDA recptor glycine site, but its selectivity for AMPA receptors is enhanced in the presence of glycine site agonists, such as D-serine, and at the concentration (10 μM) used in this study (Lester et al. 1989).

To determine the involvement of nitric oxide synthase, the vessels were also preincubated with three different nitric oxide synthase (NOS) inhibitors: N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 10 μM), 1-(2-(trifluoromethylphenyl))imidazole (TRIM; 50 μM), and N^5-(1-iminoethyl)-L-ornithine (L-NIO; 3 μM). L-NAME is a general non-selective NOS inhibitor, which inhibits all 3 forms of NOS (nNOS, iNOS, and eNOS) with a greater potency for nNOS and eNOS (K_i = 15 nM and 39 nM respectively) (Furfine et al. 1993). TRIM selectively antagonizes nNOS and iNOS (IC_{50} = 28 μM) at the concentrations used in this study, but will inhibit eNOS at higher concentrations (IC_{50} = 1 mM) (Handy et al. 1995). L-NIO is commonly used to selectively inhibit eNOS (IC_{50}= 500 nM), but can also inhibit nNOS and iNOS (Rees et al. 1990). This study also utilized MCA from eNOS knockout mice.

Endothelium Denudation

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 nM

to 10 μM). Viability of the smooth muscle layer was verified by treatment with sodium nitroprusside (SNP; 100 nM to 100 μM), a direct nitric oxide donor.

Phenolic Denervation and NADPH-diaphorase staining

Residual post-dissection 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 nM to 10 μM) and SNP (100 nM to 100 μM). To verify denervation, arteries were fixed with 4% paraformaldehyde (pH 7.6) for 10 min. NADPH-diaphorase solution, containing nitro blue tetrazolium (5 μM), the reduced form of β-nicotinamide adenine dinucleotide phosphate (β-NADPH; 10 μM), and 0.2% Triton X-100 in Tris-buffered saline (TBS; 0.05 M; pH 8.0), was applied for 30 min at 37°C to detect nNOS positive neurons.

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 was analyzed by one-way ANOVA test with Newman-Keuls *post-hoc* test.

RESULTS

NMDA receptor agonists directly dilate isolated middle cerebral arteries and penetrating cortical arterioles

Bath application of increasing concentrations (0.1 µM to 10 mM) of NMDA receptor agonists, glutamate and NMDA, to pre-constricted middle cerebral arteries (MCAs) had no significant effect on lumen diameter (Fig. 6A). In contrast, with D-serine present (100 µM), incremental concentrations of glutamate (Fig. 6B) or NMDA (Fig. 6C) produced significant concentration-dependent relaxation of MCAs, which was first statistically detectable ($p < 0.05$) at 10 µM glutamate (22.9% ± 5.7%) or NMDA (13.8% ± 2.3%). D-Serine concentrations 10-fold lower than this (10 µM) were sufficient to evoke vasodilation in the presence of 100 µM glutamate (Fig. 6D). 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 co-agonists at 10 µM and found this condition to cause a significant increase in MCA diameter (Fig. 7). D-Serine was unable to initiate vasodilation when administered with another known vasodilator, acetylcholine (Fig. 8), indicating a specific effect observed only when given with an NMDA receptor glutamate site agonist.

Glutamate and D-serine-mediated vasodilation is NMDA receptor dependent

To determine the glutamate receptor subtype involved in MCA dilation by NMDA receptor co-agonists, several antagonists were used, including a competitive glutamate binding site NMDA receptor antagonist, AP5, a glycine/D-serine co-agonist site NMDA receptor antagonist, DCKA, and an AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). AP5 (100 nM) and DCKA (300 nM) significantly blocked MCA dilation induced by glutamate and D-serine (100 µM each

combined) (Fig. 9A; $p<0.05$), indicating both glutamate and D-serine NMDA receptor binding sites are important for dilation. CNQX (up to 10 μM) had no significant effect (Fig. 9A), suggesting AMPA/kainate receptors do not mediate vasodilation by glutamate and D-serine.

Neuronal NMDA receptors require plasma membrane depolarization to expel channel-bound Mg^{2+}, whereas NMDA receptors identified on non-neuronal cell types, such as astrocytes and oligodendrocytes, have little Mg^{2+} sensitivity (Karadottir et al. 2005; Lalo et al. 2006). Removal of extracellular Mg^{2+} did not significantly change vasodilation induced by glutamate and D-serine (Fig. 9B; $p > 0.05$), indicating low or no sensitivity to Mg^{2+} block. This is consistent with other non-neuronal NMDA receptors, and could mean that NR2C, NR2D or NR3 subunits are present, since they bind Mg^{2+} with low affinity (Chan et al. 2002; Chatterton et al. 2002).

Endothelial-derived nitric oxide mediates NMDA receptor mediated vasodilation

The 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 (Fig 10A), attributable to a loss of baseline nitric oxide production, and failed to dilate in response to acetylcholine (10 nM to 10 μM; Fig. 10B), confirming endothelial destruction. Denuded arteries dilated normally in response to the nitric oxide donor, sodium nitroprusside (SNP, 100 nM to 100 μM; Fig. 10C). Exposure of denuded arteries to glutamate (0.1 μM to 10 mM) in the presence of D-serine (100 μM) produced no significant vasodilatory response (Fig. 11A), 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 non-selective NOS inhibitor, N-nitro-L-arginine methyl ester (L–NAME; 10 μM), prevented dilation induced by glutamate and D-serine together (Fig. 11B). The NOS inhibitor, 1-(2-(trifluoromethylphenyl)) imidazole (TRIM), at a concentration selective for nNOS (50 μM) (Handy et al. 1995) did not significantly alter vasodilation by glutamate and D-serine ($p>0.05$; Fig 11C). In contrast, the NOS inhibitor, $\boldsymbol{N}^5$-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO), significantly blocked relaxation induced by glutamate and D-serine ($p<0.05$; Fig. 11D) at a concentration selective for eNOS (3 μM) (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 ($p<0.05$; Fig. 11D). 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 examined for responses to glutamate and D-serine. MCAs subjected to NADPH-diaphorase staining displayed NO-positive nerve endings on the smooth muscle surface (Fig. 12A). Chemical denervation of arteries in 0.15% phenol and 1.35% ethanol removed the vast majority of NO-containing terminals (Fig. 12B). Denervated arteries were able to constrict normally in response to norepinephrine and dilate in response to

acetylcholine (10 nM to 10 μM) and SNP (100 nM to 100 μM; Fig. 10A-C), indicating full functional viability. Chemical denervation did not significantly affect the magnitude of vasodilation caused by glutamate (0.1 μM to 10 mM) in the presence of D-serine (100 μM; Fig. 12C). This indicates that NOS-containing vascular nerve endings remaining after brain arterial dissections do not contribute to vasodilation by glutamate and D-serine.

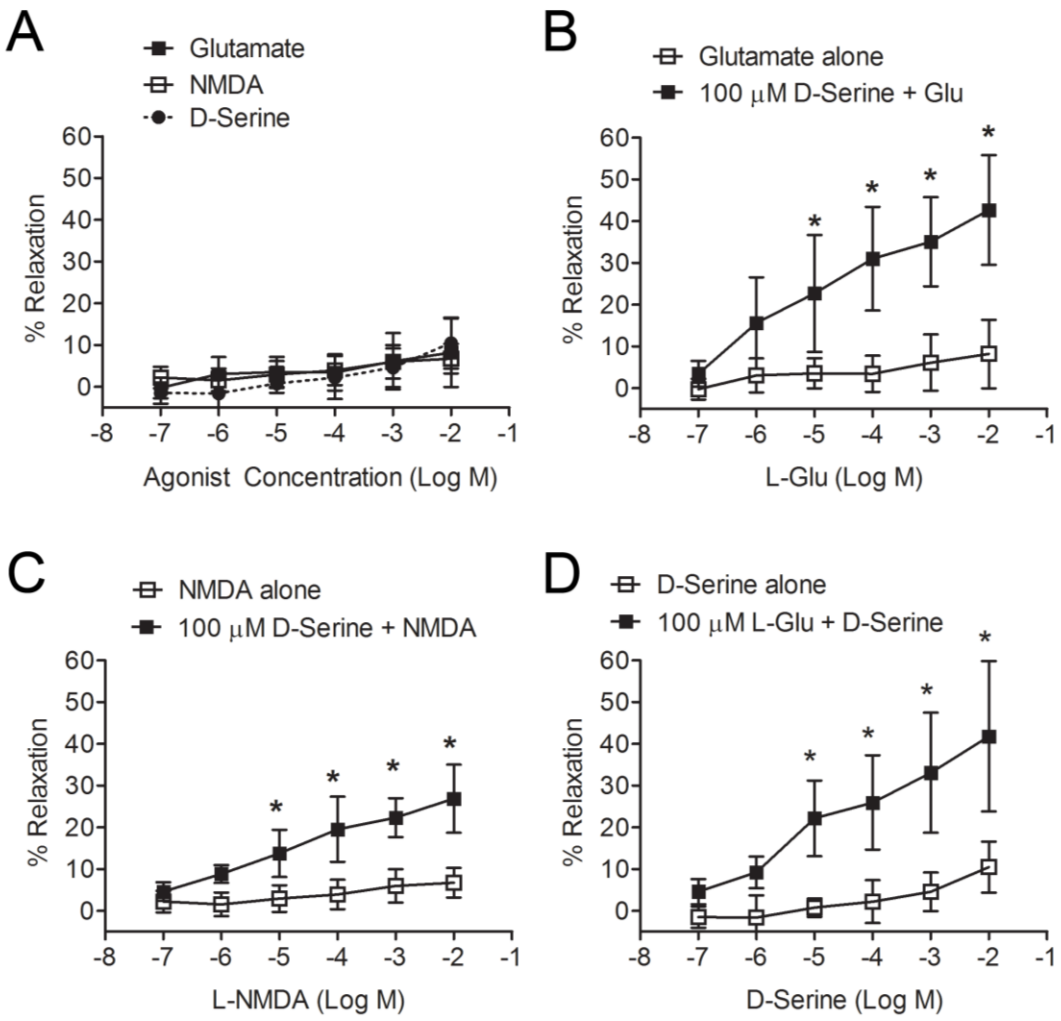

Figure 6: Dilation of isolated middle cerebral arteries (MCAs) by NMDA and glutamate requires D-serine.

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 μM) D-serine (n= 6). D, D-Serine caused concentration-dependent increases in MCA lumen diameter in the presence of fixed glutamate (100 μM, n= 6). Data are presented as mean ± SD; *$p<0.05$ compared to other groups using two-way ANOVA for repeated measures and Bonferroni post hoc test.

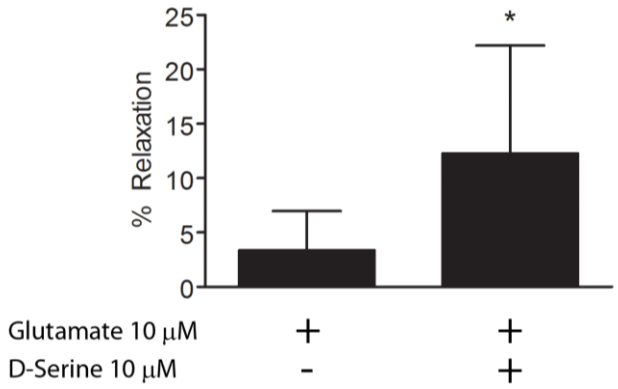

Figure 7: Glutamate and D-serine increase lumen diameter in brain arterioles.

Combinations of glutamate and D-serine, as low as 10 μM, caused significant MCA dilation (n= 6). *$p<0.05$ compared to glutamate only group using a t-test.

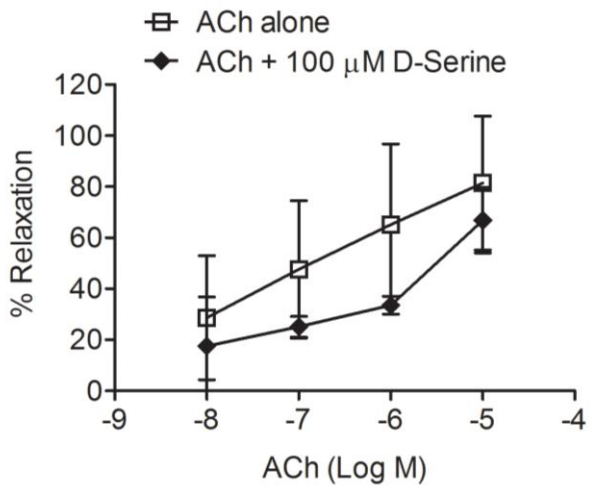

Figure 8: D-serine does not affect dilation caused by acetylcholine (ACh).

Concentration-dependent dilation of MCAs to ACh was not significantly altered by the addition of 100 μM D-serine (n= 3). Data are mean ± SD; two-way ANOVA for repeated measures and Bonferroni *post hoc* test.

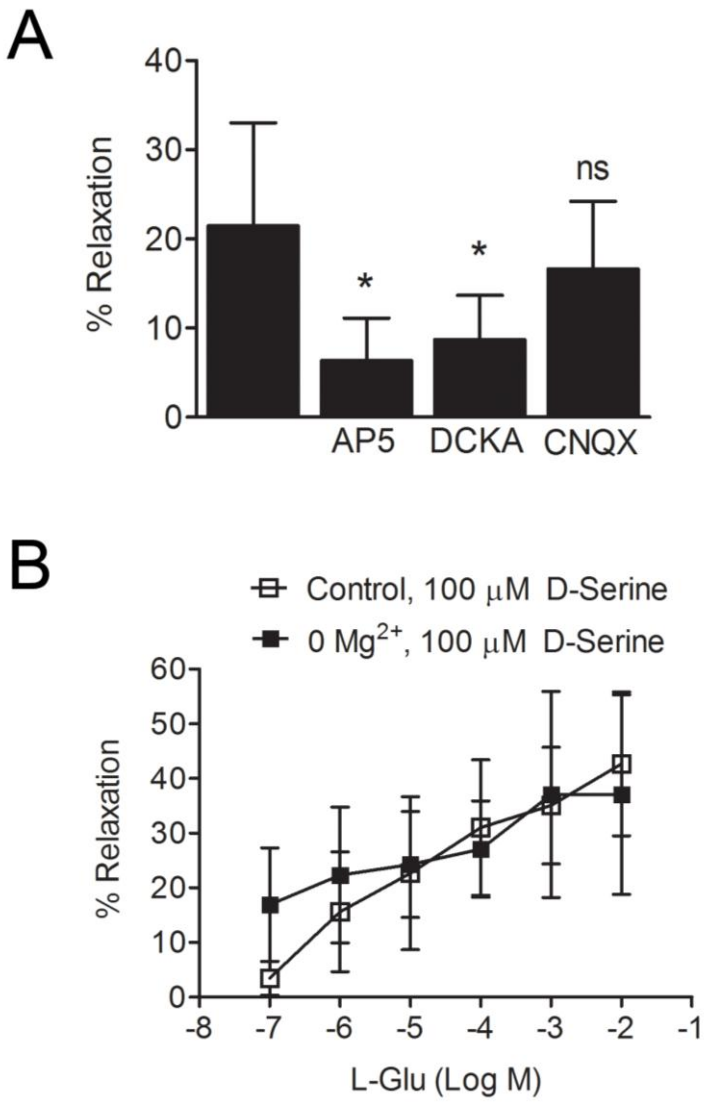

Figure 9: NMDA receptors mediate vasodilation in response to glutamate and D-serine.

A, Treatment of MCAs with glutamate and D-serine together (both 100 μM) caused a significant increase in lumen diameter. NMDA receptor antagonists, D-2-amino-5-phosphonopentanoate (AP5; 100 nM) and 5,7-dichlorokynurenic acid (DCKA; 300 nM) attenuated vasodilation (n= 6). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA/kainate receptor antagonist, did not significantly inhibit vasodilation up to 10 μM

(n= 6). Data are mean ± SD; *p < 0.05 compared to glutamate/D-serine control using one-way ANOVA with Newman-Keuls *post hoc* test. B, No significant differences in vasodilatory response to incremental glutamate in the presence of D-serine (100 μM) were detected between control arteries and arteries incubated in Mg^{2+} free buffer. Data are mean ± SD and were analyzed using two-way ANOVA for repeated measures and Bonferroni *post hoc* test.

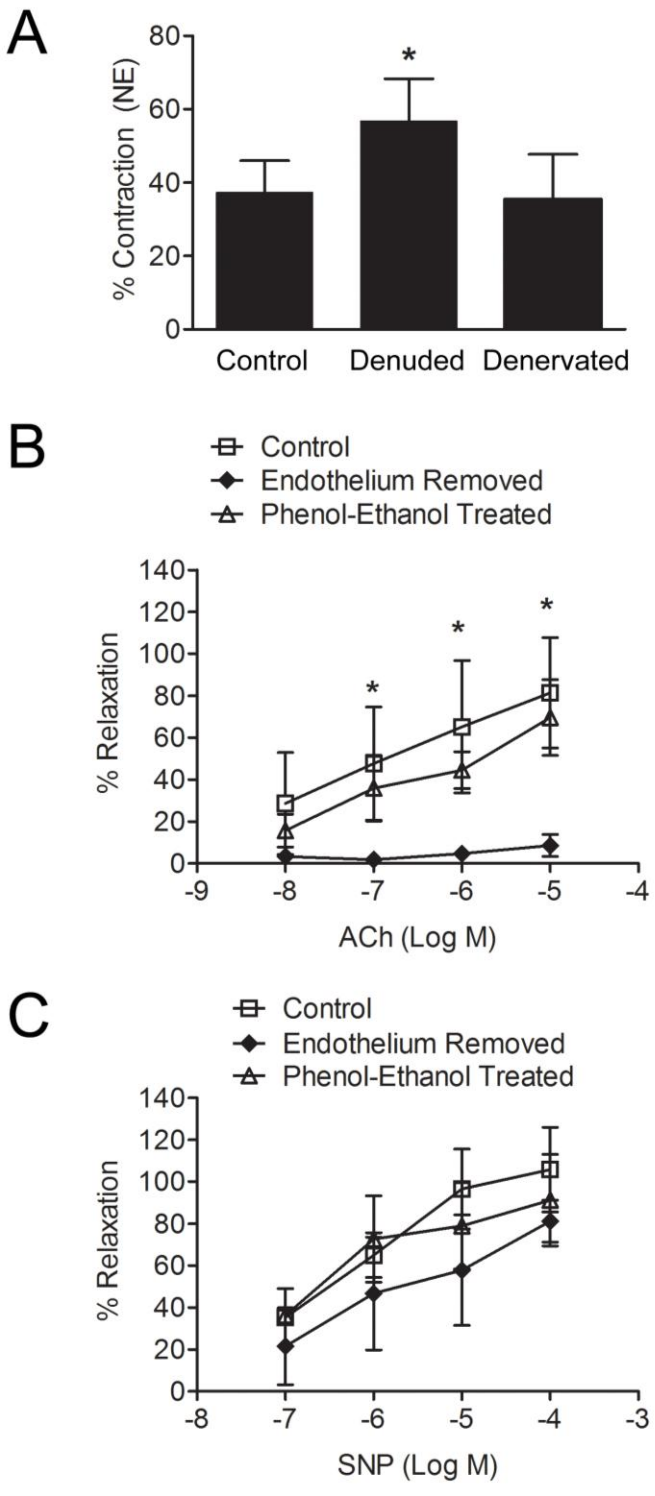

Figure 10: Denuded and denervated vessels respond to known vasoconstrictors and vasodilators.

A, Pre-constriction to 1 μM NE was similar in both denervated and control MCAs, but denuded arteries displayed a greater contraction (n= 9). This is likely due to loss of basal eNOS activity. B, Dilation by ACh, a known eNOS activator, was not significantly different between phenol-treated (denervated) and control arteries (n= 3). Endothelium denuded arteries did not significantly dilate to ACh, suggesting the endothelium had been completely destroyed (n= 3). C, There was no significant difference between dilation to nitric oxide donor, SNP, in phenol-ethanol treated, denuded and control arteries (n= 3). Data are mean ± SD; * $p < 0.05$; two-way ANOVA for repeated measures and Bonferroni *post hoc* test.

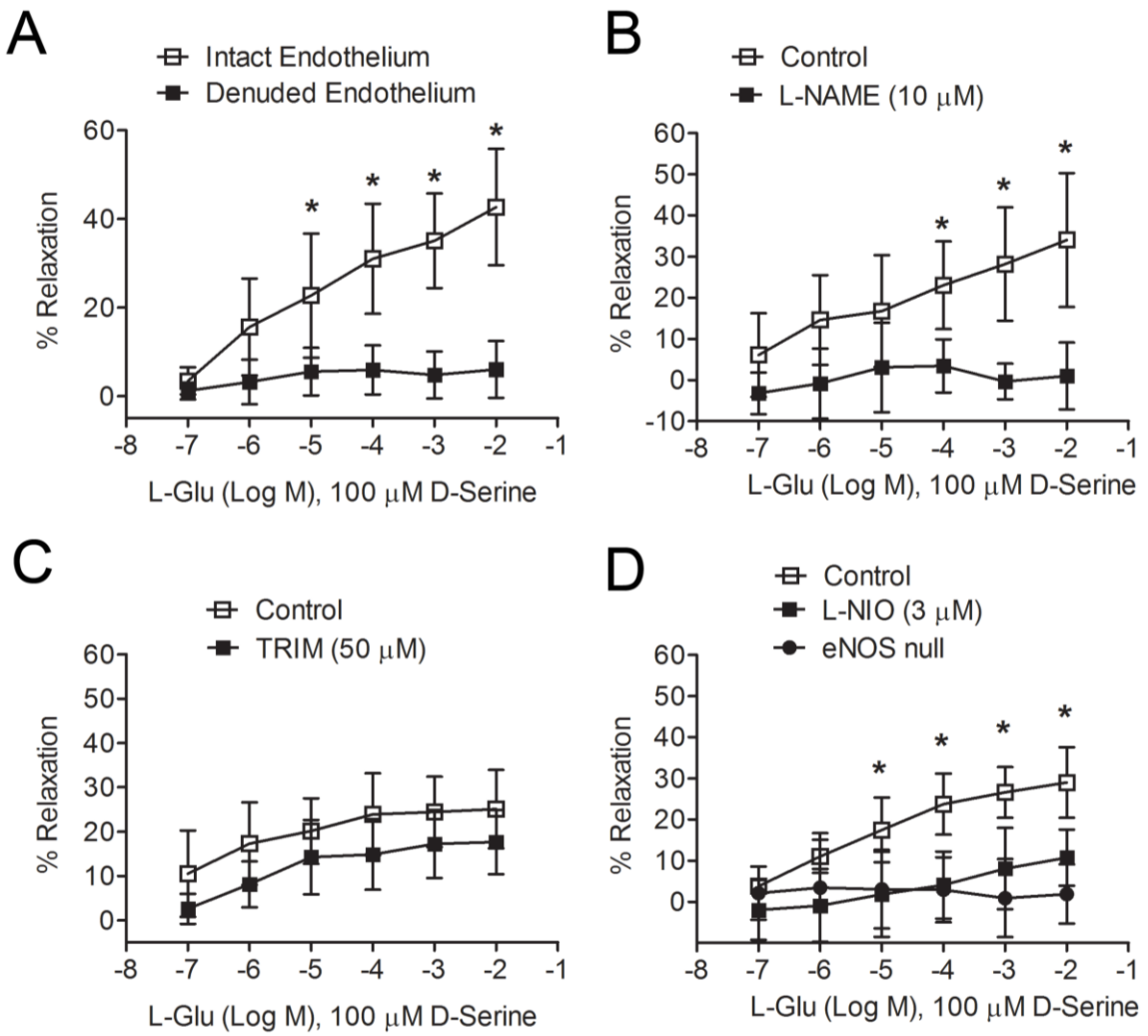

Figure 11: Vasodilation by glutamate and D-serine requires intact endothelium and endothelial nitric oxide synthase (eNOS).

A, Removal of the endothelium isolated middle cerebral arteries an air bolus significantly attenuated smooth muscle relaxation caused by incremental glutamate concentrations in the presence of 100 μM D-serine (n= 6). B, N-Nitro-L-arginine methyl ester (L-NAME, 10 μM) blocked concentration-dependent vasodilation caused by glutamate and D-serine (n= 6). C, Glutamate/D-serine-induced MCA relaxation was not affected by the neuronal NOS inhibitor, 1-(2-(trifluoromethylphenyl)) imidazole (TRIM, 50 μM). D, N5-(1-

iminoethyl)-L-ornithine (L-NIO, 3 μM), an eNOS-selective antagonist, and eNOS deletion prevented MCA relaxation by incremental glutamate increases in the presence of 100 μM D-serine (n= 6). Data are mean ± SD; *$p<0.05$ using two-way ANOVA for repeated measures and Bonferroni *post hoc* test.

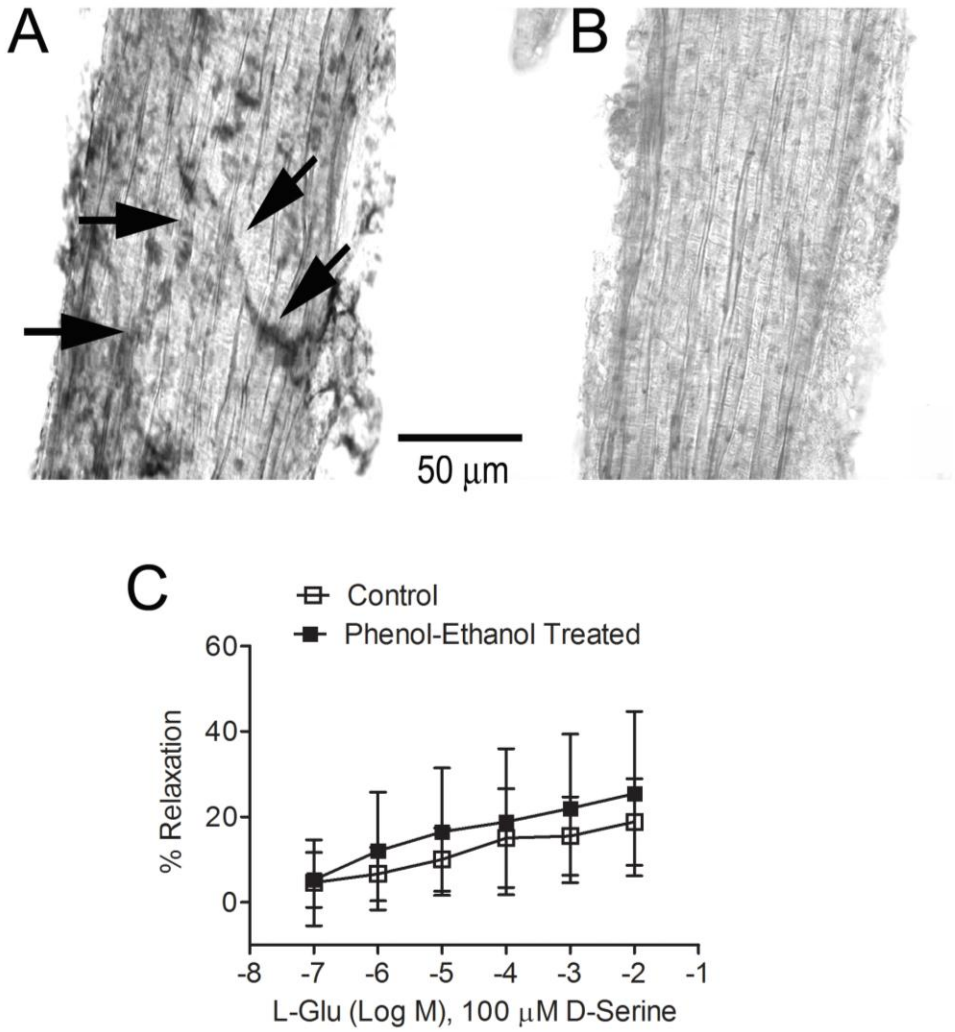

Figure 12: NMDA receptor-mediated vasodilation persists following chemical denervation of MCAs.

Isolated mouse MCAs were mechanically stripped and treated with vehicle (A) or 1.35% ethanol with 0.15% phenol for 3 min (B) to cause chemical denervation. Vessels were either analyzed for NO-containing nerve-endings by NADPH-diaphorase staining (A and 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 μM vs 10 mM) and there were no significant differences between the groups at any dose. Data are means ± SD for 6 MCAs. Data were compared using 2-way ANOVA for repeated measures, followed by the Bonferroni test for selected pairs.

DISCUSSION

Here, we present evidence supporting vasodilation of mouse brain arteries by endothelial NMDA receptors and eNOS. NMDA receptor co-agonists glutamate and D-serine stimulated vasodilation of isolated, denervated mouse MCAs in a manner sensitive to NMDA receptor antagonists and eNOS inhibition or deletion.

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. 1996b; Busija and Leffler 1989; Faraci and Breese 1993; Faraci and Breese 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 diameter. However, for the first time we found significant vasodilation when D-serine was co-applied with glutamate or NMDA. The question of whether this observation could translate into a physiologically relevant dilatory mechanism *in vivo* as our experimental glutamate and D-serine concentrations is important. Local extracellular glutamate levels can approach low millimolar levels (Clements et al. 1992; Diamond and Jahr 2000), and the binding affinity of glutamate varies with NMDA receptor subunit composition (Kutsuwada et al. 1992). Receptor kinetic studies suggest a mixed population of NMDA receptors has a K_d ~ 100 nM (Fritz et al. 1996). Basal D-serine levels at rest *in vivo* have been reported in the 5-8 μM

range by microdialysis(Ciriacks and Bowser 2006; Hashimoto et al. 1995). D-serine also has a EC_{50} (170-320 nM depending on the subunit composition) which is three fold lower than glycine, making it possible for saturation of the NMDA receptor binding sites by basal D-serine levels (Matsui et al. 1995). We found that glutamate and D-serine concentrations in these ranges (100 µM glutamate, 10 µM 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 ~2 fold. We further showed that 10 µM D-serine caused vasodilation in combination with glutamate concentrations as low as 10 µM and 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.

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 co-agonist 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. Finally, 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 further insight into the NMDA receptor subunit configuration. We found that removing Mg^{2+} had no significant effect on vasodilation induced by glutamate and D-serine, suggesting the NMDA receptors involved in dilation may contain NR2C, NR2D or NR3 subunits, which confer much lower Mg^{2+} sensitivity to NMDA receptor function (Chatterton et al. 2002; Monyer et al. 1994). This is consistent with NMDA receptors identified in non-neuronal 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 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 μM) (Handy et al. 1995), did not significantly alter vasodilation by glutamate and D-serine. In contrast, dilation was inhibited by the non-selective NOS inhibitor, L-NAME, by the NOS inhibitor, L-NIO, at a concentration selective for eNOS (3 μM) (Chinellato et al. 1998; Rees et al. 1990), and in $eNOS^{-/-}$ MCAs. These findings strongly indicate eNOS activation through endothelial 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. eNOS plays a major role in regulation of brain vascular tone (Iadecola

1993) and baseline brain blood flow (Ma et al. 1996), and while eNOS does not play 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 endothelial NMDA 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. Our results agree with previous studies 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 a functional linkage between vascular NMDA receptors and eNOS activation 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 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.

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 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 hyperemia 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*.

REFERENCES

Ayata C, Ma J, Meng W, Huang P, Moskowitz MA. (1996) L-NA-sensitive rCBF augmentation during vibrissal stimulation in type III nitric oxide synthase mutant mice. *J Cereb Blood Flow Metab* 16:539-541

Bari F, Errico RA, Louis TM, Busija DW. (1996a) Differential effects of short-term hypoxia and hypercapnia on N-methyl-D-aspartate-induced cerebral vasodilatation in piglets. *Stroke* 27:1634-1639; discussion 1639-1640

Bari F, Errico RA, Louis TM, Busija DW. (1996b) Interaction between ATP-sensitive K^+ channels and nitric oxide on pial arterioles in piglets. *J Cereb Blood Flow Metab* 16:1158-1164

Boldyrev AA, Carpenter DO, Johnson P. (2005) Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. *J Neurochem* 95:913-918

Busija DW, Bari F, Domoki F, Louis T. (2007) Mechanisms involved in the cerebrovascular dilator effects of N-methyl-d-aspartate in cerebral cortex. *Brain Res Rev* 56:89-100

Busija DW, Leffler CW. (1989) Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol* 257:H1200-1203

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 415:793-798

Chen H, Fitzgerald R, Brown AT, Qureshi I, Breckenridge J, Kazi R, Wang Y, Wu Y, Zhang X, Mukunyadzi P, Eidt J, Moursi MM. (2005) Identification of a homocysteine receptor in the peripheral endothelium and its role in proliferation.

J Vasc Surg 41:853-860

Chi OZ, Liu X, Weiss HR. (2003) Effects of inhibition of neuronal nitric oxide synthase on NMDA-induced changes in cerebral blood flow and oxygen consumption. *Exp Brain Res* 148:256-260

Chinellato A, Froldi G, Caparrotta L, Ragazzi E. (1998) Pharmacological characterization of endothelial cell nitric oxide synthase inhibitors in isolated rabbit aorta. *Life Sci* 62:479-490

Choi DW, Koh JY, Peters S. (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* 8:185-196

Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. (1992) The time course of glutamate in the synaptic cleft. *Science* 258:1498-1501

Collingridge GL, Singer W. (1990) Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol Sci* 11:290-296

Domoki F, Kis B, Gaspar T, Bari F, Busija DW. (2008) Cerebromicrovascular endothelial cells are resistant to L-glutamate. *Am J Physiol Regul Integr Comp Physiol* 295:R1099-1108

Endres M, Laufs U, Liao JK, Moskowitz MA. (2004) Targeting eNOS for stroke protection. *Trends Neurosci* 27:283-289

Faraci FM, Breese KR. (1993) Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. *Circ Res* 72:476-480

Faraci FM, Breese KR. (1994) Dilatation of cerebral arterioles in response to N-methyl-D-aspartate: role of CGRP and acetylcholine. *Brain Res* 640:93-97

Faraci FM, Brian JE, Jr. (1995) 7-Nitroindazole inhibits brain nitric oxide synthase and cerebral vasodilatation in response to N-methyl-D-aspartate. *Stroke* 26:2172-2175; discussion 2176

Fergus A, Lee KS. (1997) Regulation of cerebral microvessels by glutamatergic mechanisms. *Brain Res* 754:35-45

Fiumana E, Parfenova H, Jaggar JH, Leffler CW. (2003) Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am J Physiol Heart Circ Physiol* 284:H1073-1079

Handy RL, Wallace P, Gaffen ZA, Whitehead KJ, Moore PK. (1995) The antinociceptive effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase in vitro, in the mouse. *Br J Pharmacol* 116:2349-2350

Hardebo JE, Wieloch T, Kahrstrom J. (1989) Excitatory amino acids and cerebrovascular tone. *Acta Physiol Scand* 136:483-485

Hashimoto A, Oka T, Nishikawa T. (1995) Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialysis. *Neuroscience* 66:635-643

Hlatky R, Lui H, Cherian L, Goodman JC, O'Brien WE, Contant CF, Robertson CS. (2003) The role of endothelial nitric oxide synthase in the cerebral hemodynamics after controlled cortical impact injury in mice. *J Neurotrauma* 20:995-1006

Iadecola C. (1993) Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? *Trends Neurosci* 16:206-214

Iadecola C. (2004) Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat Rev Neurosci* 5:347-360

Jung SS, Levy E. (2005) Murine cerebrovascular cells as a cell culture model for cerebral amyloid angiopathy: isolation of smooth muscle and endothelial cells from mouse brain. *Methods Mol Biol* 299:211-219

Karadottir R, Cavelier P, Bergersen LH, Attwell D. (2005) NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature* 438:1162-1166

Krizbai IA, Deli MA, Pestenacz A, Siklos L, Szabo CA, Andras I, Joo F. (1998) Expression of glutamate receptors on cultured cerebral endothelial cells. *J Neurosci Res* 54:814-819

Lalo U, Pankratov Y, Kirchhoff F, North RA, Verkhratsky A. (2006) NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. *J Neurosci* 26:2673-2683

Ma J, Meng W, Ayata C, Huang PL, Fishman MC, Moskowitz MA. (1996) L-NNA-sensitive regional cerebral blood flow augmentation during hypercapnia in type III NOS mutant mice. *Am J Physiol* 271:H1717-1719

Martineau M, Galli T, Baux G, Mothet JP. (2008) Confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission. *Glia* 56:1271-1284

Meng W, Tobin JR, Busija DW. (1995) Glutamate-induced cerebral vasodilation is mediated by nitric oxide through N-methyl-D-aspartate receptors. *Stroke* 26:857-862; discussion 863

Metea MR, Newman EA. (2006) Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. *J Neurosci* 26:2862-2870

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA

receptors. *Neuron* 12:529-540

Morley P, Small DL, Murray CL, Mealing GA, Poulter MO, Durkin JP, Stanimirovic DB. (1998) Evidence that functional glutamate receptors are not expressed on rat or human cerebromicrovascular endothelial cells. *J Cereb Blood Flow Metab* 18:396-406

Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH. (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A* 97:4926-4931

Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G. (2005) Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. *Proc Natl Acad Sci U S A* 102:5606-5611

Parfenova H, Fedinec A, Leffler CW. (2003) Ionotropic glutamate receptors in cerebral microvascular endothelium are functionally linked to heme oxygenase. *J Cereb Blood Flow Metab* 23:190-197

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747

Philip S, Armstead WM. (2004) NMDA dilates pial arteries by KATP and Kca channel activation. *Brain Res Bull* 63:127-131

Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. (1990) Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 101:746-752

Reijerkerk A, Kooij G, van der Pol SM, Leyen T, Lakeman K, van Het Hof B, Vivien D, de Vries HE. (2010) The NR1 subunit of NMDA receptor regulates monocyte transmigration through the brain endothelial cell barrier. *J Neurochem* 113:447-453

Schell MJ, Molliver ME, Snyder SH. (1995) D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. *Proc Natl Acad Sci U S A* 92:3948-3952

Schipke CG, Ohlemeyer C, Matyash M, Nolte C, Kettenmann H, Kirchhoff F. (2001) Astrocytes of the mouse neocortex express functional N-methyl-D-aspartate receptors. *FASEB J* 15:1270-1272

Scott GS, Bowman SR, Smith T, Flower RJ, Bolton C. (2007) Glutamate-stimulated peroxynitrite production in a brain-derived endothelial cell line is dependent on N-methyl-D-aspartate (NMDA) receptor activation. *Biochem Pharmacol* 73:228-236

Sharp CD, Hines, I, Houghton, J, Warren, A, Jackson, T H, Jawahar, A, Nanda, A, Elrod, J W, Long, A, Chi, A, Minagar, A, and Alexander, J S. (2003) Glutamate causes a loss in human cerebral endothelial barrier integrity through activation of NMDA receptor. *Am J Physiol Heart Circ Physiol* 285:H2592–H2598

Simandle SA, Kerr BA, Lacza Z, Eckman DM, Busija DW, Bari F. (2005) Piglet pial arteries respond to N-methyl-D-aspartate in vivo but not in vitro. *Microvasc Res* 70:76-83

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. (2006) Astrocyte-mediated control of cerebral blood flow. *Nat Neurosci* 9:260-267

Wang Y, Bukoski RD. (1999) Use of acute phenolic denervation to show the neuronal dependence of Ca2+-induced relaxation of isolated arteries. *Life Sci* 64:887-894

Wendling WW, Chen D, Daniels FB, Monteforte MR, Fischer MB, Harakal C, Carlsson C. (1996) The effects of N-methyl-D-aspartate agonists and antagonists on isolated bovine cerebral arteries. *Anesth Analg* 82:264-268

Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S. (2003) Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. *Proc Natl Acad Sci U S A* 100:15194-15199

CHAPTER 3: Endothelial cells express NMDA receptors *in vitro* and *in situ*

SUMMARY

NMDA receptors are expressed in numerous non-neuronal cell types suggesting glutamate signalling could mediate pathways other than neurotransmission. Several studies have demonstrated endothelial cell expression of NMDA receptors which may mediate the blood-brain barrier, oxidative stress, and NO and/or CO production. However, the composition of NMDA receptor subunits in mouse endothelial cells is unknown. Through PCR and immunostaining, we found evidence for expression of NMDA receptor subunits, NR1 and NR2C/D in mouse primary brain endothelial cells, and NR2C/D in cortical vascular endothelium *in situ*. This suggests these receptors contain both glutamate and D-serine binding sites and may have reduced Mg^{2+} sensitivity, but the precise role of these receptors in endothelial calcium changes and activation of eNOS is not yet clear.

INTRODUCTION

NMDA receptors are important ionotropic glutamate receptors which influence neuronal synaptic plasticity and excitotoxicity. These receptors are tetramers composed of seven different subunits (NR1, NR2A-D, and NR3A and 3B) of which there are numerous splice variants, forming a heterogeneous receptor population. This proteomic

variability confers different receptor properties such as reduced sensitivity to Mg^{2+} blockage in the presence of NR2C/D or NR3A/B subunits (Chatterton et al. 2002; Monyer et al. 1994). NMDA receptors are generally composed of two NR1 and two NR2 subunits with NR2 containing the glutamate binding site and NR1 containing the D-serine binding site. However, NR1/NR2/NR3 and NR1/NR3 receptors are also expressed (Chatterton et al. 2002; Salter and Fern 2005). NR1 is a critical subunit for receptor formation as it is conserved across all NMDA receptors.

NMDA receptor expression has been detected in cells other than neurons, such as oligodendrocytes (Salter and Fern 2005), microglia (Murugan et al. 2011) and astrocytes (Lee et al. 2010; Palygin et al. 2010) of the CNS. Peripheral cells including lymphocytes (Boldyrev et al. 2004), cardiomyocytes (Gao et al. 2007), lung cells (Said et al. 1996), keratinocytes (Genever et al. 1999), platelets (Franconi et al. 1996) and vascular endothelial cells (Andras et al. 2007; Sharp 2003) also express NMDA receptors. The function of these NMDA receptors has not been identified in many cases, but suggests glutamate signalling is also important outside the CNS.

Endothelial NMDA receptors (eNMDA) are linked to regulation of the blood-brain barrier (BBB) (Zhu and Liu 2004), and may cause barrier disruption (Andras et al. 2007; Liu et al. 2010; Neuhaus et al. 2011b), oxidative stress and increased free radical production (Kuhlmann et al. 2009; Sharp et al. 2005) during glutamate excitotoxicity. Therefore, endothelial NMDA receptors may mediate endothelial damage during pathological conditions when extracellular glutamate rises. Also, NMDA receptors may increase activity of heme oxygenase (HO) and eNOS, elevating levels of vasodilators,

CO and NO (Parfenova et al. 2003; Scott et al. 2007), suggesting endothelial cells could be involved in glutamate-mediated vasodilation.

To date, most studies reporting eNMDA receptor expression have used cultured endothelial cells, including immortalized endothelial cell lines from human, mouse, cow, and pig (Betzen et al. 2009; Kuhlmann et al. 2008; Kuhlmann et al. 2009; Neuhaus et al. 2011a; Neuhaus et al. 2011b; Reijerkerk et al. 2010; Sharp 2003). All of these groups have detected NR1 expression, but expression of other subunits varies greatly across species and cell lines. Very few studies utilized primary endothelial cells (Andras et al. 2007; Krizbai et al. 1998; Parfenova et al. 2003), and the NMDA receptor subunit composition in mouse primary endothelial cells is unknown. Here, we report NR1 and NR2C/2D subunits are expressed in primary mouse brain endothelial cells and these results were used as a basis for immunohistochemistry studies of cerebrovascular NMDA receptors *in situ*.

MATERIALS AND METHODS

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.

Primary Endothelial Cell Culture

Endothelial cells were isolated from adult (15 week old male) or juvenile (14-19

day old) CD1 mouse brains, as described previously (Jung and Levy 2005). Briefly, 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 x*g* for 30 min at 4°C, which resulted in an endothelial cell pellet. Isolated endothelial cells were collected on glass beads and cultured in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin, and 150 µg/ml endothelial cell growth supplement. Cells were passaged twice before reaching confluence. These cultures mainly contained endothelial cells as demonstrated by positive staining using *Griffonia simplicifolia* 1 Isolectin B_4 tagged with Alexa Fluor® 488 (Invitrogen, Burlington, ON) which labels endothelial cells. These cells were negative immunostained for other cell type markers, such as glial acidic fibrillary protein (GFAP; astrocytes). They also expressed very low levels of neuronal marker microtubule associated protein 2 by real-time PCR (Fig. 13).

Preparation of cDNA from Total RNA

Total RNA was isolated from primary endothelial cell cultures, unpurified brain cultures or whole mouse brain tissue using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON). RNA was DNase digested according to the Turbo DNA-free protocol (Applied Biosystems, Carlsbad, CA), and reverse transcribed to cDNA according to the SuperScript III RNase H^- Reverse Transcriptase protocol (Invitrogen, Burlington, ON). Complementary mRNA was digested using RNase H and the remaining cDNA was purified and diluted to 10 ng/µl, according to the PCR Purification Kit (Qiagen, Mississauga, ON).

Polymerase Chain Reaction (PCR)

Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal control) and NMDA receptor subunit genes (NR1, NR2A-2D) were amplified using primers listed in Table 1. All gene targets were PCR amplified according to the DreamTaq protocol (Fermentas, Burlington, ON) using 10 ng of cDNA from brain tissue or endothelial cultures as template. The cDNA was denatured for 2 minutes at 95°C, followed by 40-60 cycles of 95°C for 30 seconds, 60°C (GAPDH), 63°C (NR1), 60°C (NR2A), 62°C (NR2B), 60°C (NR2C), or 62°C (NR2D) 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 with ethidium bromide stain.

Generation of Calibrators and Standard Curves for Quantitative PCR

To permit relative quantification of target gene expression, calibrator plasmids were generated (Stobart et al. 2007). Mouse GAPDH, von Willebrand Factor (vWF; endothelial cell marker), NR1, and NR2D genes were amplified on an MJ Research PTC-200 thermocycler. All primers were designed to span an intron-exon junction or include an intron to limit amplification of genomic DNA. All gene targets were PCR amplified according to the DreamTaq protocol (Fermentas, Burlington, ON) with primers and annealing temperatures detailed in Table 1. Amplicons were run on a 2% agarose gel at 80V for 90 minutes and bands were visualized by ethidium bromide staining and extracted from the gel according to the QIAEX II Gel Extraction kit (Qiagen, Mississauga, ON). These nucleic acids were ligated into the pCR2.1 vector at a 3:1 ratio

Table 1: PCR primers for NMDA receptor subunits and housekeeping genes.

Gene	Primer Pairs (5'--->3')	Size (bp)	Anneal °C	Extension (sec)	Cycles
GAPDH	CACGGCAAATTCAACGGCACAGT TGGGGGCATCGGCAGAAGG	232	60	10	40
vWF	TCATCGCTCCAGCCACATTCCATA AGCCACGCTCACAGTGGTTATACA	189	65	7	45
GFAP	GAGCGAGCGTGCAGAGATGATGG CTCCCGAAGCTCCGCCTGGTAGA	166	60	7	45
MAP2	AAGAGAAGGAAGCCCAATACAAGGA GCTGGCGATGGTGGTGGGGAAGGT	97	65	6	45
NR1	TGTGCGGGACAACAAGCTCCA ATGCCGATGCCAAAGCCGGA	126	63	6	45
NR2D	CTGTGTGGGTGATGATGTTCGT GTGAAGGTAGAGCCTCCGGG	133	62	6	45
NR2A	TACCTGGTGACCAATGGGAAGCAT AGAAGTCCACCACTTCTGAACGCT	142	60	7	60
NR2B	ATATTCCCAACATGCGCTCTCCCT TTCAGCTCGTCGACTCTCTTGGTT	80	62	6	45
NR2C	ACCAGAATCTCACCAAGGGCAAGA AGCGAGGAAGATGACAGCGAAGAA	176	60	8	50

(Invitrogen). Ligation products were transformed into TOP10 *E. coli* by electroporation (Invitrogen), and transformants were selected for with carbenecillin. Individual colonies confirmed to carry the fragment of interest were grown in LB broth + 200 µg/mL carbenecillin overnight at 37°C with shaking. Plasmid was isolated from bacterial cultures according to the QIAprep Spin Miniprep kit (Qiagen).

To generate standard curves, plasmids encoding target gene fragments (at a known concentration) were used as PCR template with primers in Table 1 for amplification on a LightCycler 480 using SybrGreen MasterMix (Roche Diagnostics). These standard curves were analyzed on the LightCycler 480 software and stored as standard curve files for further analysis.

Gene Expression Profiling by Quantitative PCR

To analyze the relative expression profiles of mouse GAPDH, vWF, NR1 and NR2D in primary cell cultures and whole brain, real-time quantitative PCR was conducted on a LightCycler 480 (Roche Diagnostics) amplifying 2µL (20 ng) of cDNA from each preparation, as well as 2µL of calibrator plasmid (20 pg) in triplicate according to the conditions in Table 1. Using the LightCycler 480 software (Roche Diagnostics), the relative expression levels of NR1, NR2D, and vWF were calculated based upon previously generated standard curves, and normalized to GAPDH expression.

Table 2: Antibodies for immunostaining.

Company	Antigen	Species	Procedure	Dilution
Santa Cruz	NR1	goat polyclonal	Immunocytochemistry	1 in 100
Santa Cruz	NR2C	rabbit polyclonal	Immunocytochemistry Immunohistochemistry	1 in 50 1 in 50
Santa Cruz	NR2D	rabbit polyclonal	Immunocytochemistry Immunohistochemistry	1 in 50
Santa Cruz Abcam	P-gp	mouse monoclonal	Immunohistochemistry	1 in 20
Sigma	GFAP	mouse monoclonal	Immunohistochemistry	1 in 250
Invitrogen	*Griffonia simplicifolia* 1 Isolectin B_4		Immunocytochemistry Immunohistochemistry 2-photon microscopy	5 µg/ml

Immunocytochemistry

Primary endothelial cells from adult (15 week old) or juvenile (14-19 day old) cultures were rinsed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 hour. Cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS and blocked at room temperature for 1 hour with 1% bovine serum albumin in PBS. Goat anti-NR1 polyclonal primary antibody, rabbit anti-NR2C polyclonal antibody, and rabbit anti-NR2D polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA; details in Table 2) were applied overnight at 4°C. Secondary antibodies Alexa Fluor® 568 donkey anti-goat IgG (H+L) or Alexa Fluor® 568 goat anti-rabbit IgG (H+L) were applied with endothelial cell marker, *Griffonia simplicifolia* 1 Isolectin B_4 tagged with Alexa Fluor® 488 (5 µg/ml; Invitrogen, Burlington, ON) in 1% BSA in PBS for 1 hour at room temperature. Staining was visualized with a Zeiss LSM510 laser-scanning microscope (Oberkochen, Germany).

Immunohistochemistry

For NMDA receptor subunit immunostaining in brain slices, adult (15 weeks old) or juvenile (14-19 day old) mouse brains were rinsed with PBS (pH 7.2) and placed in 4% paraformaldehyde for 24 hours. Fixed tissue was cryo-protected in 30% sucrose for 3 days at 4°C and snap frozen in liquid nitrogen-cooled n-hexane. Frozen sagittal sections (50 µm) were cut and free-floating sections were exposed to an antigen retrieval step with 10 mM citrate (pH 8.5) at 80°C for 30 min. Sections were rinsed in 0.1 M TBS (pH 7.6), treated for 2 hours with Mouse-on-Mouse blocking reagent (Vector Laboratories) to reduce non-specific staining and incubated with rabbit anti-NR2C or rabbit anti-NR2D

(Santa Cruz Biotechnologies) with either mouse anti-GFAP (Sigma) or mouse anti-p-glycoprotein (P-gp, Santa Cruz Biotechnology or Abcam) for 48 hours at 4°C. After washing, sections were incubated with secondary antibodies for 2 hours at room temperature. Alexa Fluor® 568 goat anti-rabbit IgG (H+L) was used to detect NR2C or NR2D signal, while Alexa Fluor® 488 goat anti-mouse IgG (H+L) was used to detect GFAP and P-gp. Stained sections were visualized with a Zeiss LSM510 laser-scanning microscope.

RESULTS

Mouse brain endothelial cells express mRNA for NMDA receptor subunits

As shown in Figure 14(A, B), PCR products for NR1 and NR2D were detected in adult and juvenile cultures, indicating that mRNA for these subunits is expressed. However, NR2A, 2B, and 2C did not appear to be expressed, since several different primer sets, including those listed in Table 1, failed to produce a PCR product from endothelial cDNA. Real-time PCR (Fig. 14B) suggested juvenile primary endothelial cultures display 12-fold (1240 ± 370%) vWF (endothelial marker) enrichment compared to unpurified cultures containing neurons, glia, and other brain cells. mRNA for NMDA receptor subunits NR1 and NR2D were identified in purified endothelial cells at levels less than unpurified brain cell cultures (90 ± 3% and 31 ± 2% respectively). This further confirms the presence of NR1 and NR2D transcripts and points to the relative expression levels in brain endothelial cells compared to other brain cells (Fig. 14).

NMDA receptor subunit localization in endothelial cells in vitro and in situ

NR1 was localized in cultured adult and juvenile brain endothelial cells that express isolectin B_4 (Fig. 15A-F), confirming NR1 protein expression in these cells. Antibodies for NR2A and 2B did not significantly label endothelial cultures (not shown), suggesting these subunits are not expressed. However, immunoreactivity for NR2C and 2D co-localized with isolectin B_4 in juvenile endothelial cultures (Fig. 15G-L), indicating the presence of these subunits. This staining was specific, since application of secondary antibody alone did not label the cells (Fig. 15M-O).

We also investigated whether NMDA receptor subunits are expressed by adult brain slice cortical arteries and capillaries near the pial surface. In Figure 16 A-C and G-I, vessels with diameters consistent with those of MCAs used in pressure myography experiments in Chapter 2 (50-80 μm) were analyzed. Immunoreactivity for the NMDA receptor subunit, NR2C, which is largely silent in adult cortical neurons, co-localized with the endothelial cell marker, P-gp (Fig. 16A-C). In contrast, NR2C signal was not co-localized with perivascular astrocytes that express GFAP (Fig. 16G-I). Similarly, NR2C staining also co-localized with P-gp, but not GFAP, in smaller vessels near capillary size (Fig. 16D-F, J-L). Overall, these results indicate endothelial expression of NR2C immunoreactivity in adult mouse cortical arteries and capillaries *in situ*. Staining with primary antibodies was specific and had low background, since application of secondary antibodies alone, did not generate staining patterns (Fig. 20). Similar results for NR2D immunostaining were also seen, where NR2D co-localized with P-gp (Fig.

17A-C), but was located inside GFAP staining around arterioles (Fig. 17D-F) in adult cortical slices. In brain slices from juvenile mice, NR2C and NR2D staining overlapped with endothelial marker, P-gp (Fig. 18A-F), near arterioles and was also located inside GFAP labelling (Fig. 18G-L). These images provide strong evidence NR2C/2D is expressed in endothelial cells of cortical blood vessels in both adult and juvenile brain tissue.

Some NR2C and NR2D staining in juvenile cortex (Fig. 19) also co-localized with GFAP, suggesting expression of these subunits in protoplasmic astrocytes, which fits with other evidence reporting sensitivity of astrocyte NMDA receptors to NR2C/2D antagonist, UBP141 (Palygin et al. 2011; Palygin et al. 2010). Astrocytic staining was more prevalent in juvenile brain, possibly because regional expression of NR2C/2D changes with age (Monyer et al. 1994; Takai et al. 2003).

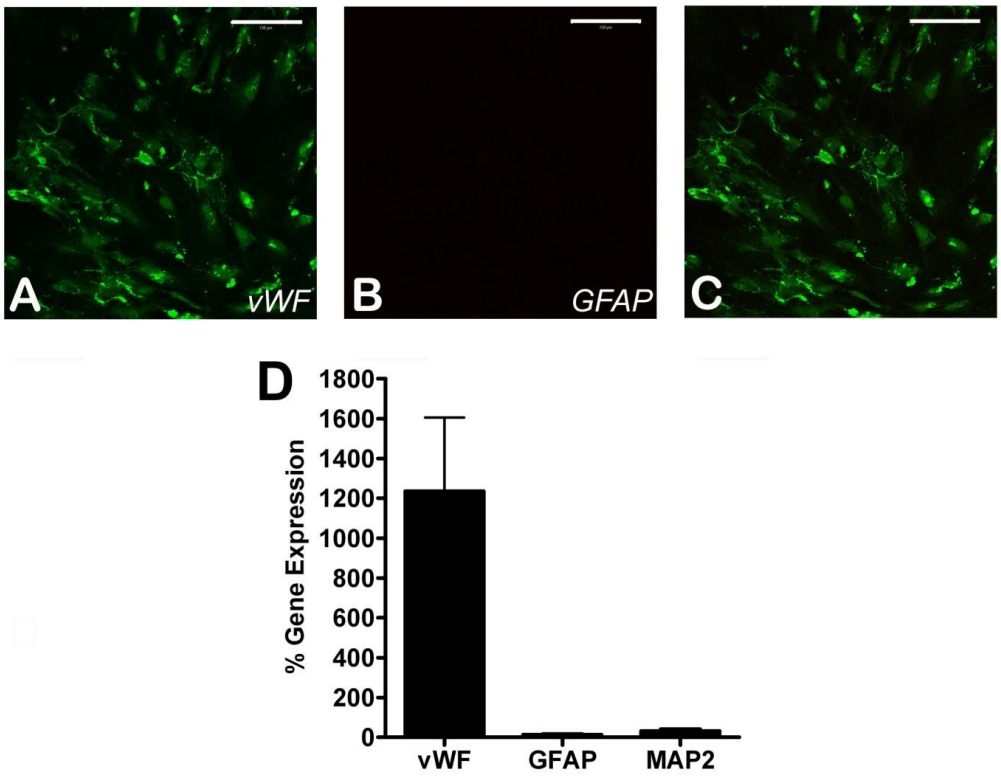

Figure 13: Primary brain endothelial cell express an endothelial marker, but not other cell type markers.

A-C, Immunostaining for endothelial cell marker, vWF, localized to cultured cells, but staining for astrocyte marker, GFAP, was undetectable. Scale bars are 100 μm. D, When compared to unpurified mixed brain cell cultures by quantitative PCR, juvenile endothelial cultures were 12-fold enriched in mRNA for vWF and very low expression of GFAP and MAP2 (neuronal marker). Data are mean ± SD; n= 3.

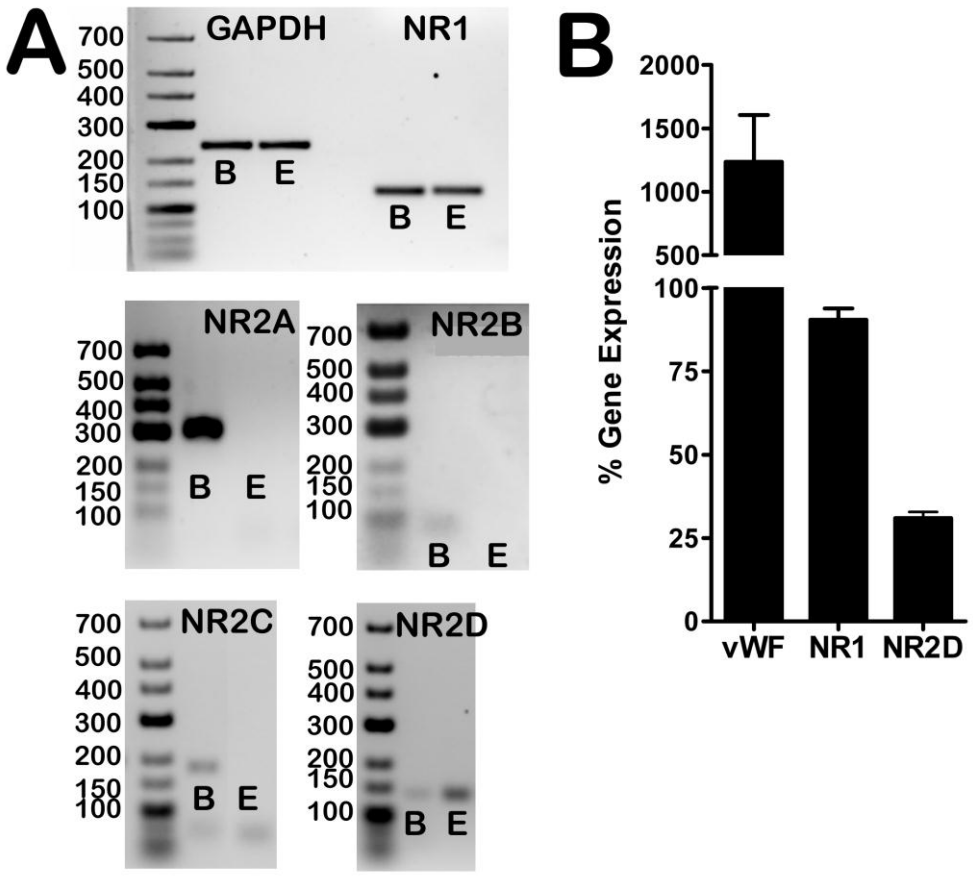

Figure 14: Primary endothelial cells express mRNA for NMDA receptor subunits.

A, Primary brain endothelial cells from adult CD1 mice expressed RNA transcripts for NR1, NR2D, and housekeeping gene, GAPDH (red arrows). Several different primer sets were tested for NR2A, 2B, or 2C, which produced a product from brain tissue, but not endothelial cells even after numerous PCR cycles. B= whole brain, E= endothelial culture. B, Brain endothelial cells from juvenile mice also displayed NR1 and NR2D expression. When compared to unpurified mixed brain cell cultures by quantitative PCR, juvenile endothelial cultures were 12-fold enriched in mRNA for the endothelial marker, von Willebrand factor (vWF; 1236%), and express NR1 (90%) and NR2D (31%). Data are mean ± SD; n= 3.

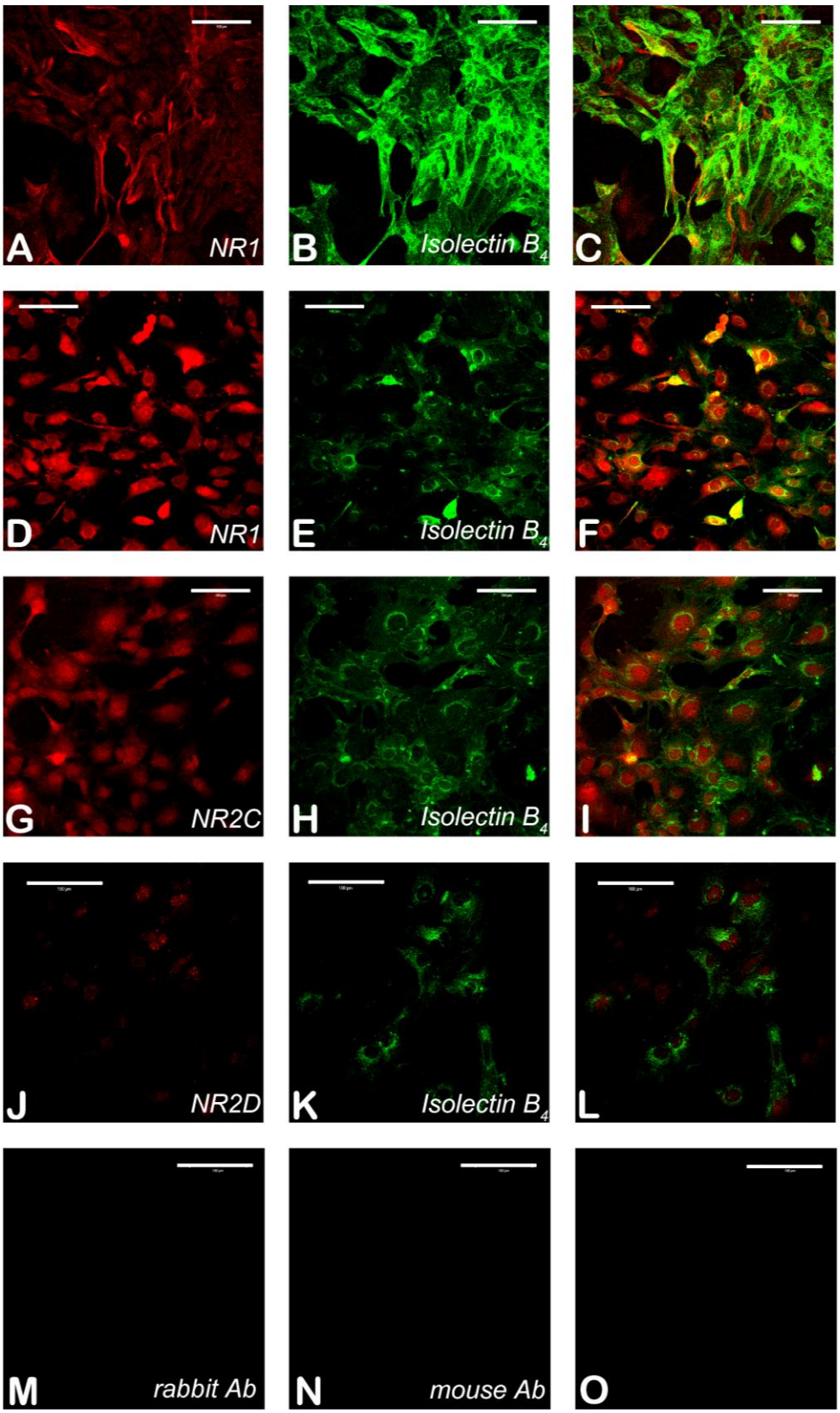
A
NR1
B
Isolectin B4
C
D
NR1
E
Isolectin B4
F
G
NR2C
H
Isolectin B4
I
J
NR2D
K
Isolectin B4
L
M
rabbit Ab
N
mouse Ab
O

Figure 15: Immunocytochemistry of primary endothelial cells displays NMDA receptor subunit distribution.

A-F, Immunofluorescence shows co-localization of NR1 (red) and the endothelial cell marker, isolectin B_4 (green) in adult endothelial cultures (A-C) and juvenile endothelial cultures (D-F). G-L, Also, both NR2C (G, red) and NR2D (J, red) co-localized with isolectin B_4 (H & K, green) in juvenile cultures. Antibodies for NR2A and 2B were investigated, but staining was very faint or non-existent, suggesting these subunits were not expressed. Also, secondary antibodies did not non-specifically bind cells, as noted by the absence of staining in M-O. Scale bars are 100 μm.

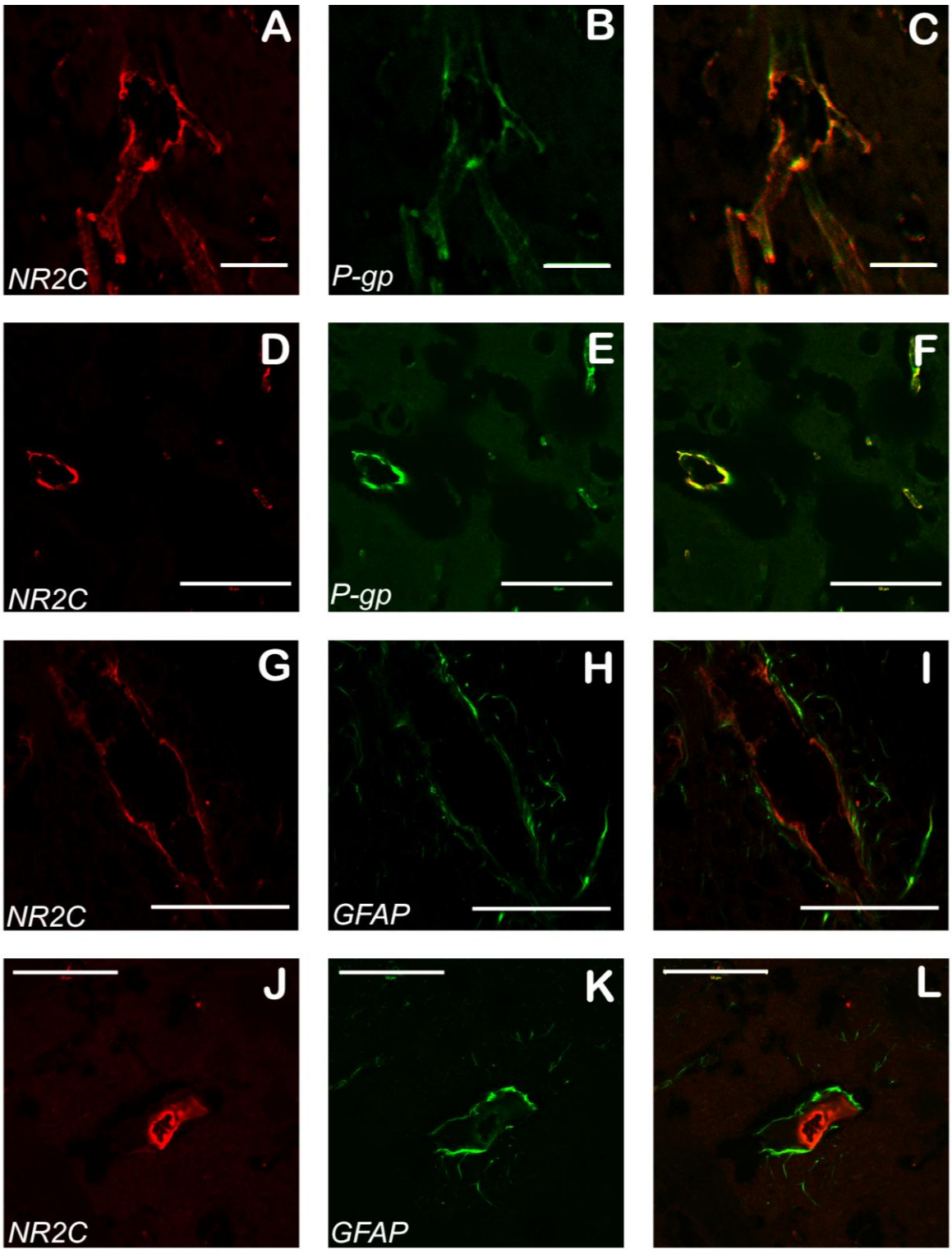

Figure 16: NR2C is localized to endothelial cells of adult mouse cortical slices.

A-F, Co-immunofluorescence of NR2C (red) and p-glycoprotein (green) localized in large cortical arteries (A-C) and smaller arterioles or capillaries (D-F). G-K, Immunofluorescence for NR2C (red) and astrocyte glial fibrillary acidic protein (GFAP; green) did not overlap (I & L) in cortical arteries near the pial surface (G-I) or smaller blood vessels (J-L). Scale bars are 50 μm.

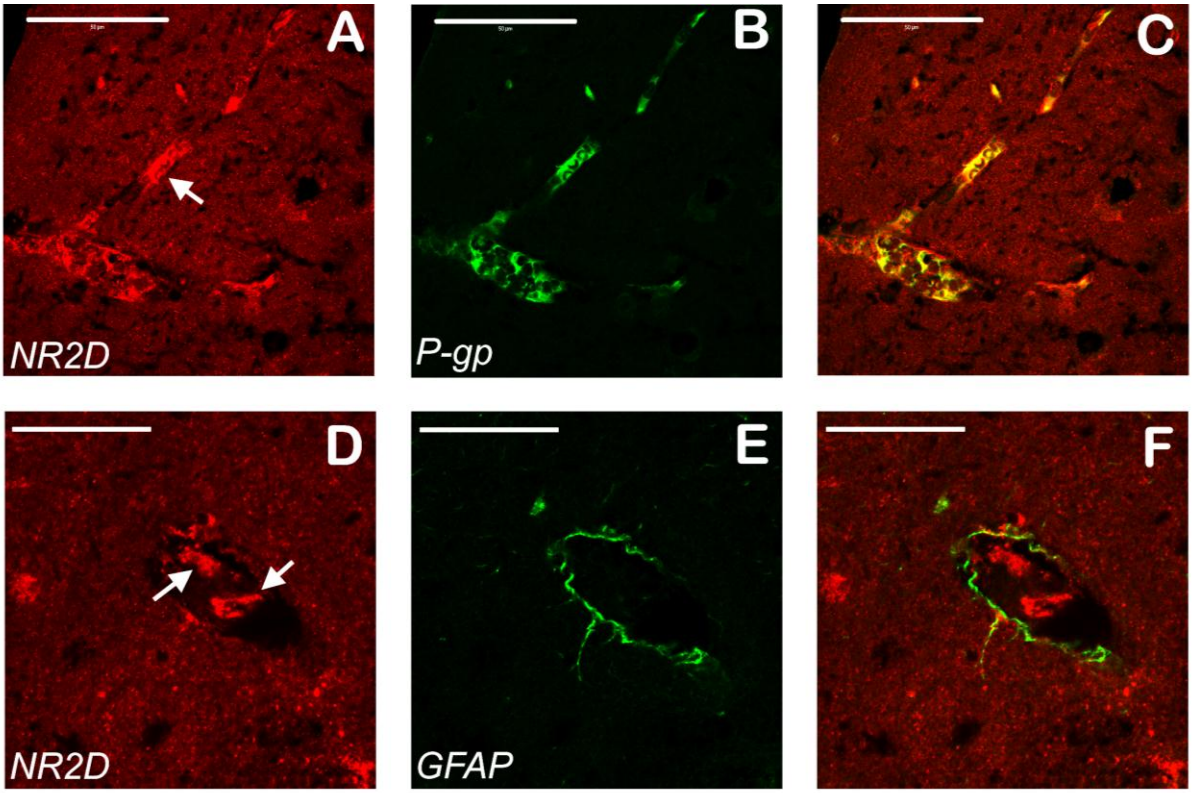

Figure 17: NR2D is localized to endothelial cells of adult mouse cortical slices.

A-C, Co-immunofluorescence of NR2D (red, white arrows) and P-gp (green) localized in adult cerebral arterioles. Scale bars are 50 μm. D-F, Immunofluorescence for NR2D (red) was located inside astrocyte GFAP (green) staining around arterioles. Scale bars are 100 μm.

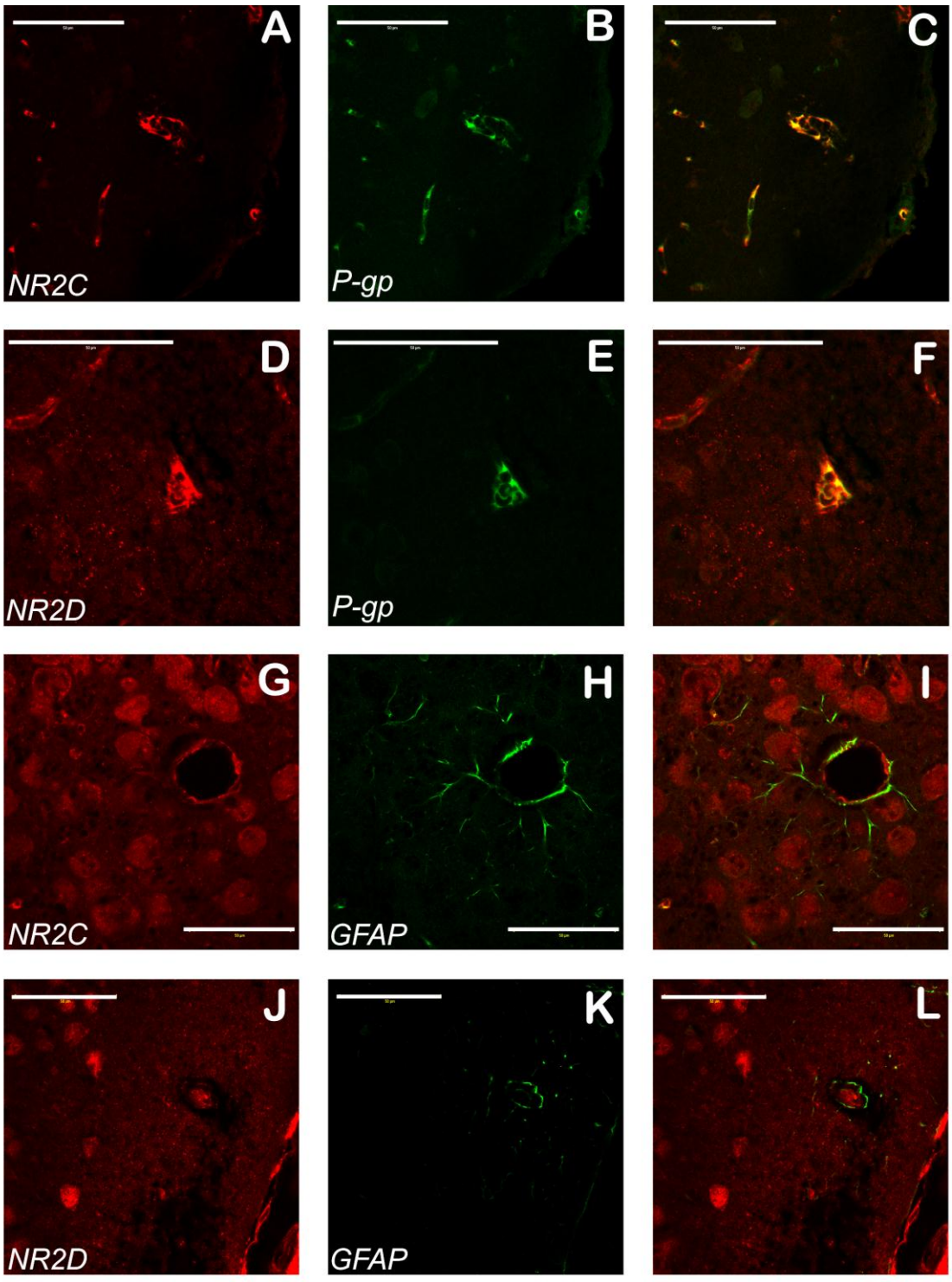

Figure 18: NR2C and 2D are localized to endothelial cells of juvenile mouse cortex.

A-F, Co-immunofluorescence of NR2C (A; red) or NR2D (D; red) with P-gp (green)

localized in juvenile cerebral arterioles. G-L, Immunofluorescence for NR2C (G; red) or NR2D (J; red) was located inside astrocyte GFAP (green) around arterioles. Both NR2C and NR2D also appeared to stain cortical neurons (large red cells). Scale bars are 50 μm.

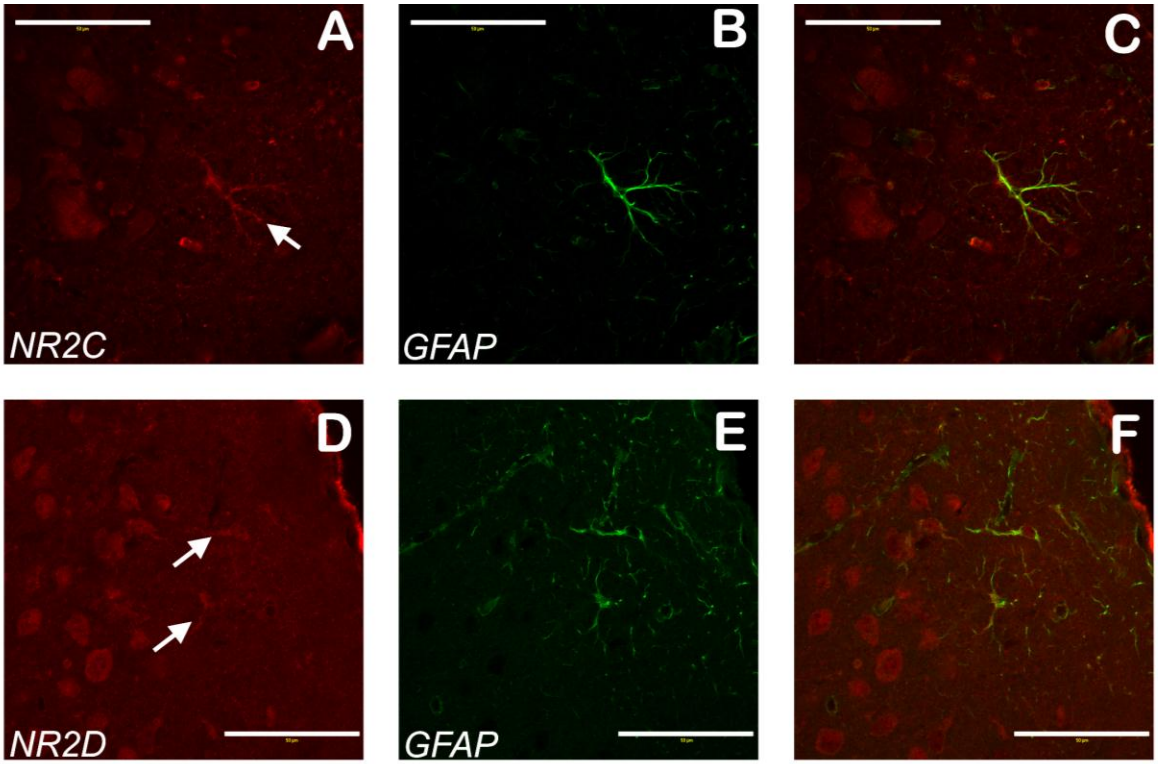

Figure 19: NR2C and 2D are localized to cortical astrocytes of juvenile mouse brain slices.

A-F, Co-immunofluorescence of NR2C (A; red) or NR2D (D; red) overlapped with GFAP in some cortical astrocytes, as indicated by arrows. Scale bars are 50 μm.

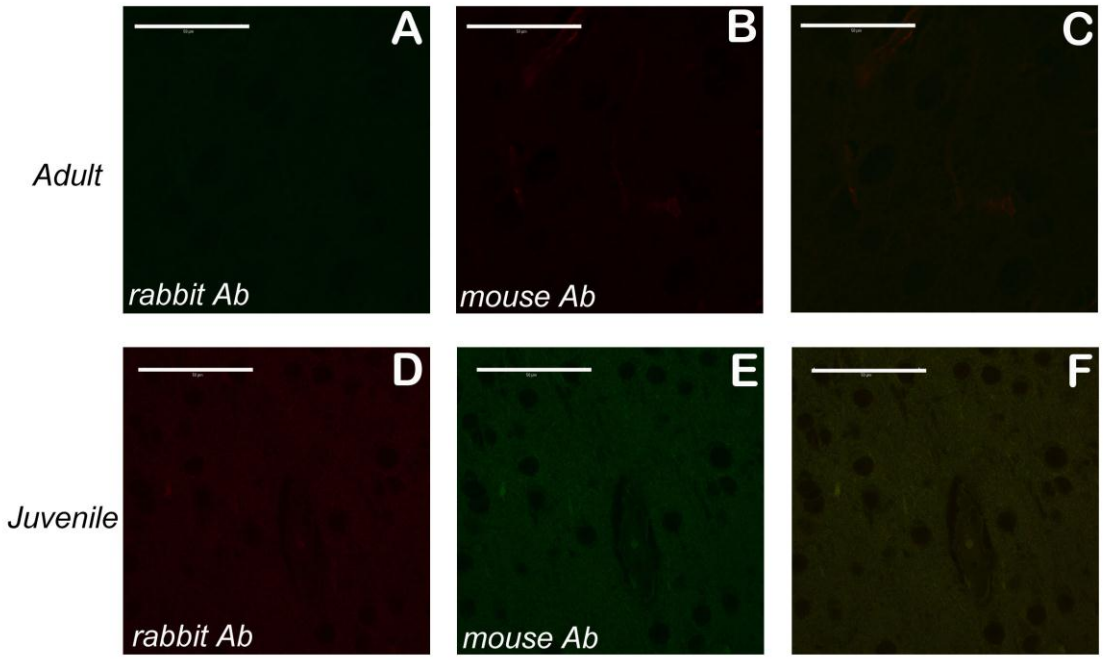

Figure 20: Secondary antibodies for mouse and rabbit have minimal background staining when applied to tissue alone.

A-C, Co-immunofluorescence of goat anti mouse and goat anti rabbit applied to adult mouse slices without primary antibodies display reduced background. D-F, Background was slightly higher from images of juvenile tissue, but staining did not give the same pattern as primary antibody staining suggesting the secondary antibodies were specific. Scale bars are 50 μm.

DISCUSSION

Here, we present evidence of NMDA receptor expression in mouse endothelial cells *in vitro* by PCR and immunocytochemistry, and *in situ* by immunohistochemistry. Our evidence suggests mouse primary cultured brain endothelial cells express NMDA receptor subunits, NR1 and NR2C/D, which was verified *in situ* by immunoreactivity for NR2C/2D in mouse brain blood vessels.

To date, nearly all studies of eNMDA receptors have used cultured endothelial cells, both from primary and immortalized cell line sources, to examine NMDA receptor expression and effects at the cellular level (Betzen et al. 2009; Kuhlmann et al. 2008; Kuhlmann et al. 2009; Neuhaus et al. 2011a; Neuhaus et al. 2011b; Reijerkerk et al. 2010; Sharp 2003). While cultured cells are useful for studying individual cell types, cell characteristics may change with culture conditions and immortalization compared to cells *in situ*. For example, brain endothelial cells *in situ* form the BBB through tight junction development generating polarization of membrane protein expression on the luminal or abluminal side of endothelial cells. Cultured and immortalized endothelial cells can display reduced expression of tight junctions and other proteins (Wolburg et al. 1994), thereby differing from native brain microvascular endothelium. While cell culture is a useful model for initial studies, interpretation of results requires caution to accurately characterize expression of NMDA receptor subunits and function within these cells, and it is important to confirm results in whole tissue. We are the first to report NR2C/D subunit expression in primary mouse endothelial cultures and confirm this subunit distribution in brain endothelial cells *in situ*.

Most studies of eNMDA receptors have reported expression of NR1, which is the conserved subunit across all NMDA receptors (Andras et al. 2007; Betzen et al. 2009; Krizbai et al. 1998; Kuhlmann et al. 2008; Kuhlmann et al. 2009; Neuhaus et al. 2011a; Neuhaus et al. 2011b; Parfenova et al. 2003; Sharp et al. 2005). Our results in adult and juvenile mouse primary endothelial cells agree with these findings, since NR1 was detected by PCR and immunocytochemistry.

Endothelial NR2 subunit composition varies between species and type of cultured cell (primary or cell line). For example, reports indicate NR2A/B, NR2B only, NR2B-D, or all four subunits (NR2A-D) are expressed in different brain endothelial cultures (Andras et al. 2007; Betzen et al. 2009; Neuhaus et al. 2011a; Neuhaus et al. 2011b; Reijerkerk et al. 2010; Sharp 2003). Two studies have also demonstrated expression of NR3A and NR3B in human and mouse cell lines (Neuhaus et al. 2011a; Reijerkerk et al. 2010). While we have not yet examined NR3A/B, we are the first to study NR2A-D subunit expression in mouse primary brain endothelial cells and mouse cortical tissue. mRNA transcripts for NR2A-C could not be detected by PCR in endothelial cultures from adult or juvenile mouse brains, even though several primer sets spanning each gene were tested. However, we did identify a PCR product for NR2D. Immunocytochemistry of endothelial cells produced similar results, where staining for NR2A and 2B was negligible (not shown), and NR2D staining co-localized with endothelial marker, isolectin B_4. The presence of NR2D was confirmed *in situ* by endothelial localization with P-gp in fixed brain sections from adult and juvenile mice. Contrary to our PCR results where NR2C transcripts were not detected, protein for NR2C was identified in primary cultures by immunocytochemistry. Also, we found strong

immunoreactivity for NR2C *in situ* in adult and juvenile cortical vasculature, both larger arterioles and smaller vessels. The NR2C signal overlapped with the endothelial marker, P-gp, and was distinctly more luminal than GFAP, supporting an endothelial distribution. This disparity between our PCR and immunostaining results could be due to amino acid sequence homology between NR2C and NR2D. These subunits have similar sequences and molecular weights, making it difficult to use antibodies to differentiate between them. Many commercially available antibodies are not specific and are known to bind both subunits. While not confirmed for the NR2C antibody we have used in this study, it may also bind NR2D. Future experiments using mass spectrometry for protein identification will help elucidate the presence or absence of NR2C and/or 2D in cerebral endothelial cells.

In considering other cell types, NR2C and 2D staining was absent in neurons of adult tissue, but stained juvenile neurons, which is consistent with known neuronal expression patterns that change during development (Monyer et al. 1994; Takai et al. 2003). We also report NR2C/2D expression in juvenile cortical astrocytes, which fits with recent pharmacological evidence (Palygin et al. 2011; Palygin et al. 2010), but is the first time such expression has been demonstrated by immunostaining. Astrocyte NMDA receptors are also resistant to blockage by Mg^{2+} (Palygin et al. 2010), which further supports the presence of NR2C/D subunits (Chatterton et al. 2002; Monyer et al. 1994). NR2C/D expression in eNMDA receptors may confer similar resistance, as shown in our pressure myography results (Chapter 2) where dilation of MCAs by glutamate and D-serine was mediated by direct vascular effects not susceptible to Mg^{2+}. Thus, NR2C/D

expression in eNMDA could impact receptor properties and function.

Other groups have specifically argued against endothelial cell NMDA receptor expression in cultured cells (Domoki et al. 2008; Morley et al. 1998). The reason for contrasting results between these studies and those supporting endothelial NMDA receptor expression is not clear, but it is possible endothelial cells do not express all known subunit splice variants. Also, it is unknown if cerebral eNMDA receptors are expressed on both sides of the BBB, or if they are localized to the luminal or abluminal side of the barrier. Comprehensive immunochemical analyses and electron microscopy of brain endothelium in large arterioles and small capillaries *in situ* are required to address this further.

While we have provided evidence of mouse eNMDA receptor expression, the precise function of these receptors remains uncertain. Treatment of immortalized human cerebral microvascular endothelial cells with glutamate increases intracellular Ca^{2+} levels (Kuhlmann et al. 2008), which can trigger second messenger pathways and bind calmodulin, activating enzymes such as eNOS. This is true in immortalized mouse brain endothelial where glutamate treatment activates eNOS, increasing NO production (Scott et al. 2007). We have not yet investigated the function of eNMDA receptors in our culture model, but based on eNOS-dependent NMDA receptor-mediated vasodilation in MCAs, we believe eNOS could be directly activated by glutamate and D-serine through eNMDA receptors. Future experiments will include intracellular Ca^{2+} imaging of cultured mouse endothelial cells and measurement of eNOS activity and NO production in response to glutamate and D-serine.

[2]CHAPTER 4: Astrocyte-mediated dilation of mouse cortical slice arterioles involves D-serine and NMDA receptor activation

SUMMARY

Increased neuronal energy demand is coupled to matched increases in blood supply by an endogenous regulatory pathway known as functional hyperemia. This process is mediated by gliotransmitter release from astrocyte endfeet, which have direct dilatory or constrictor effects on local arterioles in response to neuronal activity. These molecules include arachidonic acid (AA) metabolites, but other gliotransmitters, such as D-serine, are also produced by astrocytes in response to neuronal activity. D-Serine is a co-agonist of the glycine regulatory site at NMDA-type glutamate receptors, and NMDA receptors play a role in functional hyperemia *in vivo*. Previously, we determined that NMDA receptors dilate isolated brain arteries in a manner that requires D-serine. Thus, we hypothesized that astrocyte activation leads to perivascular D-serine release and NMDA receptor-mediated vasodilation *in situ*. To test this, we used two-photon imaging of mouse cortical slices bubbled with 20% O_2 and loaded with an astrocyte calcium indicator. By simulating glutamatergic neurotransmission through exposure to mGluR agonist, 1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (trans-ACPD), transient elevations of astrocyte Ca^{2+} were induced and corresponding dilation of neighbouring arterioles was observed. Vasodilation was blocked by D-amino acid oxidase (DAAO), which selectively degrades D-serine and addition of D-serine with DAAO rescued the

[2] LeMaistre JL, Lu L, Anderson CM. (2012). Manuscript in preparation.

dilatory response, suggesting D-serine was involved. We confirmed direct astrocyte involvement in this pathway by stimulating astrocytes via two-photon flash photolysis of caged Ca^{2+} compound, o-nitrophenyl EGTA/AM (NP-EGTA) within perivascular endfeet, which also caused arteriole vasodilation mediated by D-serine. Dilation after Ca^{2+} uncaging was also attenuated significantly by competitive NMDA receptor antagonists D-2-amino-5-phosphonopentanoate (AP5) and 5,7-dichlorokynurenic acid (DCKA), indicating a response dependent on NMDA receptor glutamate and D-serine binding sites, respectively. These effects were not affected by inhibition of neuronal activity through tetrodotoxin (TTX) treatment. We also report evidence of eNOS involvement in dilation by inhibiting 20-HETE production, permitting COX-mediated dilation. This suggests an interaction between the COX, nitric oxide and D-serine pathways, which warrants further investigation. Overall, our results provide the first evidence that D-serine is involved in astrocyte-induced vasodilation and implicate an effect mediated by NMDA receptors.

INTRODUCTION

Brain activity elevates local blood flow in a process known as functional hyperemia (Iadecola 2004; Roy and Sherrington 1890). This homeostatic mechanism links neuronal energy supply and demand, and involves signalling between neurons, glial cells and blood vessels, which is known as neurovascular coupling. Astrocytes are central to this process, since neuronal glutamate and ATP elevate intracellular astrocyte Ca^{2+}, inducing release of vasoactive molecules with variable vascular effects (Gordon et

al. 2008; Leffler et al. 2006; Metea and Newman 2006; Mulligan and MacVicar 2004; Zonta et al. 2003). These molecules include vasodilators such as metabolites of arachidonic acid (AA): prostaglandin E_2 (PGE_2), produced by cyclooxygenase (COX) activity, and epoxyeicosatrienoic acids (EETs), produced by cytochrome P450 epoxygenase, which diffuse from astrocyte endfeet to neighbouring blood vessels (Gordon et al. 2008; Metea and Newman 2006; Mishra et al. 2011; Mulligan and MacVicar 2004; Takano et al. 2006). AA from astrocytes can also be metabolized within smooth muscle by cytochrome P450 ω-hydroxylase to the vasoconstrictor 20-hydroxyeicosatetranoic acid (20-HETE) (Gordon et al. 2008; Metea and Newman 2006; Mishra et al. 2011; Mulligan and MacVicar 2004).

Recent studies have shown oxygen levels dictate neurovascular coupling in brain slices and retina (Gordon et al. 2008; Mishra et al. 2011). When tissue is incubated in high O_2 (95-100%) and the partial pressure of O_2 (pO_2) increases, neurovascular coupling stimulates 20-HETE production and vasoconstriction of blood vessels (Gordon et al. 2008; Mishra et al. 2011; Mulligan and MacVicar 2004). At a pO_2 closer to physiologic levels (20% O_2), neuronal and glial stimulation induce vasodilation, which has been correlated with reduced prostaglandin-lactate transporter activity and higher extracellular PGE_2 levels (Gordon et al. 2008), as well as reduced 20-HETE synthesis (Mishra et al. 2011). It has been suggested the polarity of astrocyte-induced vascular responses is also dictated by nitric oxide (NO), which influences AA metabolite activity (Metea and Newman 2006) by inhibiting the enzymes which produce 20-HETE and EETs (Alonso-Galicia et al. 1999; Alonso-Galicia et al. 1998; Udosen et al. 2003). NO modulation may also be affected by oxygen levels, since at 95% O_2, NO increases constriction of

retinal vessels during neurovascular coupling, possibly by inhibiting EET production, thereby permitting 20-HETE constriction (Metea and Newman 2006). However, at physiologic O_2 levels *in vivo*, NO reduces 20-HETE production, since 20-HETE inhibition reverses vasoconstriction by a NOS antagonist (Liu et al. 2008). Further studies are required to better understand the effects of NO on AA metabolite signalling and determine the contribution of neuronal nitric oxide synthase (nNOS) and/or endothelial nitric oxide synthase (eNOS) to this mechanism.

Other mechanisms of functional hyperemia have been identified, which may involve astrocytes. Specifically, application of glutamate or NMDA directly to the brain surface dilates cerebral circulation (Busija and Leffler 1989; Faraci and Breese 1993) by a mechanism involving NMDA receptors and NO (Bari et al. 1996a; Faraci and Brian 1995). This is believed to be linked to neuronal NMDA receptor Ca^{2+} influx, which activates nNOS (Roman et al. 2000), since NMDA receptors physically associate with nNOS through PSD-95 proteins (Brenman et al. 1996). Given that NO is also involved in modulation of AA metabolite signalling and switching between vasodilation and vasoconstriction, NMDA receptors may influence astrocyte-mediated neurovascular coupling through NO production. Also, NMDA receptor function is modulated by co-agonists, glycine or D-serine. The role of these compounds in NMDA receptor-mediated vasodilation is unknown, but D-serine immunostaining is localized to astrocytes, including regions near blood vessels (Schell et al. 1995), and astrocytes release D-serine by a Ca^{2+} and SNARE protein-mediated mechanism (Martineau et al. 2008; Mothet et al. 2005). Therefore, we postulated NMDA receptor-mediated functional hyperemia could

be modulated by astrocytes through D-serine release.

In the present work, we used a brain slice model treated with 20% O_2 to investigate the role of D-serine and NMDA receptors in astrocyte-induced arteriole vasodilation. We report that degradation of extracellular D-serine and NMDA receptor inhibition both reduced blood vessel dilation after astrocyte stimulation, and neuronal excitability did not mediate this effect. Also, in our previous work with isolated middle cerebral arteries (Chapter 2), we found evidence of eNOS activation during NMDA receptor-mediated vasodilation. We investigated the participation of eNOS in brain slice arteriole vasodilation and found endothelial-derived NO modulates 20-HETE and AA signalling from astrocytes, but the correlation between this pathway and D-serine signalling has yet to be determined.

MATERIALS AND METHODS

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.

D-serine Immunohistochemistry

D-Serine immunostaining was modified from the procedure by Schell *et al.* (1995). Brains from 14-19 day old CD1 mice were placed in fixative (5% glutaraldehyde, 0.5% PFA, 0.2% $Na_2S_2O_5$, 0.1 M Na_2PO_4 buffer at pH 7.4) for 24 hours

and then 30% sucrose for 3 days at 4°C. Tissue was then snap-frozen in liquid nitrogen cooled n-hexane and cut into 30 µm sections on a cryotome. Slices were reduced for 20 min at room temperature in 0.2% $Na_2S_2O_5$ and 0.5% $NaBH_4$ in 0.1 M TBS (pH 7.4) and rinsed with 0.2% $Na_2S_2O_5$ in TBS for 45 min. Tissue was blocked in 4% goat serum, 0.2% Triton X-100, and 0.2% $Na_2S_2O_5$ in 0.1 M TBS for 2 hours and then incubated in 0.1 M TBS (pH 7.2) with 2% goat serum, 0.1% Triton X-100 and primary antibodies: rabbit anti-D-serine (Millipore, Billerica, MA), mouse anti-GFAP and *Griffonia simplicifolia* 1 Isolectin B_4 tagged with Alexa Fluor® 488 for 48 hours. Secondary antibodies Alexa Fluor® 633 goat anti-rabbit IgG (H+L) and Alexa Fluor® 568 goat anti-mouse IgG (H+L) were used for visualization of D-serine and GFAP antibodies. Images were collected with a Zeiss LSM510 laser-scanning microscope.

Brain Slice Preparation and Two-photon Imaging

Brains from CD1 mice (14-19 days old) were dissected into ice cold cutting buffer (2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, 5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, 230 mM sucrose) that was bubbled with 95% O_2, 5% CO_2. Animals of this age were necessary to ensure proper dye loading. Tissue was then cut on a vibrating blade microtome (Vibratome, Bannockburn, IL) into 350 µm slices, which were incubated in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose) oxygenated with 95% O_2, 5% CO_2 and heated to 35°C. After 1 hour equilibration, slices were loaded with Ca^{2+} indicator- Rhod-2 AM (10 µM; Invitrogen, Burlington, ON) and *Griffonia simplicifolia* 1 Isolectin B_4 tagged with Alexa Fluor® 488 (5 µg/ml). Brain

slices from animals of this age were necessary Slices were incubated with aCSF aerated with 20% O_2, 5% CO_2 and 75% N_2 while imaged at 800 nm on an upright microscope with scanhead for two-photon imaging (Prairie Technologies, Middleton, WI) attached to a pulsed Ti-sapphire laser (Coherent Inc., Santa Clara, CA). Cortical astrocyte Ca^{2+} increases were stimulated by two methods: a) bath application of (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*t*ACPD, 100 µM, Tocris Bioscience, Ellisville, Missouri), which activates both group I and group II mGluRs and is commonly used (Filosa et al. 2004; Mulligan and MacVicar 2004; Zonta et al. 2003) to stimulate astrocytic Ca^{2+} via $mGluR_5$ receptors (EC_{50}~ 23µM), b) incubation of slices with caged Ca^{2+}- o-nitrophenyl-EGTA AM (10 µM, Invitrogen, Burlington, ON), which binds Ca^{2+} with high affinity (K_d~ 80 nM) and is taken up by cells. Light at 700 nm disrupted the molecule, decreasing its affinity for Ca^{2+} (K_d~ >1mM), and liberating Ca^{2+} within the cytoplasm (Ellis-Davies and Kaplan 1994). Astrocyte Ca^{2+} changes and arteriole diameter in the cortex were monitored by imaging. Slice arteriole vasodilation was also recorded in response to bath application of glutamate (10 µM) with D-serine (10 µM).

Brain slices were treated for 30 minutes with D-amino acid oxidase (DAAO; 0.1 Units/ml), which degrades extracellular D-serine. Also, to investigate the role of NMDA receptors, slices were incubated for 15 minutes with glutamate-site competitive antagonist, 2-amino-5-phosphonopentanoate (AP5; 50 µM), or glycine (D-serine)-site competitive antagonist, 5,7-dichlorokynurenic acid (DCKA; 100 µM). Neuronal activity was eliminated in slices by treatment with voltage-gated Na^+ channel blocker, tetrodotoxin (TTX; 1 µM; Tocris Bioscience, Ellisville, Missouri). Activity of cyclooxygenase (COX) was reduced by 20 minute incubation with indomethacin

(100μM). To determine the involvement of nitric oxide synthase, slices were also preincubated for 15 minutes with eNOS-selective inhibitor, N^5-(1-iminoethyl)-L-ornithine (L-NIO; 3 μM). 20-HETE synthesis was inhibited by N-hydroxy-N'-(4-n-butyl-2-methylphenyl)formamidine (HET0016; 100 nM).

Data Analysis

Area under the curve (AUC) was calculated from the percent vessel relaxation versus time graphs. AUC from antagonist treatments were analyzed compared to AUC from controls by t-test for two groups or one-way ANOVA (analysis of variance) with Newman-Keuls *post-hoc* test for three or more groups. For analysis of time course of dilation during glutamate and D-serine treatments (Fig. 21), two-way ANOVA for repeated measures were conducted with Bonferroni *post-hoc* tests.

RESULTS

D-serine is located in astrocytes of the neurovascular unit

Immunostaining for D-serine in cortical brain slices (Fig. 21A) co-localized with astrocyte marker, GFAP (Fig. 21B), in protoplasmic endfeet around arterioles labelled with isolectin B_4 (Fig. 21C). This indicates D-serine is readily available for release in close proximity to cortical blood vessels.

D-serine and NMDA receptors contribute to dilation of brain slice blood vessels

To explore potential vasomodulatory effects of D-serine on blood vessels in brain parenchyma, we determined whether the combination of D-serine and glutamate dilates cortical arterioles in mouse brain slices maintained at 20% O_2. Bath application of glutamate and D-serine (each 10 µM) dilated brain slice arterioles (maximum 4.0 ± 3.1%) over 400 seconds (Fig. 22). Adding TTX to block neuronal excitability had no significant effect on dilation (maximum 2.8 ± 3.0%), thus eliminating the possibility that signalling through neuronal excitation is responsible for this effect of glutamate and D-serine.

Using acute cortical slices, glutamatergic neurotransmission was mimicked by application of *t*ACPD, which is an agonist of $mGluR_5$ and causes inositol-3-phosphate-mediated Ca^{2+} release in astrocytes detectable by increased Rhod-2 fluorescence. When slices were incubated in aCSF aerated with 20% O_2, *t*ACPD consistently elevated astrocyte Ca^{2+} and induced vasodilation of neighbouring cortical blood vessels (Fig. 23A). From 15 blood vessels measured, *t*ACPD treatment resulted in an average vasodilation of 3.7 ± 2.1% which peaked 75 seconds after application (Fig. 23B). Astrocytes release D-serine by a vesicle-dependent mechanism when cytoplasmic Ca^{2+} rises (Martineau et al. 2008; Mothet et al. 2005). The role of D-serine was tested by incubating slices with D-amino acid oxidase (DAAO; 0.1 Units/ml), an enzyme that degrades extracellular D-serine, and it significantly reduced dilation in response to *t*ACPD application ($p<0.01$; Fig 23C). Addition of exogenous D-serine to compete for DAAO enzyme activity rescued the vasodilatory effect ($p>0.05$). This suggests D-serine plays a role in cortical vasodilation after simulated neurotransmission via *t*ACPD application. This arteriole vasodilation is also mediated by NMDA receptors,

since treatment with the competitive NMDA receptor antagonist, DCKA (100 μM), significantly inhibited the vascular response. DCKA acts at the glycine/D-serine binding site, further supporting a role for NMDA receptor co-agonists in this dilation mechanism.

Simulation of neurotransmission through tACPD treatment elevates brain slice astrocyte Ca^{2+} and induces blood vessel dilation. However, to directly link astrocyte Ca^{2+} increases with vasodilation we liberated Ca^{2+} ions via flash photolysis of o-nitrophenyl-EGTA AM, a caged Ca^{2+} compound, in the cytoplasm of astrocytes labelled with Rhod 2. This resulted in elevated astrocyte Ca^{2+}, which consistently correlated with vasodilation of neighbouring cortical blood vessels (Fig. 24A). Vasodilation peaked at 5.7 ± 4.3%, 105 seconds after stimulation. Involvement of D-serine in this dilation mechanism was tested by incubating slices with DAAO before Ca^{2+} uncaging. DAAO significantly reduced the resulting dilation ($p<0.01$; Fig. 24C), and addition of exogenous D-serine to compete for DAAO enzyme activity recovered dilation after flash photolysis ($p>0.05$). This suggests D-serine plays a role in cortical vasodilation after both direct (uncaging) or indirect (*t*ACPD application) astrocyte Ca^{2+} stimulation.

To test if astrocytes were directly involved in NMDA receptor-mediated dilation, cytoplasmic Ca^{2+} was increased via Ca^{2+} uncaging, while exposing slices to DCKA (100 μM) or competitive glutamate-site NMDA receptor antagonist, AP5 (50 μM). These antagonists both significantly reduced dilation triggered by astrocyte stimulation ($p<0.05$, $p<0.01$; Fig. 24E), indicating astrocytes directly influence NMDA receptor-mediated vasodilation in this pathway. Also, we investigated whether neuronal excitation downstream of astrocyte activation accounts for vasodilation. Flash photolysis of

astrocyte caged Ca^{2+} was performed in the presence of neuronal Na^{+} channel blocker, tetrodotoxin (TTX; 1 μM). TTX did not significantly alter the vasodilatory response ($p>0.05$; Fig. 24D), suggesting neuronal excitation does not play a role in dilation after astrocyte stimulation. Furthermore, TTX did not modify inhibition by AP5 or DCKA ($p>0.05$; Fig. 24E), confirming neurotransmission and neuronal NMDA receptors are not involved in blood vessel dilation after astrocyte Ca^{2+} uncaging.

Nitric oxide inhibits constriction pathways permitting vasodilation

Astrocytes produce AA when intracellular calcium increases, which is metabolized to PGE_2, a vasodilator produced by COX activity, and 20-HETE, a vasoconstrictor produced by cytochrome P450 enzyme (CYP4A), ω-hydroxylase (Gordon et al. 2008; Metea and Newman 2006). Evidence suggests PGE_2 and 20-HETE production are in equilibrium, where dilation by PGE_2 is favoured at lower oxygen levels (20%), while constriction by 20-HETE prevails at high oxygen concentrations (95%) (Gordon et al. 2008; Mishra et al. 2011). This equilibrium appears to favour PGE_2-induced vasodilation in our brain slice model, since indomethacin (INM; 100 μM) significantly reduced dilation at 20% O_2 ($p<0.05$; Fig. 25A), indicating COX activity (and therefore PGE_2) was involved in astrocyte-mediated vasodilation. However, the CYP4A (ω-hydroxylase) inhibitor, HET0016 (HET; 100 nM), did not significantly alter dilation ($p>0.05$; Fig. 25A), suggesting 20-HETE levels are low under control conditions in our model.

NO potentially inhibits 20-HETE production to permit vasodilation at 20% O_2 (Attwell et al. 2010), as suggested by *in vivo* results (Liu et al. 2008). Since eNOS was

shown to influence NMDA receptor-mediated dilation in Chapter 2, we examined the contribution of eNOS in our brain slice model by incubation with eNOS selective antagonist, L-NIO (3 μM). L-NIO significantly inhibited vasodilation induced by Ca^{2+} uncaging ($p<0.01$; Fig. 25B). Endothelial-derived NO and PGE_2 act independently on blood vessels and appear to have an additive effect on vasodilation in this model, since inhibition of eNOS and COX through co-treatment of L-NIO and indomethacin converted vasodilation to vasoconstriction ($p<0.001$; Fig. 25B). L-NIO was unable to block vasodilation when the 20-HETE synthesis pathway had been disabled simultaneously with HET0016 ($p>0.05$; Fig. 25B). This suggests that eNOS-induced vasodilation is dependent on suppression of 20-HETE formation.

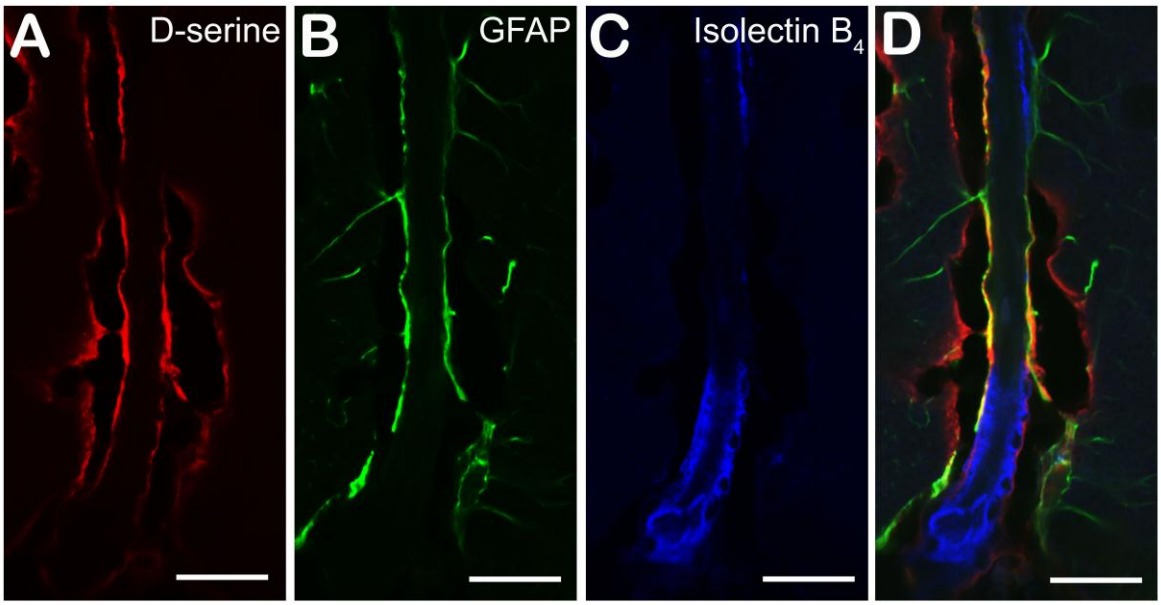

Figure 21: D-serine is localized to astrocyte endfeet near arterioles.

A-D, In 30 μm sections of 5% glutaraldehyde-fixed brain from 14-19 day old mouse pups, D-serine immunostaining (A, red) co-localized with astrocyte marker, GFAP (B, green) in cortical perivascular astrocyte endfeet near blood vessels labelled with isolectin B_4 (C, blue). Scale bars are 20 μm.

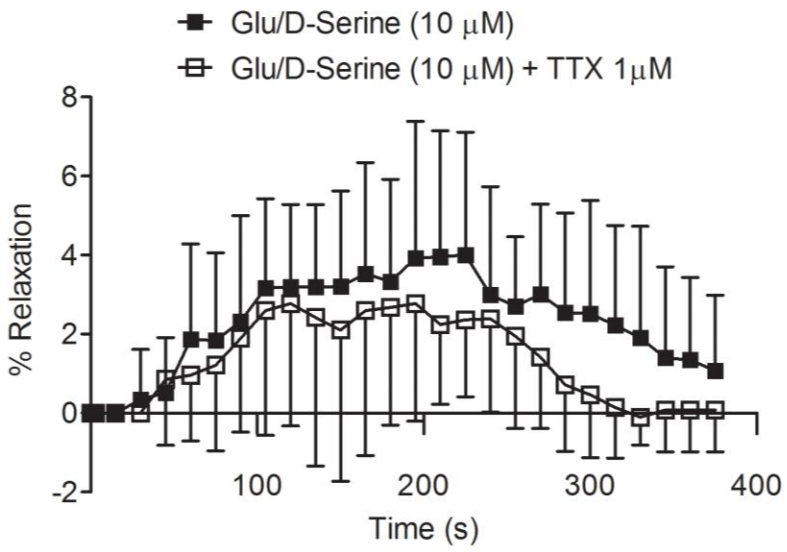

Figure 22: Glutamate and D-serine increase lumen diameter in brain slice arterioles. Combination of glutamate and D-serine causes significant dilation of penetrating arterioles in cortical slices in the presence and absence of the neuronal excitability blocker, TTX. Data are means ± SD of 14 (control) or 18 (TTX group) brain slices and were analyzed by two-way ANOVA for repeated measures and Bonferroni *post hoc* test.

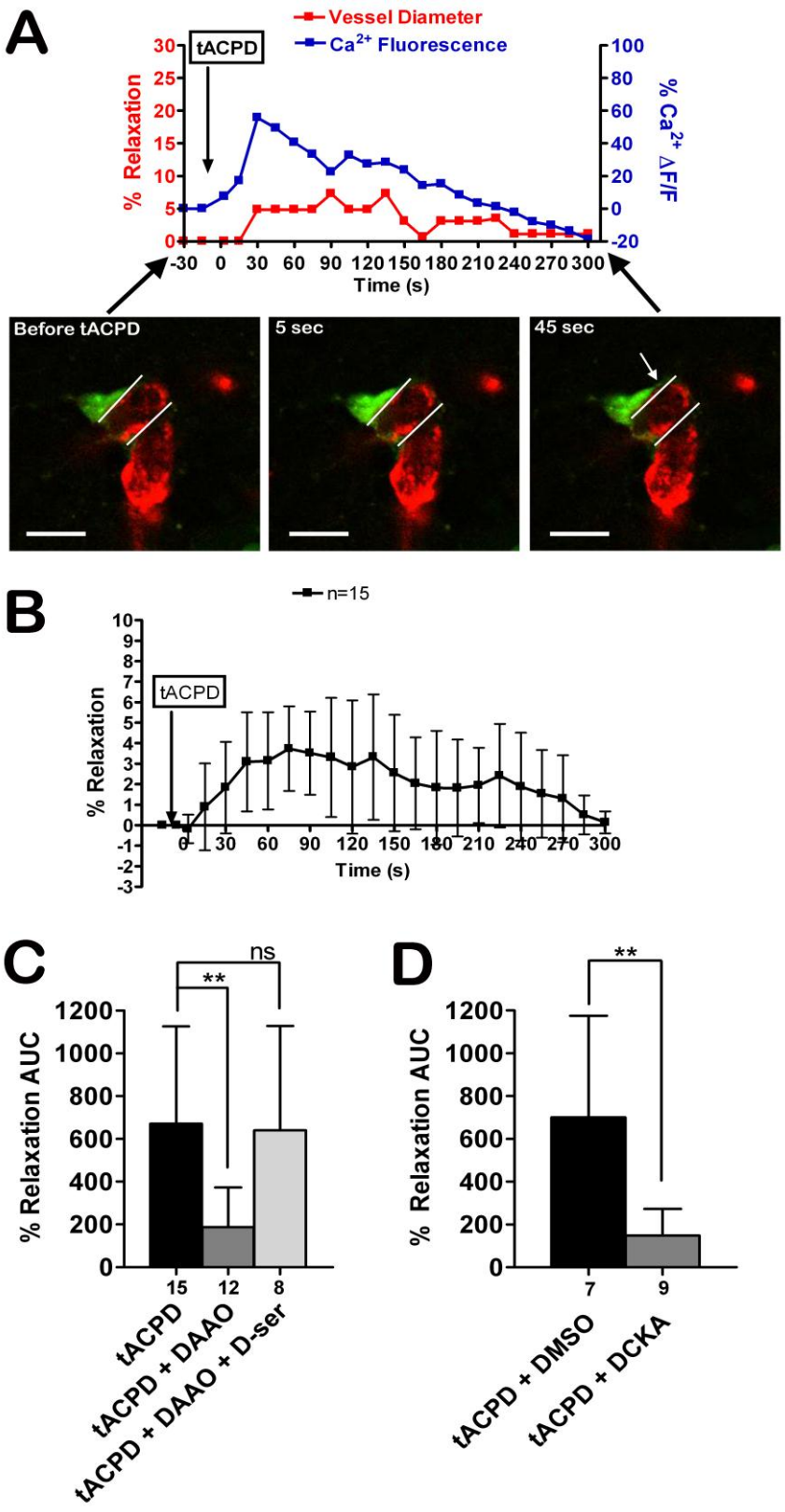
A
Vessel Diameter
Ca2+ Fluorescence
tACPD
% Relaxation
% Ca2+ ΔF/F
Time (s)
Before tACPD
5 sec
45 sec
B
n=15
tACPD
% Relaxation
Time (s)
C
ns
**
% Relaxation AUC
15
12
8
tACPD
tACPD + DAAO
tACPD + DAAO + D-ser
D
**
% Relaxation AUC
7
9
tACPD + DMSO
tACPD + DCKA

Figure 23: mGluR agonist, tACPD, simulates glutamatergic neurotransmission, inducing brain slice astrocyte Ca^{2+} elevations and vasodilation of arterioles by a D-serine and NMDA receptor-mediated mechanism.

A, Application of mGluR agonist, tACPD (100 μM), simulates glutamatergic neurotransmission, elevating astrocyte Ca^{2+} and triggering arteriolar vasodilation over time, as indicated by the representative images which correspond to times on the graph of vessel diameter and Ca^{2+} fluorescence. The green signal is Rhod 2 fluorescence and the signal is fluorescent isolectin B_4, which stain blood vessels. Scale bar represents 15 μm. B, Plot of average dilation of 15 vessels after tACPD treatment over 300 seconds. C, D-amino acid oxidase (DAAO; 0.1 Units/ml) significantly inhibited vasodilation after tACPD application. Addition of D-serine (100 μM) with DAAO recovered dilation to *t*ACPD treatment and increased AUC. This graph represents area under the curve (AUC) data calculated from % relaxation over time. **p<0.01 compared to control tACPD AUC using one-way ANOVA with Newman-Keuls *post hoc* test. D, Competitive NMDA receptor antagonist, DCKA (100 μM; dissolved in DMSO), significantly inhibited brain slice arteriole vasodilation induced via astrocyte activation by simulated neurotransmission, through tACPD treatment. **p<0.01 compared to control tACPD AUC using a t-test. All data are presented as mean ± SD.

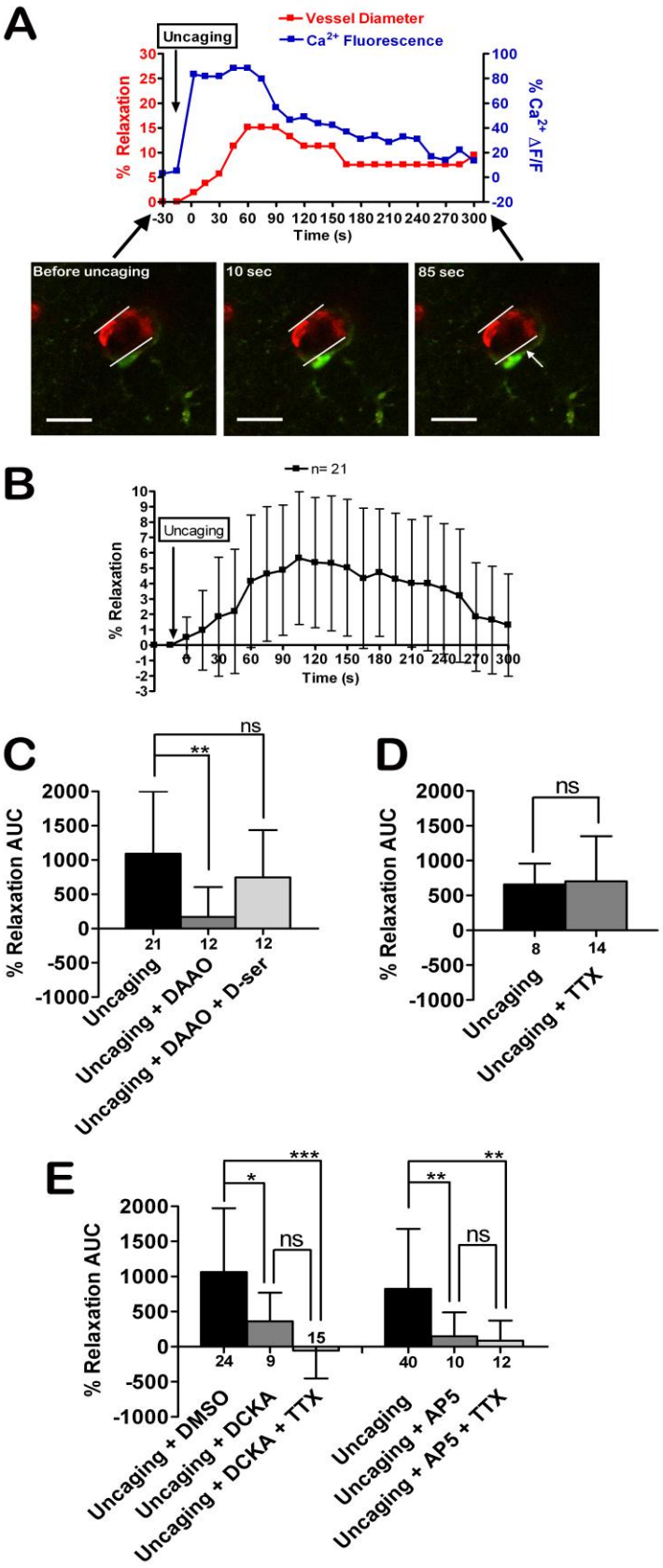
A
Uncaging
Vessel Diameter
Ca2+ Fluorescence
% Relaxation
% Ca2+ ΔF/F
Time (s)
Before uncaging
10 sec
85 sec
B
n= 21
Uncaging
% Relaxation
Time (s)
C
% Relaxation AUC
ns
**
21
12
12
Uncaging
Uncaging + DAAO
Uncaging + DAAO + D-ser
D
% Relaxation AUC
ns
8
14
Uncaging
Uncaging + TTX
E
% Relaxation AUC
***
*
ns
**
**
ns
24
9
15
40
10
12
Uncaging + DMSO
Uncaging + DCKA
Uncaging + DCKA + TTX
Uncaging
Uncaging + AP5
Uncaging + AP5 + TTX

Figure 24: Astrocyte Ca^{2+} elevations directly mediate arteriole vasodilation involving D-serine and NMDA receptors.

A, Liberation of caged Ca^{2+} in a perivascular astrocyte directly stimulates cytoplasmic Ca^{2+} which corresponds to vasodilation of a neighbouring arteriole over time, as demonstrated in the representative images. The green signal is Rhod 2 fluorescence and the signal is fluorescent isolectin B_4, which stain blood vessels. Scale bar represents 15 μm. B, Plot of average dilation of 21 vessels after astrocyte uncaging over 300 seconds. C, D-amino acid oxidase (DAAO; 0.1 Units/ml) significantly inhibited vasodilation after astrocyte Ca^{2+} uncaging, but addition of D-serine (100 μM) with DAAO recovered the dilation. **$p<0.01$ compared to control uncaging AUC using one-way ANOVA with Newman-Keuls *post hoc* test. D, Treatment with tetrodotoxin (TTX; 1 μM) to block neuronal excitation did not significantly affect blood vessel dilation after astrocyte Ca^{2+} uncaging, as analyzed by t-test. E, Application of NMDA receptor antagonist DCKA (100 μM) significantly blocked arteriole vasodilation induced by direct astrocyte Ca^{2+} uncaging. TTX (1 μM) treatment with DCKA also inhibited dilation induced by Ca^{2+} uncaging, but not significantly more than DCKA treatment alone. Similarly, application of another competitive NMDA receptor antagonist, AP5 (50 μM), significantly reduced vasodilation after uncaging. Also, TTX treatment with AP5 inhibited dilation at a similar level to AP5 alone. Data were analyzed using one-way ANOVA with Newman-Keuls post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ All data are presented as mean ± SD.

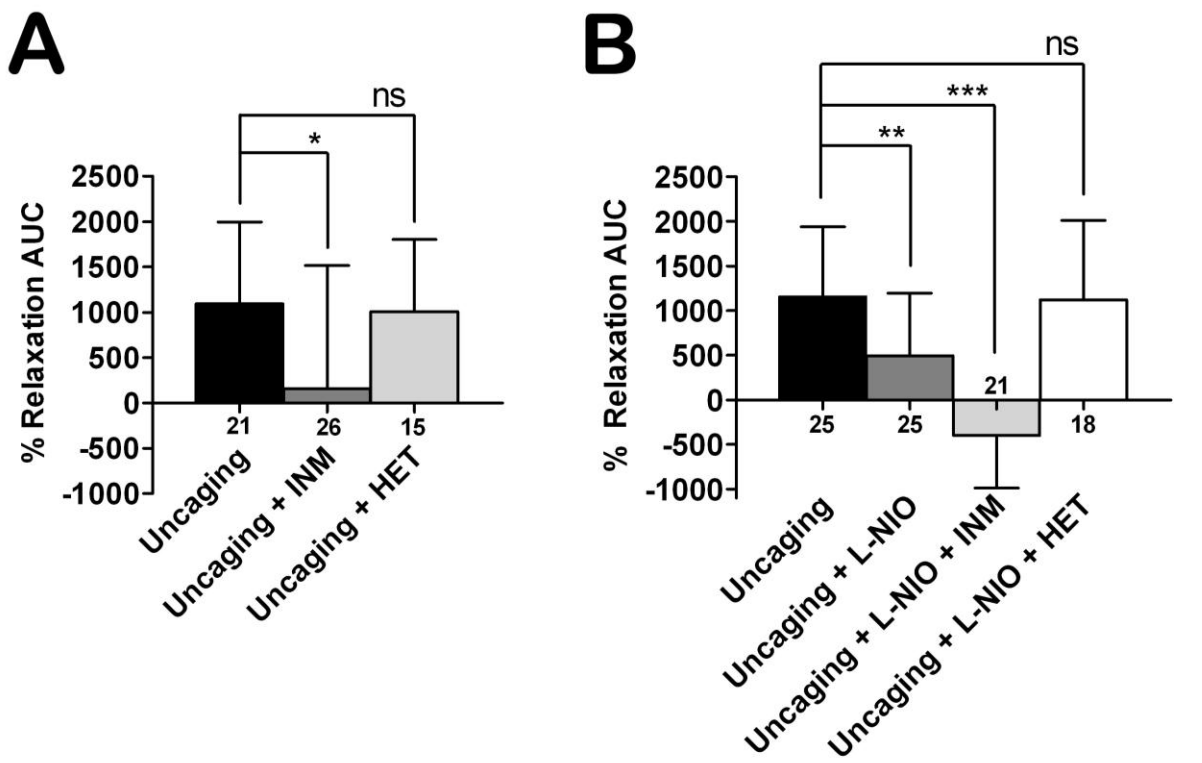

Figure 25: AA metabolites and eNOS are also involved in brain slice arteriole dilation after astrocyte Ca^{2+} uncaging.

A, Treatment of slices with COX inhibitor, indomethacin (INM; 100 µM) significantly blocked arteriole dilation after astrocyte Ca^{2+} uncaging, as indicated by reduced % relaxation AUC compared to control AUC. The contribution of 20-HETE was tested by treatment with HET0016 (HET; 100 nM), a 20-HETE synthesis inhibitor, which did not significantly change dilation. B, Application of eNOS-selective antagonist, L-NIO (3 µM), also significantly reduced vasodilation induced by uncaging. Treatment with L-NIO and INM (100 µM) caused significant vasoconstriction after astrocyte Ca^{2+} uncaging. Dilation was recovered by addition of L-NIO and HET (100 nM). Data are mean ± SD and where analyzed by one-way ANOVA with Newman-Keuls *post hoc* test; *p<0.05; **p<0.01; ***p<0.001

DISCUSSION

Cerebral functional hyperemia is influenced by astrocytes, and these mechanisms have been examined in brain slices and *in vivo* (Gordon et al. 2008; Liu et al. 2008; Metea and Newman 2006; Mishra et al. 2011; Mulligan and MacVicar 2004; Takano et al. 2006; Zonta et al. 2003). Tissue oxygen concentration appears to dictate astrocyte responses, as treatment with 20% O_2, which provides pO_2 near physiologic levels, favours vasodilation, while high O_2 (95-100%) provokes vasoconstriction (Gordon et al. 2008; Mishra et al. 2011). To study vasodilation during neurovascular coupling, we used a mouse brain slice model aerated with 20% O_2 and applied tACPD to model glutamatergic neurotransmission, which triggered astrocyte Ca^{2+} and vasodilation. To confirm astrocytes were directly involved in vasodilation, astrocyte Ca^{2+} was stimulated by photo-liberation of caged Ca^{2+}, which also induced vasodilation. Both D-serine and NMDA receptors were involved in dilation after tACPD and Ca^{2+} uncaging, independent of neuronal excitability. Known vasodilator and AA metabolite, PGE_2, released from astrocytes, also mediates vasodilation in our slices, but we have new evidence that eNOS can act independently of PGE_2 to influence vasodilation by inhibiting 20-HETE production, as outlined in Figure 26.

While numerous studies have indicated topical application of glutamate and NMDA through cranial windows dilate extraparenchymal pial arteries (Bari et al. 1996b; Busija and Leffler 1989; Faraci and Breese 1993; Faraci and Breese 1994; Philip and Armstead 2004), the contribution of co-agonists, D-serine and glycine, to NMDA receptor controlled neurovascular coupling has not been considered. To address the

involvement of D-serine in signalling within the neurovascular unit, we used three approaches. First, immunostaining localized D-serine to astrocyte endfeet around cortical blood vessels, as suggested by Schell et al. (1995), which indicates D-serine is readily available for release within the neurovascular unit. Second, 10 µM D-serine in combination with 10 µM glutamate induced dilation of penetrating arterioles in cortical slices, and neuronal excitability (blocked by TTX) was not necessary to induce dilation. D-Serine and glutamate (10 µM) were near physiologically attainable concentration ranges, since basal extracellular D-serine has been detected between 5-8 µM (Hashimoto et al. 1995) and local glutamate levels can approach low millimolar amounts (Clements et al. 1992). Third, we demonstrated vasodilation of arterioles after simulation of glutamatergic neurotransmission by mGluR agonist, tACPD, was significantly inhibited by the D-serine degrading enzyme, DAAO. To confirm astrocytes were central to this effect, cytoplasmic Ca^{2+} was directly stimulated by Ca^{2+} flash photolysis, and the resulting vasodilation was also blocked by DAAO. By treating slices with D-serine (100 µM) to compete for DAAO binding, dilation after tACPD and uncaging was recovered. These results suggest D-serine is released upon elevation of astrocyte Ca^{2+} and participates in vasodilation of neighbouring blood vessels. Taken together, our experiments support a novel role for D-serine in neurovascular coupling. D-Serine modulates other NMDA receptor mediated processes, such as LTP and excitotoxicity (Fossat et al. 2011; Henneberger et al. 2010; Shleper et al. 2005) and is not known to act at any other receptors, thus, a role for D-serine in vasodilation is indicative of an NMDA-receptor mediated response.

NMDA receptors are involved in functional hyperemia *in vivo* (Bari et al. 1996b; Busija and Leffler 1989; Faraci and Breese 1993; Faraci and Breese 1994; Philip and Armstead 2004), since dilation is inhibited by NMDA receptor antagonists (Bhardwaj et al. 2000; Faraci and Breese 1993; Meng et al. 1995). In our brain slice model, blood vessel vasodilation after indirect astrocyte activation by tACPD was significantly inhibited by the competitive NMDA receptor antagonist, DCKA, which blocks the D-serine/glycine binding site. Direct involvement of astrocytes was verified by Ca^{2+} uncaging, and the resulting vasodilation was also blocked by DCKA and competitive glutamate binding site antagonist, AP5. Furthermore, NMDA receptor-mediated dilation was independent of neuronal activation, since TTX did not significantly affect dilation or alter inhibition by AP5 and DCKA. This suggests NMDA receptors play a role in our model of neurovascular coupling, but do not require neuronal activation. Cerebral blood vessels are not only surrounded by astrocyte endfeet, but also receive direct innervation from neuronal terminals, which can release neurovascular signals (such as NO) upon neuronal activation (Tong and Hamel 2000). However, since TTX blocks neurotransmission, but not vasodilation in our model, these neuronal varicosities do not appear to be involved. It is tempting to speculate that vascular NMDA receptors, and not neuronal NMDA receptors, contribute to dilation in this model, but further studies targeting vascular receptors are required to confirm their involvement. In contrast with our results, a study of brain slices treated with tACPD showed astrocyte-Ca^{2+} induced vasodilation was not affected by AP5 (Zonta et al. 2003). However, these slices were aerated with 95% O_2, which alters vascular responses (Gordon et al. 2008; Metea and Newman 2006; Mishra et al. 2011). Additional experiments at 95% O_2 in our model may

produce similar effects. Our results also suggest astrocytes directly influence NMDA receptor-mediated vasodilation, since dilation after direct astrocyte stimulation (Ca^{2+} uncaging) is blocked by NMDA receptor antagonists. This agrees with a previous study where gliotoxin application to the cerebral surface disrupted glutamate and NMDA receptor-induced dilation (Leffler et al. 2006). This study and our experiments are currently the only evidence indicating astrocytes can influence NMDA receptor-mediated vasodilation.

Astrocytes are known to mediate neurovascular coupling in slices, retina and *in vivo* through production of AA, which is metabolized to PGE_2 and EETs (vasodilators) or 20-HETE (a vasoconstrictor) (Gordon et al. 2008; Metea and Newman 2006; Mulligan and MacVicar 2004; Takano et al. 2006; Zonta et al. 2003). We tested the involvement of prostaglandins and 20-HETE in our model of dilation by treating slices with indomethacin (INM), a COX inhibitor, or HET0016 (HET), a CYP4A ω-hydroxylase blocker, to prevent PGE_2 and 20-HETE production. INM significantly blocked blood vessel dilation after astrocyte Ca^{2+} uncaging, signifying that PGE_2 mediates vasodilation under these conditions (20% O_2 treated brain slice), as shown in previous studies (Gordon et al. 2008; Mishra et al. 2011; Takano et al. 2006). HET did not significantly change vasodilation after Ca^{2+} uncaging in our slices, suggesting 20-HETE production is not prevalent, which agrees with results from retina treated with 21% O_2 where 20-HETE contribution to neurovascular coupling was not significant (Mishra et al. 2011). Also, similar results were demonstrated *in vivo* where HET treatment had no significant effect on functional hyperemia in response to whisker stimulation at physiologic pO_2 levels (Liu et al. 2008). Thus, our results confirm known vasodilator PGE_2 is involved in our

dilation mechanism, while 20-HETE does not appear to reduce neurovascular coupling at 20% O_2.

Nitric oxide (NO) can modulate vascular effects of AA metabolites by inhibiting cytochrome P450 enzymes which produce 20-HETE and EETs (Metea and Newman 2006). NMDA receptors are coupled to NO production, since Ca^{2+} influx through these cation receptors can stimulate NOS activity (Roman et al. 2000), and nNOS activation is central to NMDA receptor-mediated functional hyperemia *in vivo* (Bari et al. 1996b; Chi et al. 2003; Faraci and Breese 1993; Meng et al. 1995). Thus, it is possible NMDA receptor activation in our brain slice model may trigger NO production and modulate AA metabolite formation. Our previous study of isolated arteries demonstrated eNOS is also involved in NMDA receptor-mediated vasodilation, but the contribution of eNOS to modulation of AA metabolites is unknown. Therefore, we treated our brain slices with eNOS-selective inhibitor, L-NIO, which significantly reduced vasodilation after Ca^{2+} uncaging. Subsequent administration of INM with L-NIO had additive effects on the dilatory response, resulting in vasoconstriction. This indicates dilation induced by eNOS is independent of PGE_2 signals. Importantly, endothelial-derived NO inhibits 20-HETE production, since co-administration of HET and L-NIO recovered dilation after uncaging, suggesting 20-HETE levels increased in the presence of L-NIO and reduced vasodilation. This points to a new role for eNOS in neurovascular coupling and provides the first evidence of modulation of 20-HETE production by endothelial NO, where increased eNOS activation suppresses 20-HETE synthesis and permits dilation by PGE_2. In contrast with our results and others which indicate a role for NO in astrocyte-mediated neurovascular coupling (Liu et al. 2008; Metea and Newman 2006), Zonta et al.

(2003) found L-NAME, a general NOS inhibitor, does not inhibit vasodilation after tACPD application. Again, their slices were incubated with 95% O_2, and NOS activity is dictated by oxygen concentration, so the contribution of NO modulation of neurovascular coupling may vary with oxygen content. A recent *in vivo* study in cats demonstrated both nNOS and eNOS participate in functional hyperemia, but involvement of eNOS was greater at lower levels of neuronal activity, while higher stimulation activated nNOS (de Labra et al. 2009). While we have not yet considered the contribution of nNOS in our model, it is possible eNOS effects are more prevalent during astrocyte Ca^{2+} uncaging, when neuronal activation is low, and nNOS is more involved in models of greater neuronal stimulation. Further studies with eNOS and nNOS knockout mice may also help determine the characteristics of eNOS and nNOS-mediated neurovascular coupling and correlate these with NMDA receptor activation.

The work presented in this chapter supports an exciting new link between astrocyte D-serine release, NMDA receptor activation, and NO-mediated vasodilation in mouse cortical brain slices. Our results agree with previous studies of NMDA receptor-mediated functional hyperemia, which suggest involvement of NO (Chi et al. 2003; Faraci and Breese 1993; Faraci and Brian 1995; Meng et al. 1995). Further work is required to fully characterize this mechanism and link D-serine and NMDA recpetors with eNOS modulation of AA metabolites, but we have provided important evidence of D-serine involvement in neurovascular coupling.

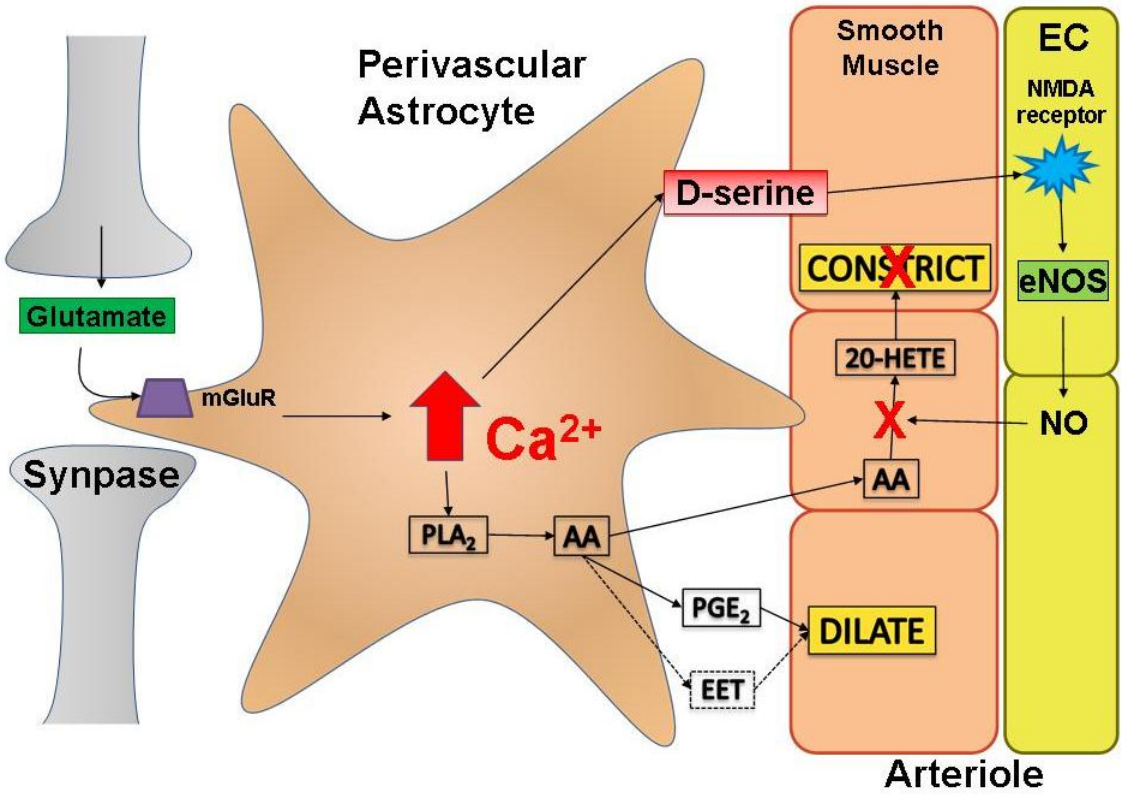

Figure 26: Astrocyte D-serine mediates arteriole vasodilation.

We have shown D-serine is involved in vasodilation induced by mGluR activation or direct astrocyte stimulation by Ca^{2+} uncaging. We have also demonstrated interaction between eNOS and 20-HETE, where eNOS appears to reduce 20-HETE synthesis. Thus, we speculate, based on our results in Chapter 2, D-serine may have direct vascular effects and stimulate eNMDA recpetors and eNOS activation permitting prostaglandin-mediated dilation by inhibiting 20-HETE production. However, we have not yet linked D-serine with eNOS activation, or investigated the localization of eNMDA receptors within endothelial cells.

CHAPTER 5: General Discussion

Functional hyperemia couples neuronal energy supply with demand generated by neurotransmission in a complex process mediated by numerous signalling pathways and cell types within the neurovascular unit. This includes astrocytes, which release vasoactive molecules: AA metabolites, K^+, and possibly CO when intracellular Ca^{2+} rises in response to synaptic activity (Filosa et al. 2006; Gordon et al. 2008; Li et al. 2008). Astrocyte Ca^{2+} elevations also trigger release of gliotransmitters: glutamate, ATP, and D-serine (Mothet et al. 2005; Parpura et al. 1994). The role of these molecules in neurovascular coupling is unknown. However, in this thesis we present evidence pointing to a novel mechanism of D-serine-induced, NMDA receptor-mediated cerebral vasodilation. First, we demonstrated NMDA receptor agonists, glutamate and D-serine, together dilated pressurized, isolated middle cerebral arteries through NMDA receptor-mediated direct vascular effects. Second, we examined the intact neurovascular unit by simultaneous two-photon imaging of astrocyte Ca^{2+} elevations and blood vessel diameter in cortical brain slices incubated with 20% O_2 (to favour vasodilation) to determine the contribution of D-serine to NMDA receptor-mediated blood flow regulation. We localized D-serine to astrocyte endfeet around arterioles, suggesting it is readily available for release, and we demonstrated that application of glutamate and D-serine to brain slices dilated cortical arterioles. Treatment with DAAO, a D-serine degrading enzyme, implicated D-serine involvement in vasodilation of arterioles when astrocyte Ca^{2+} increased after tACPD application, which imitates glutamatergic neurotransmission, or Ca^{2+} uncaging, which provides a direct link to astrocyte activation. This mechanism was also mediated by NMDA receptors, but was not dependent on neuronal excitation. This

is the first indication in brain slices that D-serine, likely released during astrocyte stimulation, can modulate vascular tone through NMDA receptors.

Here, we provide evidence of eNOS participation in brain vasodilation in both isolated arteries and brain slices. Substantial literature argues that neuronal NMDA receptor activation is coupled to nNOS and vasodilation by direct smooth muscle effects of NO (Faraci and Breese 1993; Faraci and Brian 1995). However, in our pressure myography studies, D-serine and glutamate, stimulated NMDA receptor-mediated vasodilation by direct vascular action, dependent on intact endothelial function and eNOS activation. This suggests a novel pathway connecting D-serine with eNOS-mediated dilation in intact brain arteries, possibly through eNMDA receptors. We further investigated eNOS activation within the intact neurovascular unit of brain slices and found endothelial-derived NO can inhibit the vasoconstrictor, 20-HETE, facilitating dilation by PGE_2. eNOS has been theorized to modulate AA metabolites in this manner (Attwell et al. 2010), but this is the first direct confirmation of such a mechanism in brain tissue. However, we have not yet directly linked D-serine with eNOS activation in brain slices or demonstrated direct dilation of cortical arteriole smooth muscle by endothelial NO, as suggested by our work with isolated MCAs. The presence of numerous cell types in brain slices complicates determination of signalling pathways because multiple pathways, such as AA metabolites and K^+ occur at once. Also, we have not yet determined the contribution of nNOS by treating brain slices with a nNOS-selective antagonist, such as TRIM, before Ca^{2+} uncaging, since nNOS may also influence AA metabolites and play a role in NMDA receptor-mediated vasodilation. One study suggests endothelial NO mediates vasodilation during low frequency neuronal

stimulation, while nNOS is more greatly involved during high frequency stimulation (de Labra et al. 2009). Therefore, we expect little involvement of nNOS, as direct astrocyte stimulation by Ca^{2+} photolysis provides minimal neuronal stimuation and should favour eNOS pathways. At this point, our work supports participation of eNOS in neurovascular coupling through modulation of AA metabolites and possibly direct vascular effects, but further work is required to better characterize this pathway.

While we have provided valuable new evidence for cerebral vasodilation mechanisms, there are limitations to the models employed in these studies. Blood vessels have normal intrinsic properties that influence diameter, such as shear stress stimulated by luminal blood flow and myogenic tone induced by blood pressure. These cause endogenous release of signals, such as nitric oxide or endothelin (a vasoconstrictor) from endothelial cells, which alter smooth muscle tone (Ngai and Winn 1995). Thus, it is important to consider these factors when studying vascular coupling. Utilizing isolated, pressurized arteries for experiments permits study of direct vascular pharmacological effect and allows complete control of arterial pressure and environment. However, we used a "blind sac" preparation where the distal pipette was closed with a stopcock, maintaining vascular pressure, but preventing luminal flow. Lack of blood flow reduces normal vascular shear stress and other endogenous signals, and may change smooth muscle responses (Ngai and Winn 1995). Also, arteries are dilated when mounted in the apparatus and require pre-constriction (we used norepinephrine) to induce greater vascular tone and facilitate measurement of vasodilation. Thus, pressure myography is a useful tool to study direct vascular effects, but experimental conditions do not entirely mimic physiological situations and alter normal arterial responses. Brain slices

are a useful model because they permit access to all brain regions, maintain the cellular structure of the neurovascular unit, and allow pharmacological study of cellular mechanisms, such as intercellular communication (Filosa 2010). However, brain slices lack both blood pressure and flow, which is a drawback for studying vascular responses (Ngai and Winn 1995), and requires caution when interpreting results regarding neurovascular coupling. While we have evidence of D-serine-induced vasodilation of isolated MCAs and arterioles in brain slices, further physiologic studies *in vivo* are required to properly characterize this pathway. Also, functional hyperemia studies *in vivo* display a faster time course of dilation compared to brain slices. This could be due to: a) altered vascular properties in slices, b) the use of young animals for brain slices, which may have under developed neurovascular coupling compared to adults in *in vivo* experiments, and c) other pathways are more prevalent *in vivo*, which do not require diffusion of AA metabolites (Dunn and Nelson 2010).

Traditional brain slice experiments employ conditions suited to neuronal survival, such as aerating aCSF with 95% O_2. This concentration of O_2 results in superoxide free radicals and cell damage (D'Agostino et al. 2007; Hajos and Mody 2009). Moreover, brain O_2 *in vivo* is much lower, and these artificially high conditions may not only alter astrocyte AA metabolite pathways (Gordon et al. 2008), but may change endogenous vascular signalling. Previous brain slice and retinal preparations using 95% O_2 report a mixture of vascular responses to astrocyte stimulation, such as dilation, constriction, or both dilation and constriction (Metea and Newman 2006; Mulligan and MacVicar 2004; Straub et al. 2006; Zonta et al. 2003). However, perfusion of slices with 20% O_2 solution favours dilation (Gordon et al. 2008), so we chose these conditions for our

experiments, which should provide O_2 concentrations close to physiologic levels and limit superoxide induced cell death (D'Agostino et al. 2007). Oxygen levels also affect NO production (Abu-Soud et al. 1996; Elayan et al. 2000), which is important for modulating vascular responses and astrocyte-blood vessel AA metabolite signalling. Thus, it is important when studying neurovascular coupling in slices to consider conditions which maintain neuronal survival but also optimize vascular responses and NO release (Filosa 2010). Furthermore, oxygen diffusion into tissue forms a gradient, suggesting the effects of oxygen on neurovascular coupling may vary between outer and inner layers of brain slices. In our study, we were limited to the top 100 µm of slices, based on the penetration and loading of dyes (Rhod 2, isolectin B_4), so the oxygen tension was likely constant throughout experiments. However, it would be valuable in the future to measure oxygen at different layers during our experiments to confirm pO_2 is near physiological levels.

While we have made progress to better understand the role of D-serine, NMDA receptors, and NO in cerebral blood flow regulation, there remains several areas to address in the future. We have demonstrated that D-serine and NMDA receptors can influence astrocyte-mediated vasodilation and we have also shown NO is involved in this blood vessel dilation, through eNOS. However, we have not confirmed whether D-serine can contribute to eNOS activation directly in brain slices. D-Serine-mediated dilation and NOS-mediated dilation may occur independently, but we plan to show glutamate and D-serine can elevate eNOS activation within endothelial cells of a brain slice by NO fluorescence imaging and correlate this fluorescence with arteriole vasodilation. Also, we plan to demonstrate a correlation between D-serine and 20-HETE levels using

co-administration of DAAO and HET on brain slices during astrocyte Ca^{2+} uncaging.

Also, it is conceivable blood glutamate and D-serine may activate eNMDA receptors expressed on the luminal side of the BBB. Plasma glutamate concentrations in healthy patients are near 400 µM (Filho et al. 1999), and serum D-serine is near 1-2 µM (Ohnuma et al. 2008). Both of these concentrations are lower than local levels in brain tissue (Clements et al. 1992; Hashimoto et al. 1995), but high enough to activate eNMDA receptors. However, we find D-serine-mediated dilation is induced by astrocyte stimulation, so the mechanism is activity-dependent and likely requires larger local agonist concentrations instead of continuous blood concentrations. Further work is required to determine the localization of eNMDA receptors within the BBB and potential effects of blood glutamate and D-serine on this dilation mechanism.

We have not considered the involvement of other endogenous NMDA receptor agonists, glutamate and glycine, in astrocyte-induced vasodilation. Glutamate activates NMDA receptors in combination with a co-agonist (D-serine or glycine). In brain slices aerated with 20% O_2, bath application of glutamate (10 µM) and D-serine (10 µM) induced arteriole relaxation, but the contribution of endogenous glutamate to NMDA receptor mediated dilation after astrocyte stimulation is unknown. Extracellular glutamate could be sufficient in our model to activate NMDA receptors, and pre-treating brain slices with glutamate dehydrogenase to degrade glutamate before Ca^{2+} uncaging would help determine the role of glutamate in this mechanism. Glycine, the other known NMDA receptor co-agonist, may also modulate vasodilation similar to D-serine. Application of glycine oxidase to slices would indicate the involvement of extracellular

glycine in blood flow control, but we expect glycine to have a minimal role, since other studies have shown D-serine is the principle endogenous co-agonist of neuronal NMDA receptors in several brain regions, including the pre-frontal cortex (Fossat et al. 2011; Panatier et al. 2006). Moreover, our brain slice experiments have been limited to observation of cortical arterioles. Several studies indicate cerebral blood flow can be regulated at the capillary level through pericyte contraction (Peppiatt et al. 2006; Yemisci et al. 2009), and it is also possible neurovascular coupling mechanisms may vary between brain regions depending on the populations of neurons and glia present. Thus, it could be beneficial to study a variety of blood vessels, arteries and capillaries, in different brain regions to further characterize the role of D-serine and NMDA receptors in functional hyperemia.

We have also not yet considered the involvement of other astrocytic pathways, such as EETs, in our model of neurovascular coupling. EETs mediate vasodilation in retina after light and glial-evoked stimulation and *in vivo* after whisker barrel activation (Liu et al. 2008; Liu et al. 2011; Metea and Newman 2006). Inhibition of EETs production also reduces cerebral vasodilation induced by microdialysis perfusion of NMDA *in vivo* (Bhardwaj et al. 2000). Thus, EETs may contribute to our vasodilation mechanism, and further studies are required. Also, we have not judged other ways astrocytes could be involved in D-serine-mediated vasodilation. For example, astrocytes also express NMDA receptors (Lalo et al. 2006; Palygin et al. 2011; Palygin et al. 2010), which could be activated by D-serine release in an autocrine manner. Astrocyte NMDA receptor activation may increase intracellular Ca^{2+} and induce AA metabolite production contributing to neurovascular coupling. However, the only method to selectively

target astrocyte NMDA receptors is to create a new mouse strain utilizing Cre/loxP recombination. By crossing transgenic mice which have lox P sites within the NR1 gene (floxed NR1) with mice expressing Cre recombinase under control of the GFAP (astrocyte specific) promoter (GFAP-cre), an astrocyte-NMDA receptor knockout mouse line would be generated where Cre recombinase is expressed only in astrocytes and excises part of NR1 gene. Brain slice studies with these animals will permit further investigation of astrocyte function in neurovascular coupling and may further elucidate D-serine signalling capabilities. Further complicating NO mediated neurovascular coupling pathways is the possibility of astrocyte eNOS activity, since eNOS expression in astrocytes has been identified by immunostaining (Wiencken and Casagrande 1999). Astrocyte eNOS can be targeted by the same means as astrocyte NMDA receptors by crossing floxed eNOS mice with GFAP-cre mice. This is potentially important, as the involvement of this eNOS form in dynamic vascular interactions is unknown.

We are also the first to suggest endothelial cell NMDA receptors mediate blood flow and provide characterization of mouse endothelial cell NMDA receptor subunit expression of NR1 and NR2C/D in primary endothelial cultures and *in situ*. Our results from pressure myography of MCAs suggest NMDA receptors influence dilation by direct vascular effects. MCA dilation in response to glutamate and D-serine was not affected by the presence of Mg^{2+}, suggesting the presence of NR2C/2D and/or NR3A/3B, which reduce NMDA receptor sensitivity to Mg^{2+} (Monyer et al. 1994). Several studies of cultured endothelial cells have reported expression of NMDA receptors (Andras et al. 2007; Scott et al. 2007; Sharp et al. 2005), but the role of these receptors in blood flow has not been investigated. Our results with isolated arteries suggest glutamate and

D-serine stimulate endothelium-dependent dilation by direct activation of eNOS, which lead us to investigate NMDA receptor subunit distribution in primary mouse endothelial cells. PCR analysis of eNMDA receptor subunits only detected expression of NR1, the conserved subunit, and NR2D. Further, we have shown expression of NR2C/2D in cultured cells and endothelial cells *in situ*. While we have evidence of eNMDA receptor expression, we do not have tangible results indicating functionality of eNMDA receptors and eNOS activation. Further studies of eNOS and calcium signalling in cultured endothelial cells and blood vessels in brain slices may help link eNMDA receptors and blood flow changes. In the future, we will investigate the role of eNMDA receptors in neurovascular coupling via brain slices from endothelial-NR1 knockout mice. These will be produced by breeding floxed NR1 mice with Tek-cre recombinase mice to disrupt NR1 expression in endothelial cells since Tek is an endothelial specific promoter. Two-photon experiments of brain slices from these animals will help to determine the contribution of eNMDA to vasodilation.

The novel mechanism of neurovascular coupling outlined in this thesis involving astrocyte D-serine release, NMDA receptor activation, and possibly eNOS-mediated modulation of 20-HETE is potentially important in disease situations where astrocytes are dysfunctional. During Alzheimer's disease (AD), functional hyperemia and resting blood flow are reduced (Iadecola 2004; Mentis et al. 1996; Niwa et al. 2000; Warkentin and Passant 1997), and the cerebral vasculature is transformed by endothelial cell deformation, smooth muscle degradation and decreased NO levels (Farkas and Luiten 2001; Niwa et al. 2001; Thomas et al. 1996). These vascular effects may impact eNOS activation and NO modulation of AA metabolites, contributing to reduction of

functional hyperemia. Also, astrocytes from transgenic AD mice display dysfunctional Ca^{2+} signalling, with spontaneous Ca^{2+} responses that do not correlate with neuronal activity (Kuchibhotla et al. 2009; Takano et al. 2007), which could affect Ca^{2+}-induced D-serine release and reduce vasodilation. Future studies with AD mouse models may help elucidate the impact of AD on the neurovascular coupling mechanism outlined in this thesis.

Ischemia damages the neurovascular unit, reducing blood flow and functional hyperemia during reperfusion (del Zoppo 2010), including dilation of pial arteries to NMDA receptor agonists (Bari et al. 1996a; Bari et al. 1998a; Bari et al. 1998b; Busija et al. 1996). Part of this altered neurovascular coupling is attributed to distorted concentrations of 20-HETE and EETs, and increasing EETs and decreasing 20-HETE production has been a new therapeutic target (Dunn et al. 2008; Miyata et al. 2005; Tanaka et al. 2007; Zhang et al. 2007; Zhang et al. 2008a). Elevated 20-HETE concentrations could indicate reduced eNOS activation, since we have shown endothelial-derived NO permits vasodilation by reducing 20-HETE. Thus, repeating some of our studies in an ischemia-reperfusion model may show changes in eNOS activation and possibly D-serine signalling.

During epileptiform activity, neurons consume large amounts of energy and oxygen, and blood flow is elevated to meet the demand. However, there is a prolonged delay in vascular responses after astrocyte Ca^{2+} is elevated, suggesting normal neurovascular coupling is altered. eNOS is essential for vasodilation under these conditions (Gomez-Gonzalo et al. 2011; Pereira de Vasconcelos et al. 2006). It is

feasible that high extracellular glutamate concentrations during epileptiform activity may further activate NMDA receptors and eNOS to induce greater vasodilation, and future studies inducing seizures in brain slices would permit investigation of the contribution of D-serine, NMDA receptors and eNOS to neurovascular coupling in this disease.

To summarize, the determination of a novel role for D-serine in NMDA receptor-mediated vasodilation is a valuable contribution to understanding how functional hyperemia is regulated within the neurovascular unit. However, many studies remain in order to fully characterize this mechanism and decipher the action of D-serine and possible activation of eNOS enzyme *in vivo*.

REFERENCES

Abramov AY, Scorziello A, Duchen MR. (2007) Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J Neurosci* 27:1129-1138

Abu-Soud HM, Rousseau DL, Stuehr DJ. (1996) Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. *J Biol Chem* 271:32515-32518

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, et al. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480-1487

Alderton WK, Cooper CE, Knowles RG. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357:593-615

Alexander GE, Chen K, Pietrini P, Rapoport SI, Reiman EM. (2002) Longitudinal PET Evaluation of Cerebral Metabolic Decline in Dementia: A Potential Outcome Measure in Alzheimer's Disease Treatment Studies. *Am J Psychiatry* 159:738-745

Alonso-Galicia M, Hudetz AG, Shen H, Harder DR, Roman RJ. (1999) Contribution of 20-HETE to vasodilator actions of nitric oxide in the cerebral microcirculation. *Stroke* 30:2727-2734; discussion 2734

Alonso-Galicia M, Sun CW, Falck JR, Harder DR, Roman RJ. (1998) Contribution of 20-HETE to the vasodilator actions of nitric oxide in renal arteries. *Am J Physiol* 275:F370-378

Amedee T, Robert A, Coles JA. (1997) Potassium homeostasis and glial energy metabolism. *Glia* 21:46-55

Anderson CM, Swanson RA. (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32:1-14

Andras IE, Deli MA, Veszelka S, Hayashi K, Hennig B, Toborek M. (2007) The NMDA and AMPA/KA receptors are involved in glutamate-induced alterations of

occludin expression and phosphorylation in brain endothelial cells. *J Cereb Blood Flow Metab* 27:1431-1443

Antonetti D. (2009) Eye vessels saved by rescuing their pericyte partners. *Nat Med* 15:1248-1249

Aoki C, Rhee J, Lubin M, Dawson TM. (1997) NMDA-R1 subunit of the cerebral cortex co-localizes with neuronal nitric oxide synthase at pre- and postsynaptic sites and in spines. *Brain Res* 750:25-40

Araque A, Li N, Doyle RT, Haydon PG. (2000) SNARE protein-dependent glutamate release from astrocytes. *J Neurosci* 20:666-673

Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. (2010) Glial and neuronal control of brain blood flow. *Nature* 468:232-243

Attwell D, Laughlin SB. (2001) An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab* 21:1133-1145

Awad N, Gagnon M, Messier C. (2004) The relationship between impaired glucose tolerance, type 2 diabetes, and cognitive function. *J Clin Exp Neuropsychol* 26:1044-1080

Awobuluyi M, Yang J, Ye Y, Chatterton JE, Godzik A, Lipton SA, Zhang D. (2007) Subunit-specific roles of glycine-binding domains in activation of NR1/NR3 N-methyl-D-aspartate receptors. *Mol Pharmacol* 71:112-122

Ayata C, Ma J, Meng W, Huang P, Moskowitz MA. (1996) L-NA-sensitive rCBF augmentation during vibrissal stimulation in type III nitric oxide synthase mutant mice. *J Cereb Blood Flow Metab* 16:539-541

Bari F, Errico RA, Louis TM, Busija DW. (1996a) Differential effects of short-term hypoxia and hypercapnia on N-methyl-D-aspartate-induced cerebral vasodilatation in piglets. *Stroke* 27:1634-1639; discussion 1639-1640

Bari F, Errico RA, Louis TM, Busija DW. (1996b) Interaction between ATP-sensitive K+ channels and nitric oxide on pial arterioles in piglets. *J Cereb Blood Flow*

Metab 16:1158-1164

Bari F, Louis TM, Busija DW. (1998a) Effects of ischemia on cerebral arteriolar dilation to arterial hypoxia in piglets. *Stroke* 29:222-227; discussion 227-228

Bari F, Louis TM, Busija DW. (1998b) Factors modulating N-methyl-D-aspartate (NMDA)-induced cerebral vasodilation after hypoxia/ischemia in piglets. *Neurobiology (Bp)* 6:459-460

Bari F, Louis TM, Meng W, Busija DW. (1996c) Global ischemia impairs ATP-sensitive K+ channel function in cerebral arterioles in piglets. *Stroke* 27:1874-1880; discussion 1880-1871

Bari F, Thore CR, Louis TM, Busija DW. (1998c) Inhibitory effects of hypoxia and adenosine on N-methyl-D-aspartate-induced pial arteriolar dilation in piglets. *Brain Res* 780:237-244

Barkoudah E, Jaggar JH, Leffler CW. (2004) The permissive role of endothelial NO in CO-induced cerebrovascular dilation. *Am J Physiol Heart Circ Physiol* 287:H1459-1465

Baron BM, Harrison BL, Miller FP, McDonald IA, Salituro FG, Schmidt CJ, Sorensen SM, White HS, Palfreyman MG. (1990) Activity of 5,7-dichlorokynurenic acid, a potent antagonist at the N-methyl-D-aspartate receptor-associated glycine binding site. *Mol Pharmacol* 38:554-561

Barros LF, Deitmer JW. (2009) Glucose and lactate supply to the synapse. *Brain Res Rev*

Barros LF, Martinez C. (2007) An enquiry into metabolite domains. *Biophys J* 92:3878-3884

Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT. (2009) Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. *Mol Psychiatry* 14:719-727

Behe P, Stern P, Wyllie DJ, Nassar M, Schoepfer R, Colquhoun D. (1995) Determination of NMDA NR1 subunit copy number in recombinant NMDA receptors. *Proc Biol*

Sci 262:205-213

Benveniste M, Mayer ML. (1993) Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones. *J Physiol* 464:131-163

Bergersen L, Waerhaug O, Helm J, Thomas M, Laake P, Davies AJ, Wilson MC, Halestrap AP, Ottersen OP. (2001) A novel postsynaptic density protein: the monocarboxylate transporter MCT2 is co-localized with delta-glutamate receptors in postsynaptic densities of parallel fiber-Purkinje cell synapses. *Exp Brain Res* 136:523-534

Bernard-Helary K, Lapouble E, Ardourel M, Hevor T, Cloix JF. (2000) Correlation between brain glycogen and convulsive state in mice submitted to methionine sulfoximine. *Life Sci* 67:1773-1781

Bernardinelli Y, Magistretti PJ, Chatton JY. (2004) Astrocytes generate Na+-mediated metabolic waves. *Proc Natl Acad Sci U S A* 101:14937-14942

Betzen C, White R, Zehendner CM, Pietrowski E, Bender B, Luhmann HJ, Kuhlmann CR. (2009) Oxidative stress upregulates the NMDA receptor on cerebrovascular endothelium. *Free Radic Biol Med* 47:1212-1220

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391:281-285

Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhauser C, Pilati E, Volterra A. (2004) Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. *Nat Neurosci* 7:613-620

Bhardwaj A, Northington FJ, Carhuapoma JR, Falck JR, Harder DR, Traystman RJ, Koehler RC. (2000) P-450 epoxygenase and NO synthase inhibitors reduce cerebral blood flow response to N-methyl-D-aspartate. *Am J Physiol Heart Circ Physiol* 279:H1616-1624

Binder DK, Steinhauser C. (2006) Functional changes in astroglial cells in epilepsy. *Glia* 54:358-368

Blahos J, 2nd, Wenthold RJ. (1996) Relationship between N-methyl-D-aspartate receptor NR1 splice variants and NR2 subunits. *J Biol Chem* 271:15669-15674

Blanco VM, Stern JE, Filosa JA. (2008) Tone-dependent vascular responses to astrocyte-derived signals. *Am J Physiol Heart Circ Physiol* 294:H2855-2863

Bliss TV, Collingridge GL. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31-39

Block ML, Zecca L, Hong JS. (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 8:57-69

Blomstrand F, Aberg ND, Eriksson PS, Hansson E, Ronnback L. (1999) Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. *Neuroscience* 92:255-265

Boldyrev AA, Carpenter DO, Johnson P. (2005) Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. *J Neurochem* 95:913-918

Boldyrev AA, Kazey VI, Leinsoo TA, Mashkina AP, Tyulina OV, Johnson P, Tuneva JO, Chittur S, Carpenter DO. (2004) Rodent lymphocytes express functionally active glutamate receptors. *Biochem Biophys Res Commun* 324:133-139

Bonde C, Sarup A, Schousboe A, Gegelashvili G, Zimmer J, Noraberg J. (2003) Neurotoxic and neuroprotective effects of the glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) during physiological and ischemia-like conditions. *Neurochem Int* 43:371-380

Bonvento G, Sibson N, Pellerin L. (2002) Does glutamate image your thoughts? *Trends Neurosci* 25:359-364

Bouzier-Sore AK, Merle M, Magistretti PJ, Pellerin L. (2002) Feeding active neurons: (re)emergence of a nursing role for astrocytes. *J Physiol Paris* 96:273-282

Bradley KK, Jaggar JH, Bonev AD, Heppner TJ, Flynn ER, Nelson MT, Horowitz B. (1999) Kir2.1 encodes the inward rectifier potassium channel in rat arterial

smooth muscle cells. *J Physiol* 515 (Pt 3):639-651

Braunewell KH, Manahan-Vaughan D. (2001) Long-term depression: a cellular basis for learning? *Rev Neurosci* 12:121-140

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84:757-767

Bringmann A, Pannicke T, Uhlmann S, Kohen L, Wiedemann P, Reichenbach A. (2002) Membrane conductance of Muller glial cells in proliferative diabetic retinopathy. *Can J Ophthalmol* 37:221-227

Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287:C817-833

Brown AM, Ransom BR. (2007) Astrocyte glycogen and brain energy metabolism. *Glia* 55:1263-1271

Brown AM, Sickmann HM, Fosgerau K, Lund TM, Schousboe A, Waagepetersen HS, Ransom BR. (2005) Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. *J Neurosci Res* 79:74-80

Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, Monaghan DT. (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. *J Neurosci* 14:5471-5484

Bushong EA, Martone ME, Jones YZ, Ellisman MH. (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci* 22:183-192

Busija DW, Bari F, Domoki F, Louis T. (2007) Mechanisms involved in the cerebrovascular dilator effects of N-methyl-d-aspartate in cerebral cortex. *Brain Res Rev* 56:89-100

Busija DW, Leffler CW. (1989) Dilator effects of amino acid neurotransmitters on piglet

pial arterioles. *Am J Physiol* 257:H1200-1203

Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM. (1996) Effects of ischemia on cerebrovascular responses to N-methyl-D-aspartate in piglets. *Am J Physiol* 270:H1225-1230

Butt AM, Colquhoun K, Berry M. (1994) Confocal imaging of glial cells in the intact rat optic nerve. *Glia* 10:315-322

Butt AM, Kalsi A. (2006) Inwardly rectifying potassium channels (Kir) in central nervous system glia: a special role for Kir4.1 in glial functions. *J Cell Mol Med* 10:33-44

Byrnes KR, Loane DJ, Faden AI. (2009) Metabotropic glutamate receptors as targets for multipotential treatment of neurological disorders. *Neurotherapeutics* 6:94-107

Caesar K, Hashemi P, Douhou A, Bonvento G, Boutelle MG, Walls AB, Lauritzen M. (2008) Glutamate receptor-dependent increments in lactate, glucose and oxygen metabolism evoked in rat cerebellum in vivo. *J Physiol* 586:1337-1349

Cater HL, Benham CD, Sundstrom LE. (2001) Neuroprotective role of monocarboxylate transport during glucose deprivation in slice cultures of rat hippocampus. *J Physiol* 531:459-466

Chan BS, Endo S, Kanai N, Schuster VL. (2002) Identification of lactate as a driving force for prostanoid transport by prostaglandin transporter PGT. *Am J Physiol Renal Physiol* 282:F1097-1102

Charles A. (2005) Reaching out beyond the synapse: glial intercellular waves coordinate metabolism. *Sci STKE* 2005:pe6

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 415:793-798

Chen L, Huang LY. (1992) Protein kinase C reduces Mg2+ block of NMDA-receptor

channels as a mechanism of modulation. *Nature* 356:521-523

Chen Y, Swanson RA. (2003) The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *J Neurochem* 84:1332-1339

Chi OZ, Liu X, Weiss HR. (2003) Effects of inhibition of neuronal nitric oxide synthase on NMDA-induced changes in cerebral blood flow and oxygen consumption. *Exp Brain Res* 148:256-260

Chih CP, Roberts Jr EL. (2003) Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron lactate shuttle hypothesis. *J Cereb Blood Flow Metab* 23:1263-1281

Chinellato A, Froldi G, Caparrotta L, Ragazzi E. (1998) Pharmacological characterization of endothelial cell nitric oxide synthase inhibitors in isolated rabbit aorta. *Life Sci* 62:479-490

Cho Y, Bannai S. (1990) Uptake of glutamate and cysteine in C-6 glioma cells and in cultured astrocytes. *J Neurochem* 55:2091-2097

Choi DW. (1987) Ionic dependence of glutamate neurotoxicity. *J Neurosci* 7:369-379

Choi DW, Koh JY, Peters S. (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* 8:185-196

Cholet N, Pellerin L, Welker E, Lacombe P, Seylaz J, Magistretti P, Bonvento G. (2001) Local injection of antisense oligonucleotides targeted to the glial glutamate transporter GLAST decreases the metabolic response to somatosensory activation. *J Cereb Blood Flow Metab* 21:404-412

Chui H. (2001) Dementia due to subcortical ischemic vascular disease. *Clin Cornerstone* 3:40-51

Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S, Sevarino KA. (1995) Cloning and characterization of chi-1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J Neurosci* 15:6498-6508

Ciriacks CM, Bowser MT. (2006) Measuring the effect of glutamate receptor agonists on

extracellular D-serine concentrations in the rat striatum using online microdialysis-capillary electrophoresis. *Neurosci Lett* 393:200-205

Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. (1992) The time course of glutamate in the synaptic cleft. *Science* 258:1498-1501

Cloix JF, Hevor T. (2009) Epilepsy, regulation of brain energy metabolism and neurotransmission. *Curr Med Chem* 16:841-853

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C. (2003) Storage and release of ATP from astrocytes in culture. *J Biol Chem* 278:1354-1362

Collingridge GL, Singer W. (1990) Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol Sci* 11:290-296

Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Saez JC. (2002) Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. *Proc Natl Acad Sci U S A* 99:495-500

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* 247:470-473

Crespi F, Lazzarini C, Andreoli M, Vecchiato E. (2000) Voltammetric and functional evidence that N-methyl-D-aspartate and substance P mediate rat vascular relaxation via nitrogen monoxide release. *Neurosci Lett* 287:219-222

Csernansky JG, Bardgett ME, Sheline YI, Morris JC, Olney JW. (1996) CSF excitatory amino acids and severity of illness in Alzheimer's disease. *Neurology* 46:1715-1720

Cull-Candy SG, Leszkiewicz DN. (2004) Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE* 2004:re16

D'Agostino DP, Putnam RW, Dean JB. (2007) Superoxide (*O2-) production in CA1 neurons of rat hippocampal slices exposed to graded levels of oxygen. *J*

Neurophysiol 98:1030-1041

D'Ambrosio R. (2004) The role of glial membrane ion channels in seizures and epileptogenesis. *Pharmacol Ther* 103:95-108

Danbolt NC. (2001) Glutamate uptake. *Prog Neurobiol* 65:1-105

Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA, Nakanishi N. (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature* 393:377-381

de Labra C, Rivadulla C, Espinosa N, Dasilva M, Cao R, Cudeiro J. (2009) Different sources of nitric oxide mediate neurovascular coupling in the lateral geniculate nucleus of the cat. *Front Syst Neurosci* 3:9

del Zoppo GJ. (2010) The neurovascular unit in the setting of stroke. *J Intern Med* 267:156-171

del Zoppo GJ, Mabuchi T. (2003) Cerebral microvessel responses to focal ischemia. *J Cereb Blood Flow Metab* 23:879-894

Deng A, Valdivielso JM, Munger KA, Blantz RC, Thomson SC. (2002) Vasodilatory N-methyl-D-aspartate receptors are constitutively expressed in rat kidney. *J Am Soc Nephrol* 13:1381-1384

Devor A, Tian P, Nishimura N, Teng IC, Hillman EM, Narayanan SN, Ulbert I, Boas DA, Kleinfeld D, Dale AM. (2007) Suppressed neuronal activity and concurrent arteriolar vasoconstriction may explain negative blood oxygenation level-dependent signal. *J Neurosci* 27:4452-4459

Di Castro MA, Chuquet J, Liaudet N, Bhaukaurally K, Santello M, Bouvier D, Tiret P, Volterra A. (2011) Local Ca2+ detection and modulation of synaptic release by astrocytes. *Nat Neurosci* 14:1276-1284

Diamond JS, Jahr CE. (2000) Synaptically released glutamate does not overwhelm transporters on hippocampal astrocytes during high-frequency stimulation. *J*

Neurophysiol 83:2835-2843

Ding X, Ma N, Nagahama M, Yamada K, Semba R. (2011) Localization of D-serine and serine racemase in neurons and neuroglias in mouse brain. *Neurol Sci* 32:263-267

Dingledine R, Borges K, Bowie D, Traynelis SF. (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61

Dirnagl U, Niwa K, Lindauer U, Villringer A. (1994) Coupling of cerebral blood flow to neuronal activation: role of adenosine and nitric oxide. *Am J Physiol* 267:H296-301

do Carmo A, Lopes C, Santos M, Proenca R, Cunha-Vaz J, Carvalho AP. (1998) Nitric oxide synthase activity and L-arginine metabolism in the retinas from streptozotocin-induced diabetic rats. *Gen Pharmacol* 30:319-324

Domoki F, Kis B, Gaspar T, Bari F, Busija DW. (2008) Cerebromicrovascular endothelial cells are resistant to L-glutamate. *Am J Physiol Regul Integr Comp Physiol* 295:R1099-1108

Domoki F, Perciaccante JV, Shimizu K, Puskar M, Busija DW, Bari F. (2002) N-methyl-D-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets. *Am J Physiol Heart Circ Physiol* 282:H1404-1409

Dong XX, Wang Y, Qin ZH. (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin* 30:379-387

Dorrance AM, Rupp N, Pollock DM, Newman JW, Hammock BD, Imig JD. (2005) An epoxide hydrolase inhibitor, 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA), reduces ischemic cerebral infarct size in stroke-prone spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 46:842-848

Dringen R, Gebhardt R, Hamprecht B. (1993) Glycogen in astrocytes: possible function as lactate supply for neighboring cells. *Brain Res* 623:208-214

Dringen R, Hamprecht B. (1993) Differences in glycogen metabolism in astroglia-rich primary cultures and sorbitol-selected astroglial cultures derived from mouse

brain. *Glia* 8:143-149

Du Y, Sarthy VP, Kern TS. (2004) Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. *Am J Physiol Regul Integr Comp Physiol* 287:R735-741

Dudek FE, Patrylo PR, Wuarin JP. (1999) Mechanisms of neuronal synchronization during epileptiform activity. *Adv Neurol* 79:699-708

Dun Y, Mysona B, Itagaki S, Martin-Studdard A, Ganapathy V, Smith SB. (2007) Functional and molecular analysis of D-serine transport in retinal Muller cells. *Exp Eye Res* 84:191-199

Dunn KM, Nelson MT. (2010) Potassium channels and neurovascular coupling. *Circ J* 74:608-616

Dunn KM, Renic M, Flasch AK, Harder DR, Falck J, Roman RJ. (2008) Elevated production of 20-HETE in the cerebral vasculature contributes to severity of ischemic stroke and oxidative stress in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 295:H2455-2465

Earley S, Heppner TJ, Nelson MT, Brayden JE. (2005) TRPV4 forms a novel Ca2+ signaling complex with ryanodine receptors and BKCa channels. *Circ Res* 97:1270-1279

Edmond J, Robbins RA, Bergstrom JD, Cole RA, de Vellis J. (1987) Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. *J Neurosci Res* 18:551-561

Edwards FR, Hirst GD, Silverberg GD. (1988) Inward rectification in rat cerebral arterioles; involvement of potassium ions in autoregulation. *J Physiol* 404:455-466

Ehlers MD, Zhang S, Bernhadt JP, Huganir RL. (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* 84:745-755

Eid T, Lee TS, Thomas MJ, Amiry-Moghaddam M, Bjornsen LP, Spencer DD, Agre P, Ottersen OP, de Lanerolle NC. (2005) Loss of perivascular aquaporin 4 may

underlie deficient water and K+ homeostasis in the human epileptogenic hippocampus. *Proc Natl Acad Sci U S A* 102:1193-1198

Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC, Ottersen OP, de Lanerolle NC. (2004) Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. *Lancet* 363:28-37

Elayan IM, Axley MJ, Prasad PV, Ahlers ST, Auker CR. (2000) Effect of hyperbaric oxygen treatment on nitric oxide and oxygen free radicals in rat brain. *J Neurophysiol* 83:2022-2029

Ellis-Davies GC, Kaplan JH. (1994) Nitrophenyl-EGTA, a photolabile chelator that selectively binds Ca2+ with high affinity and releases it rapidly upon photolysis. *Proc Natl Acad Sci U S A* 91:187-191

Endres M, Laufs U, Liao JK, Moskowitz MA. (2004) Targeting eNOS for stroke protection. *Trends Neurosci* 27:283-289

Engel J, Jr., Brown WJ, Kuhl DE, Phelps ME, Mazziotta JC, Crandall PH. (1982) Pathological findings underlying focal temporal lobe hypometabolism in partial epilepsy. *Ann Neurol* 12:518-528

Engel J, Jr., Kuhl DE, Phelps ME, Rausch R, Nuwer M. (1983) Local cerebral metabolism during partial seizures. *Neurology* 33:400-413

Fadda E, Danysz W, Wroblewski JT, Costa E. (1988) Glycine and D-serine increase the affinity of N-methyl-D-aspartate sensitive glutamate binding sites in rat brain synaptic membranes. *Neuropharmacology* 27:1183-1185

Faff-Michalak L, Albrecht J. (1993) Hyperammonemia and hepatic encephalopathy stimulate rat cerebral synaptic mitochondrial glutamate dehydrogenase activity specifically in the direction of glutamate oxidation. *Brain Res* 618:299-302

Faraci FM, Breese KR. (1993) Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. *Circ Res* 72:476-480

Faraci FM, Breese KR. (1994) Dilatation of cerebral arterioles in response to N-methyl-

D-aspartate: role of CGRP and acetylcholine. *Brain Res* 640:93-97

Faraci FM, Brian JE, Jr. (1995) 7-Nitroindazole inhibits brain nitric oxide synthase and cerebral vasodilatation in response to N-methyl-D-aspartate. *Stroke* 26:2172-2175; discussion 2176

Farkas E, Luiten PG. (2001) Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol* 64:575-611

Fergus A, Lee KS. (1997) Regulation of cerebral microvessels by glutamatergic mechanisms. *Brain Res* 754:35-45

Fernandez-Klett F, Offenhauser N, Dirnagl U, Priller J, Lindauer U. (2010) Pericytes in capillaries are contractile in vivo, but arterioles mediate functional hyperemia in the mouse brain. *Proc Natl Acad Sci U S A* 107:22290-22295

Filho JC, Hazel SJ, Anderstam B, Bergstrom J, Lewitt M, Hall K. (1999) Effect of protein intake on plasma and erythrocyte free amino acids and serum IGF-I and IGFBP-1 levels in rats. *Am J Physiol* 277:E693-701

Filosa JA. (2010) Vascular tone and neurovascular coupling: considerations toward an improved in vitro model. *Front Neuroenergetics* 2

Filosa JA, Bonev AD, Nelson MT. (2004) Calcium dynamics in cortical astrocytes and arterioles during neurovascular coupling. *Circ Res* 95:e73-81

Filosa JA, Bonev AD, Straub SV, Meredith AL, Wilkerson MK, Aldrich RW, Nelson MT. (2006) Local potassium signaling couples neuronal activity to vasodilation in the brain. *Nat Neurosci* 9:1397-1403

Fiumana E, Parfenova H, Jaggar JH, Leffler CW. (2003) Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am J Physiol Heart Circ Physiol* 284:H1073-1079

Fletcher EL, Downie LE, Ly A, Ward MM, Batcha AH, Puthussery T, Yee P, Hatzopoulos KM. (2008) A review of the role of glial cells in understanding retinal disease. *Clin Exp Optom* 91:67-77

Fletcher EL, Phipps JA, Ward MM, Puthussery T, Wilkinson-Berka JL. (2007) Neuronal and glial cell abnormality as predictors of progression of diabetic retinopathy. *Curr Pharm Des* 13:2699-2712

Fletcher EL, Phipps JA, Wilkinson-Berka JL. (2005) Dysfunction of retinal neurons and glia during diabetes. *Clin Exp Optom* 88:132-145

Fonnum F. (1984) Glutamate: a neurotransmitter in mammalian brain. *J Neurochem* 42:1-11

Fossat P, Turpin FR, Sacchi S, Dulong J, Shi T, Rivet JM, Sweedler JV, Pollegioni L, Millan MJ, Oliet SH, Mothet JP. (2011) Glial D-Serine Gates NMDA Receptors at Excitatory Synapses in Prefrontal Cortex. *Cereb Cortex*

Fotheringham J, Donati D, Akhyani N, Fogdell-Hahn A, Vortmeyer A, Heiss JD, Williams E, Weinstein S, Bruce DA, Gaillard WD, Sato S, Theodore WH, Jacobson S. (2007) Association of human herpesvirus-6B with mesial temporal lobe epilepsy. *PLoS Med* 4:e180

Fowler JC. (1993) Glucose deprivation results in a lactate preventable increase in adenosine and depression of synaptic transmission in rat hippocampal slices. *J Neurochem* 60:572-576

Franconi F, Miceli M, De Montis MG, Crisafi EL, Bennardini F, Tagliamonte A. (1996) NMDA receptors play an anti-aggregating role in human platelets. *Thromb Haemost* 76:84-87

Fritz KI, Groenendaal F, McGowan JE, Mishra OP, Delivoria-Papadopoulos M. (1996) Effect of cerebral hypoxia on NMDA receptor binding characteristics after treatment with 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) in newborn piglets. *Brain Res* 729:66-74

Fujimoto Y, Uno E, Sakuma S. (2004) Effects of reactive oxygen and nitrogen species on cyclooxygenase-1 and -2 activities. *Prostaglandins Leukot Essent Fatty Acids* 71:335-340

Fuller S, Munch G, Steele M. (2009) Activated astrocytes: a therapeutic target in Alzheimer's disease? *Expert Rev Neurother* 9:1585-1594

Furfine ES, Harmon MF, Paith JE, Garvey EP. (1993) Selective inhibition of constitutive nitric oxide synthase by L-NG-nitroarginine. *Biochemistry* 32:8512-8517

Gandhi GK, Ball KK, Cruz NF, Dienel GA. (2010) Hyperglycaemia and diabetes impair gap junctional communication among astrocytes. *ASN Neuro* 2:e00030

Gao X, Xu X, Pang J, Zhang C, Ding JM, Peng X, Liu Y, Cao JM. (2007) NMDA receptor activation induces mitochondrial dysfunction, oxidative stress and apoptosis in cultured neonatal rat cardiomyocytes. *Physiol Res* 56:559-569

Garriga-Canut M, Schoenike B, Qazi R, Bergendahl K, Daley TJ, Pfender RM, Morrison JF, Ockuly J, Stafstrom C, Sutula T, Roopra A. (2006) 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat Neurosci* 9:1382-1387

Garthwaite J. (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci* 14:60-67

Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, Harder DR. (1992) Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol* 263:H519-525

Genever PG, Maxfield SJ, Kennovin GD, Maltman J, Bowgen CJ, Raxworthy MJ, Skerry TM. (1999) Evidence for a novel glutamate-mediated signaling pathway in keratinocytes. *J Invest Dermatol* 112:337-342

Gerrits RJ, Stein EA, Greene AS. (2002) Ca(2+)-activated potassium (K(Ca)) channel inhibition decreases neuronal activity-blood flow coupling. *Brain Res* 948:108-116

Giaume C, McCarthy KD. (1996) Control of gap-junctional communication in astrocytic networks. *Trends Neurosci* 19:319-325

Gibbs ME, Anderson DG, Hertz L. (2006) Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* 54:214-222

Gibbs ME, Gibbs Z, Hertz L. (2009) Rescue of Abeta(1-42)-induced memory impairment in day-old chick by facilitation of astrocytic oxidative metabolism: implications

for Alzheimer's disease. *J Neurochem* 109 Suppl 1:230-236

Gibbs ME, Lloyd HG, Santa T, Hertz L. (2007) Glycogen is a preferred glutamate precursor during learning in 1-day-old chick: biochemical and behavioral evidence. *J Neurosci Res* 85:3326-3333

Girouard H, Bonev AD, Hannah RM, Meredith A, Aldrich RW, Nelson MT. (2010) Astrocytic endfoot Ca2+ and BK channels determine both arteriolar dilation and constriction. *Proc Natl Acad Sci U S A* 107:3811-3816

Gliddon CM, Shao Z, LeMaistre JL, Anderson CM. (2009) Cellular distribution of the neutral amino acid transporter subtype ASCT2 in mouse brain. *J Neurochem* 108:372-383

Gomez-Gonzalo M, Losi G, Brondi M, Uva L, Sato SS, de Curtis M, Ratto GM, Carmignoto G. (2011) Ictal but not interictal epileptic discharges activate astrocyte endfeet and elicit cerebral arteriole responses. *Front Cell Neurosci* 5:8

Gomez-Gonzalo M, Losi G, Chiavegato A, Zonta M, Cammarota M, Brondi M, Vetri F, Uva L, Pozzan T, de Curtis M, Ratto GM, Carmignoto G. (2010) An excitatory loop with astrocytes contributes to drive neurons to seizure threshold. *PLoS Biol* 8:e1000352

Gordon GR, Choi HB, Rungta RL, Ellis-Davies GC, Macvicar BA. (2008) Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature*

Gordon GR, Iremonger KJ, Kantevari S, Ellis-Davies GC, MacVicar BA, Bains JS. (2009) Astrocyte-mediated distributed plasticity at hypothalamic glutamate synapses. *Neuron* 64:391-403

Gracy KN, Pickel VM. (1997) Ultrastructural localization and comparative distribution of nitric oxide synthase and N-methyl-D-aspartate receptors in the shell of the rat nucleus accumbens. *Brain Res* 747:259-272

Gray AT, Buck LT, Feiner JR, Bickler PE. (1997) Interactive effects of pH and temperature on N-methyl-D-aspartate receptor activity in rat cortical brain slices. *J Neurosurg Anesthesiol* 9:180-187

Gustafson EC, Stevens ER, Wolosker H, Miller RF. (2007) Endogenous D-serine

contributes to NMDA-receptor-mediated light-evoked responses in the vertebrate retina. *J Neurophysiol* 98:122-130

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. (1999) ATP released from astrocytes mediates glial calcium waves. *J Neurosci* 19:520-528

Haglund MM, Meno JR, Hochman DW, Ngai AC, Winn HR. (2008) Correlation of intrinsic optical signal, cerebral blood flow, and evoked potentials during activation of rat somatosensory cortex. *J Neurosurg* 109:654-663

Hajos N, Mody I. (2009) Establishing a physiological environment for visualized in vitro brain slice recordings by increasing oxygen supply and modifying aCSF content. *J Neurosci Methods* 183:107-113

Hamai M, Minokoshi Y, Shimazu T. (1999) L-Glutamate and insulin enhance glycogen synthesis in cultured astrocytes from the rat brain through different intracellular mechanisms. *J Neurochem* 73:400-407

Hamilton NB, Attwell D. (2010) Do astrocytes really exocytose neurotransmitters? *Nat Rev Neurosci* 11:227-238

Hamilton NB, Attwell D, Hall CN. (2010) Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. *Front Neuroenergetics* 2

Handy RL, Wallace P, Gaffen ZA, Whitehead KJ, Moore PK. (1995) The antinociceptive effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase in vitro, in the mouse. *Br J Pharmacol* 116:2349-2350

Hardebo JE, Wieloch T, Kahrstrom J. (1989) Excitatory amino acids and cerebrovascular tone. *Acta Physiol Scand* 136:483-485

Harder DR, Narayanan J, Birks EK, Liard JF, Imig JD, Lombard JH, Lange AR, Roman RJ. (1996) Identification of a putative microvascular oxygen sensor. *Circ Res* 79:54-61

Hashimoto A, Nishikawa T, Oka T, Takahashi K. (1993) Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. *J Neurochem*

60:783-786

Hashimoto A, Oka T, Nishikawa T. (1995) Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialysis. *Neuroscience* 66:635-643

Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M. (2003) Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch Gen Psychiatry* 60:572-576

Hassinger TD, Guthrie PB, Atkinson PB, Bennett MV, Kater SB. (1996) An extracellular signaling component in propagation of astrocytic calcium waves. *Proc Natl Acad Sci U S A* 93:13268-13273

Helboe L, Egebjerg J, Moller M, Thomsen C. (2003) Distribution and pharmacology of alanine-serine-cysteine transporter 1 (asc-1) in rodent brain. *Eur J Neurosci* 18:2227-2238

Heneka MT, Feinstein DL. (2001) Expression and function of inducible nitric oxide synthase in neurons. *J Neuroimmunol* 114:8-18

Henneberger C, Papouin T, Oliet SH, Rusakov DA. (2010) Long-term potentiation depends on release of D-serine from astrocytes. *Nature* 463:232-236

Hepp R, Perraut M, Chasserot-Golaz S, Galli T, Aunis D, Langley K, Grant NJ. (1999) Cultured glial cells express the SNAP-25 analogue SNAP-23. *Glia* 27:181-187

Hertz L. (2003) Astrocytic amino acid metabolism under control conditions and during oxygen and/or glucose deprivation. *Neurochem Res* 28:243-258

Hertz L. (2008) Bioenergetics of cerebral ischemia: a cellular perspective. *Neuropharmacology* 55:289-309

Hertz L, Hertz E. (2003) Cataplerotic TCA cycle flux determined as glutamate-sustained oxygen consumption in primary cultures of astrocytes. *Neurochem Int* 43:355-361

Hertz L, Peng L, Dienel GA. (2007) Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J Cereb Blood Flow Metab* 27:219-249

Hertz L, Zielke HR. (2004) Astrocytic control of glutamatergic activity: astrocytes as stars of the show. *Trends Neurosci* 27:735-743

Hewett SJ, Csernansky CA, Choi DW. (1994) Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron* 13:487-494

Higashimori H, Blanco VM, Tuniki VR, Falck JR, Filosa JA. (2010) Role of epoxyeicosatrienoic acids as autocrine metabolites in glutamate-mediated K+ signaling in perivascular astrocytes. *Am J Physiol Cell Physiol* 299:C1068-1078

Hinschen AK, Rose'Meyer RB, Headrick JP. (2003) Adenosine receptor subtypes mediating coronary vasodilation in rat hearts. *J Cardiovasc Pharmacol* 41:73-80

Hlatky R, Lui H, Cherian L, Goodman JC, O'Brien WE, Contant CF, Robertson CS. (2003) The role of endothelial nitric oxide synthase in the cerebral hemodynamics after controlled cortical impact injury in mice. *J Neurotrauma* 20:995-1006

Holt DC, Fedinec AL, Vaughn AN, Leffler CW. (2007) Age and species dependence of pial arteriolar responses to topical carbon monoxide in vivo. *Exp Biol Med (Maywood)* 232:1465-1469

Honore T, Davies SN, Drejer J, Fletcher EJ, Jacobsen P, Lodge D, Nielsen FE. (1988) Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science* 241:701-703

Hu Y, Wilson GS. (1997) A temporary local energy pool coupled to neuronal activity: fluctuations of extracellular lactate levels in rat brain monitored with rapid-response enzyme-based sensor. *J Neurochem* 69:1484-1490

Iadecola C. (1993) Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? *Trends Neurosci* 16:206-214

Iadecola C. (2004) Neurovascular regulation in the normal brain and in Alzheimer's

disease. *Nat Rev Neurosci* 5:347-360

Iadecola C, Nedergaard M. (2007) Glial regulation of the cerebral microvasculature. *Nat Neurosci* 10:1369-1376

Idestrup CP, Salter MW. (1998) P2Y and P2U receptors differentially release intracellular Ca2+ via the phospholipase c/inositol 1,4,5-triphosphate pathway in astrocytes from the dorsal spinal cord. *Neuroscience* 86:913-923

Ido Y, Chang K, Woolsey TA, Williamson JR. (2001) NADH: sensor of blood flow need in brain, muscle, and other tissues. *FASEB J* 15:1419-1421

Ignarro LJ, Cirino G, Casini A, Napoli C. (1999) Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol* 34:879-886

Iliff JJ, D'Ambrosio R, Ngai AC, Winn HR. (2003) Adenosine receptors mediate glutamate-evoked arteriolar dilation in the rat cerebral cortex. *Am J Physiol Heart Circ Physiol* 284:H1631-1637

Imig JD, Hammock BD. (2009) Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov* 8:794-805

Inagaki N, Kuromi H, Gonoi T, Okamoto Y, Ishida H, Seino Y, Kaneko T, Iwanaga T, Seino S. (1995) Expression and role of ionotropic glutamate receptors in pancreatic islet cells. *FASEB J* 9:686-691

Inoue R, Hashimoto K, Harai T, Mori H. (2008) NMDA- and beta-amyloid1-42-induced neurotoxicity is attenuated in serine racemase knock-out mice. *J Neurosci* 28:14486-14491

Izumi Y, Katsuki H, Zorumski CF. (1997) Monocarboxylates (pyruvate and lactate) as alternative energy substrates for the induction of long-term potentiation in rat hippocampal slices. *Neurosci Lett* 232:17-20

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova D, E S, Cheng X. (2002) Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca2+ sparks to Ca2+-activated K+ channels. *Circ Res* 91:610-617

Jamieson D, Vandenbrenk HA. (1963) Measurement of Oxygen Tensions in Cerebral Tissues of Rats Exposed to High Pressures of Oxygen. *J Appl Physiol* 18:869-876

Jimenez-Jimenez FJ, Molina JA, Gomez P, Vargas C, de Bustos F, Benito-Leon J, Tallon-Barranco A, Orti-Pareja M, Gasalla T, Arenas J. (1998) Neurotransmitter amino acids in cerebrospinal fluid of patients with Alzheimer's disease. *J Neural Transm* 105:269-277

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A. (2007) Glutamate exocytosis from astrocytes controls synaptic strength. *Nat Neurosci* 10:331-339

Jung SS, Levy E. (2005) Murine cerebrovascular cells as a cell culture model for cerebral amyloid angiopathy: isolation of smooth muscle and endothelial cells from mouse brain. *Methods Mol Biol* 299:211-219

Juranek I, Suzuki H, Yamamoto S. (1999) Affinities of various mammalian arachidonate lipoxygenases and cyclooxygenases for molecular oxygen as substrate. *Biochim Biophys Acta* 1436:509-518

Juurlink BH. (1997) Response of glial cells to ischemia: roles of reactive oxygen species and glutathione. *Neurosci Biobehav Rev* 21:151-166

Kacem K, Lacombe P, Seylaz J, Bonvento G. (1998) Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. *Glia* 23:1-10

Kaku DA, Giffard RG, Choi DW. (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science* 260:1516-1518

Karadottir R, Cavelier P, Bergersen LH, Attwell D. (2005) NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature* 438:1162-1166

Karlsson GA, Chaitoff KA, Hossain S, Bohlke M, Maher TJ, Ally A. (2007) Modulation of cardiovascular responses and neurotransmission during peripheral nociception following nNOS antagonism within the periaqueductal gray. *Brain Res* 1143:150-160

Kartvelishvily E, Shleper M, Balan L, Dumin E, Wolosker H. (2006) Neuron-derived D-

serine release provides a novel means to activate N-methyl-D-aspartate receptors. *J Biol Chem* 281:14151-14162

Kasischke KA. (2008) A new pathway for lactate production in the CNS. *J Physiol* 586:1207-1208

Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. (2004) Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* 305:99-103

Katsuki H, Nonaka M, Shirakawa H, Kume T, Akaike A. (2004) Endogenous D-serine is involved in induction of neuronal death by N-methyl-D-aspartate and simulated ischemia in rat cerebrocortical slices. *J Pharmacol Exp Ther* 311:836-844

Katsuki H, Watanabe Y, Fujimoto S, Kume T, Akaike A. (2007) Contribution of endogenous glycine and d-serine to excitotoxic and ischemic cell death in rat cerebrocortical slice cultures. *Life Sci* 81:740-749

Kim E, Cho KO, Rothschild A, Sheng M. (1996) Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins. *Neuron* 17:103-113

Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH, Barrow RK, Huganir RL, Ghosh A, Snyder SH. (2005) Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. *Proc Natl Acad Sci U S A* 102:2105-2110

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA. (1990) Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J Neurosci* 10:1583-1591

Knot HJ, Nelson MT. (1998) Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure. *J Physiol* 508 (Pt 1):199-209

Knot HJ, Zimmermann PA, Nelson MT. (1996) Extracellular K(+)-induced hyperpolarizations and dilatations of rat coronary and cerebral arteries involve inward rectifier K(+) channels. *J Physiol* 492 (Pt 2):419-430

Ko KR, Ngai AC, Winn HR. (1990) Role of adenosine in regulation of regional cerebral blood flow in sensory cortex. *Am J Physiol* 259:H1703-1708

Koenig H, Trout JJ, Goldstone AD, Lu CY. (1992) Capillary NMDA receptors regulate blood-brain barrier function and breakdown. *Brain Res* 588:297-303

Kofuji P, Newman EA. (2004) Potassium buffering in the central nervous system. *Neuroscience* 129:1045-1056

Krizbai IA, Deli MA, Pestenacz A, Siklos L, Szabo CA, Andras I, Joo F. (1998) Expression of glutamate receptors on cultured cerebral endothelial cells. *J Neurosci Res* 54:814-819

Kuchibhotla KV, Lattarulo CR, Hyman BT, Bacskai BJ. (2009) Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. *Science* 323:1211-1215

Kuhl DE, Engel J, Jr., Phelps ME, Selin C. (1980) Epileptic patterns of local cerebral metabolism and perfusion in humans determined by emission computed tomography of 18FDG and 13NH3. *Ann Neurol* 8:348-360

Kuhlmann CR, Gerigk M, Bender B, Closhen D, Lessmann V, Luhmann HJ. (2008) Fluvastatin prevents glutamate-induced blood-brain-barrier disruption in vitro. *Life Sci* 82:1281-1287

Kuhlmann CR, Zehendner CM, Gerigk M, Closhen D, Bender B, Friedl P, Luhmann HJ. (2009) MK801 blocks hypoxic blood-brain-barrier disruption and leukocyte adhesion. *Neurosci Lett* 449:168-172

Kuschinsky W, Wahl M, Bosse O, Thurau K. (1972) Perivascular potassium and pH as determinants of local pial arterial diameter in cats. A microapplication study. *Circ Res* 31:240-247

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, et al. (1992) Molecular diversity of the NMDA receptor channel. *Nature* 358:36-41

Labrie V, Fukumura R, Rastogi A, Fick LJ, Wang W, Boutros PC, Kennedy JL, Semeralul MO, Lee FH, Baker GB, Belsham DD, Barger SW, Gondo Y, Wong

AH, Roder JC. (2009) Serine racemase is associated with schizophrenia susceptibility in humans and in a mouse model. *Hum Mol Genet* 18:3227-3243

Lalo U, Pankratov Y, Kirchhoff F, North RA, Verkhratsky A. (2006) NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. *J Neurosci* 26:2673-2683

Lam TK, Gutierrez-Juarez R, Pocai A, Bhanot S, Tso P, Schwartz GJ, Rossetti L. (2007) Brain glucose metabolism controls the hepatic secretion of triglyceride-rich lipoproteins. *Nat Med* 13:171-180

Lam TK, Gutierrez-Juarez R, Pocai A, Rossetti L. (2005) Regulation of blood glucose by hypothalamic pyruvate metabolism. *Science* 309:943-947

Lassen NA. (1959) Cerebral blood flow and oxygen consumption in man. *Physiol Rev* 39:183-238

Lau A, Tymianski M. (2010) Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch* 460:525-542

Lee AY. (2011) Vascular dementia. *Chonnam Med J* 47:66-71

Lee MC, Ting KK, Adams S, Brew BJ, Chung R, Guillemin GJ. (2010) Characterisation of the expression of NMDA receptors in human astrocytes. *PLoS One* 5:e14123

Leeson PD, Baker R, Carling RW, Curtis NR, Moore KW, Williams BJ, Foster AC, Donald AE, Kemp JA, Marshall GR. (1991) Kynurenic acid derivatives. Structure-activity relationships for excitatory amino acid antagonism and identification of potent and selective antagonists at the glycine site on the N-methyl-D-aspartate receptor. *J Med Chem* 34:1243-1252

Leffler CW, Balabanova L, Fedinec AL, Parfenova H. (2005a) Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. *Am J Physiol Heart Circ Physiol* 289:H1442-1447

Leffler CW, Balabanova L, Fedinec AL, Waters CM, Parfenova H. (2003) Mechanism of glutamate stimulation of CO production in cerebral microvessels. *Am J Physiol*

Heart Circ Physiol 285:H74-80

Leffler CW, Busija DW, Mirro R, Armstead WM, Beasley DG. (1989) Effects of ischemia on brain blood flow and oxygen consumption of newborn pigs. *Am J Physiol* 257:H1917-1926

Leffler CW, Fedinec AL, Parfenova H, Jaggar JH. (2005b) Permissive contributions of NO and prostacyclin in CO-induced cerebrovascular dilation in piglets. *Am J Physiol Heart Circ Physiol* 289:H432-438

Leffler CW, Nasjletti A, Yu C, Johnson RA, Fedinec AL, Walker N. (1999) Carbon monoxide and cerebral microvascular tone in newborn pigs. *Am J Physiol* 276:H1641-1646

Leffler CW, Parfenova H, Fedinec AL, Basuroy S, Tcheranova D. (2006) Contributions of astrocytes and CO to pial arteriolar dilation to glutamate in newborn pigs. *Am J Physiol Heart Circ Physiol* 291:H2897-2904

Leithner C, Royl G, Offenhauser N, Fuchtemeier M, Kohl-Bareis M, Villringer A, Dirnagl U, Lindauer U. (2010) Pharmacological uncoupling of activation induced increases in CBF and CMRO2. *J Cereb Blood Flow Metab* 30:311-322

Lerma J, Morales M, Vicente MA, Herreras O. (1997) Glutamate receptors of the kainate type and synaptic transmission. *Trends Neurosci* 20:9-12

Lester RA, Quarum ML, Parker JD, Weber E, Jahr CE. (1989) Interaction of 6-cyano-7-nitroquinoxaline-2,3-dione with the N-methyl-D-aspartate receptor-associated glycine binding site. *Mol Pharmacol* 35:565-570

Leung JC, Travis BR, Verlander JW, Sandhu SK, Yang SG, Zea AH, Weiner ID, Silverstein DM. (2002) Expression and developmental regulation of the NMDA receptor subunits in the kidney and cardiovascular system. *Am J Physiol Regul Integr Comp Physiol* 283:R964-971

Li A, Xi Q, Umstot ES, Bellner L, Schwartzman ML, Jaggar JH, Leffler CW. (2008) Astrocyte-derived CO is a diffusible messenger that mediates glutamate-induced cerebral arteriolar dilation by activating smooth muscle Cell KCa channels. *Circ Res* 102:234-241

Li PA, Siesjo BK. (1997) Role of hyperglycaemia-related acidosis in ischaemic brain damage. *Acta Physiol Scand* 161:567-580

Li S, Mallory M, Alford M, Tanaka S, Masliah E. (1997) Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression. *J Neuropathol Exp Neurol* 56:901-911

Lieberman DN, Mody I. (1994) Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. *Nature* 369:235-239

Lindauer U, Leithner C, Kaasch H, Rohrer B, Foddis M, Fuchtemeier M, Offenhauser N, Steinbrink J, Royl G, Kohl-Bareis M, Dirnagl U. (2010) Neurovascular coupling in rat brain operates independent of hemoglobin deoxygenation. *J Cereb Blood Flow Metab* 30:757-768

Liu M, Alkayed NJ. (2005) Hypoxic preconditioning and tolerance via hypoxia inducible factor (HIF) 1alpha-linked induction of P450 2C11 epoxygenase in astrocytes. *J Cereb Blood Flow Metab* 25:939-948

Liu X, Hunter C, Weiss HR, Chi OZ. (2010) Effects of blockade of ionotropic glutamate receptors on blood-brain barrier disruption in focal cerebral ischemia. *Neurol Sci* 31:699-703

Liu X, Li C, Falck JR, Roman RJ, Harder DR, Koehler RC. (2008) Interaction of nitric oxide, 20-HETE, and EETs during functional hyperemia in whisker barrel cortex. *Am J Physiol Heart Circ Physiol* 295:H619-631

Liu X, Li C, Gebremedhin D, Hwang SH, Hammock BD, Falck JR, Roman RJ, Harder DR, Koehler RC. (2011) Epoxyeicosatrienoic acid-dependent cerebral vasodilation evoked by metabotropic glutamate receptor activation in vivo. *Am J Physiol Heart Circ Physiol* 301:H373-381

Lodge D, Davies SN, Jones MG, Millar J, Manallack DT, Ornstein PL, Verberne AJ, Young N, Beart PM. (1988) A comparison between the in vivo and in vitro activity of five potent and competitive NMDA antagonists. *Br J Pharmacol* 95:957-965

Lu YM, Roder JC, Davidow J, Salter MW. (1998) Src activation in the induction of long-

term potentiation in CA1 hippocampal neurons. *Science* 279:1363-1367

Ma J, Meng W, Ayata C, Huang PL, Fishman MC, Moskowitz MA. (1996) L-NNA-sensitive regional cerebral blood flow augmentation during hypercapnia in type III NOS mutant mice. *Am J Physiol* 271:H1717-1719

Magistretti PJ. (2006) Neuron-glia metabolic coupling and plasticity. *J Exp Biol* 209:2304-2311

Magistretti PJ, Pellerin L. (1999) Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. *Philos Trans R Soc Lond B Biol Sci* 354:1155-1163

Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. (1999) Energy on demand. *Science* 283:496-497

Maienschein V, Marxen M, Volknandt W, Zimmermann H. (1999) A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. *Glia* 26:233-244

Malenka RC. (1994) Synaptic plasticity in the hippocampus: LTP and LTD. *Cell* 78:535-538

Malinow R, Schulman H, Tsien RW. (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* 245:862-866

Mangia S, Garreffa G, Bianciardi M, Giove F, Di Salle F, Maraviglia B. (2003) The aerobic brain: lactate decrease at the onset of neural activity. *Neuroscience* 118:7-10

Mangia S, Tkac I, Gruetter R, Van de Moortele PF, Maraviglia B, Ugurbil K. (2007) Sustained neuronal activation raises oxidative metabolism to a new steady-state level: evidence from 1H NMR spectroscopy in the human visual cortex. *J Cereb Blood Flow Metab* 27:1055-1063

Martineau M, Baux G, Mothet JP. (2006) D-serine signalling in the brain: friend and foe. *Trends Neurosci* 29:481-491

Martineau M, Galli T, Baux G, Mothet JP. (2008) Confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission. *Glia* 56:1271-1284

Masino SA, Dunwiddie TV. (1999) Temperature-dependent modulation of excitatory transmission in hippocampal slices is mediated by extracellular adenosine. *J Neurosci* 19:1932-1939

Masliah E, Alford M, Mallory M, Rockenstein E, Moechars D, Van Leuven F. (2000) Abnormal glutamate transport function in mutant amyloid precursor protein transgenic mice. *Exp Neurol* 163:381-387

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI. (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J Neurosci* 24:7821-7828

Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, Wada K. (1995) Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J Neurochem* 65:454-458

Maus M, Marin P, Israel M, Glowinski J, Premont J. (1999) Pyruvate and lactate protect striatal neurons against N-methyl-D-aspartate-induced neurotoxicity. *Eur J Neurosci* 11:3215-3224

Mayhan WG, Patel KP. (1995) Acute effects of glucose on reactivity of cerebral microcirculation: role of activation of protein kinase C. *Am J Physiol* 269:H1297-1302

McCarron JG, Halpern W. (1990) Potassium dilates rat cerebral arteries by two independent mechanisms. *Am J Physiol* 259:H902-908

McCormick DA, Contreras D. (2001) On the cellular and network bases of epileptic seizures. *Annu Rev Physiol* 63:815-846

McDonald JW, Johnston MV. (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Res Brain Res Rev* 15:41-70

McKenna MC. (2007) The glutamate-glutamine cycle is not stoichiometric: fates of

glutamate in brain. *J Neurosci Res* 85:3347-3358

McKenna MC, Hopkins IB, Lindauer SL, Bamford P. (2006) Aspartate aminotransferase in synaptic and nonsynaptic mitochondria: differential effect of compounds that influence transient hetero-enzyme complex (metabolon) formation. *Neurochem Int* 48:629-636

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34:939-944

Meng W, Tobin JR, Busija DW. (1995) Glutamate-induced cerebral vasodilation is mediated by nitric oxide through N-methyl-D-aspartate receptors. *Stroke* 26:857-862; discussion 863

Meno JR, Crum AV, Winn HR. (2001) Effect of adenosine receptor blockade on pial arteriolar dilation during sciatic nerve stimulation. *Am J Physiol Heart Circ Physiol* 281:H2018-2027

Meno JR, Nguyen TS, Jensen EM, Alexander West G, Groysman L, Kung DK, Ngai AC, Britz GW, Winn HR. (2005) Effect of caffeine on cerebral blood flow response to somatosensory stimulation. *J Cereb Blood Flow Metab* 25:775-784

Mentis MJ, Horwitz B, Grady CL, Alexander GE, VanMeter JW, Maisog JM, Pietrini P, Schapiro MB, Rapoport SI. (1996) Visual cortical dysfunction in Alzheimer's disease evaluated with a temporally graded "stress test" during PET. *Am J Psychiatry* 153:32-40

Metea MR, Kofuji P, Newman EA. (2007) Neurovascular coupling is not mediated by potassium siphoning from glial cells. *J Neurosci* 27:2468-2471

Metea MR, Newman EA. (2006) Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. *J Neurosci* 26:2862-2870

Metzger H, Erdmann W, Thews G. (1971) Effect of short periods of hypoxia, hyperoxia, and hypercapnia on brain O 2 supply. *J Appl Physiol* 31:751-759

Miekisiak G, Kulik T, Kusano Y, Kung D, Chen JF, Winn HR. (2008) Cerebral blood

flow response in adenosine 2a receptor knockout mice during transient hypoxic hypoxia. *J Cereb Blood Flow Metab* 28:1656-1664

Mishra A, Hamid A, Newman EA. (2011) Oxygen modulation of neurovascular coupling in the retina. *Proc Natl Acad Sci U S A*

Mishra A, Newman EA. (2010) Inhibition of inducible nitric oxide synthase reverses the loss of functional hyperemia in diabetic retinopathy. *Glia* 58:1996-2004

Miura H, Gutterman DD. (1998) Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P-450 monooxygenase and Ca2+-activated K+ channels. *Circ Res* 83:501-507

Miya K, Inoue R, Takata Y, Abe M, Natsume R, Sakimura K, Hongou K, Miyawaki T, Mori H. (2008) Serine racemase is predominantly localized in neurons in mouse brain. *J Comp Neurol* 510:641-654

Miyata N, Seki T, Tanaka Y, Omura T, Taniguchi K, Doi M, Bandou K, Kametani S, Sato M, Okuyama S, Cambj-Sapunar L, Harder DR, Roman RJ. (2005) Beneficial effects of a new 20-hydroxyeicosatetraenoic acid synthesis inhibitor, TS-011 [N-(3-chloro-4-morpholin-4-yl) phenyl-N'-hydroxyimido formamide], on hemorrhagic and ischemic stroke. *J Pharmacol Exp Ther* 314:77-85

Mogi M, Horiuchi M. (2011) Neurovascular coupling in cognitive impairment associated with diabetes mellitus. *Circ J* 75:1042-1048

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529-540

Morley P, Small DL, Murray CL, Mealing GA, Poulter MO, Durkin JP, Stanimirovic DB. (1998) Evidence that functional glutamate receptors are not expressed on rat or human cerebromicrovascular endothelial cells. *J Cereb Blood Flow Metab* 18:396-406

Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH. (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A* 97:4926-4931

Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G. (2005) Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. *Proc Natl Acad Sci U S A* 102:5606-5611

Mothet JP, Rouaud E, Sinet PM, Potier B, Jouvenceau A, Dutar P, Videau C, Epelbaum J, Billard JM. (2006) A critical role for the glial-derived neuromodulator D-serine in the age-related deficits of cellular mechanisms of learning and memory. *Aging Cell* 5:267-274

Mulkey RM, Endo S, Shenolikar S, Malenka RC. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* 369:486-488

Mulligan SJ, MacVicar BA. (2004) Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. *Nature* 431:195-199

Murugan M, Sivakumar V, Lu J, Ling EA, Kaur C. (2011) Expression of N-methyl D-aspartate receptor subunits in amoeboid microglia mediates production of nitric oxide via NF-kappaB signaling pathway and oligodendrocyte cell death in hypoxic postnatal rats. *Glia* 59:521-539

Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT, Barrow RK, Coyle JT, Snyder SH, Dore S. (2010) Serine racemase deletion protects against cerebral ischemia and excitotoxicity. *J Neurosci* 30:1413-1416

Nedergaard M. (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263:1768-1771

Nelson MT, Patlak JB, Worley JF, Standen NB. (1990) Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol* 259:C3-18

Neuhaus W, Burek M, Djuzenova CS, Thal S, Koepsell H, Roewer N, Forster CY. (2011a) Addition of NMDA-receptor antagonist MK801 during oxygen/glucose deprivation moderately attenuates the up-regulation of glucose uptake after subsequent reoxygenation in brain endothelial cells. *Neurosci Lett*

Neuhaus W, Freidl M, Szkokan P, Berger M, Wirth M, Winkler J, Gabor F, Pifl C, Noe CR. (2011b) Effects of NMDA receptor modulators on a blood-brain barrier in

vitro model. *Brain Res* 1394:49-61

Newman EA. (2001) Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. *J Neurosci* 21:2215-2223

Newman EA, Frambach DA, Odette LL. (1984) Control of extracellular potassium levels by retinal glial cell K+ siphoning. *Science* 225:1174-1175

Ngai AC, Winn HR. (1995) Modulation of cerebral arteriolar diameter by intraluminal flow and pressure. *Circ Res* 77:832-840

Nguyen-Duong H. (2001) Vasorelaxation induced by L-glutamate in porcine coronary arteries. *J Toxicol Environ Health A* 62:643-653

Nicholls DG. (2004) Mitochondrial dysfunction and glutamate excitotoxicity studied in primary neuronal cultures. *Curr Mol Med* 4:149-177

Nicholls DG, Johnson-Cadwell L, Vesce S, Jekabsons M, Yadava N. (2007) Bioenergetics of mitochondria in cultured neurons and their role in glutamate excitotoxicity. *J Neurosci Res* 85:3206-3212

Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. (1997) Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J Neurosci* 17:171-180

Niswender CM, Conn PJ. (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* 50:295-322

Niwa K, Porter VA, Kazama K, Cornfield D, Carlson GA, Iadecola C. (2001) A beta-peptides enhance vasoconstriction in cerebral circulation. *Am J Physiol Heart Circ Physiol* 281:H2417-2424

Niwa K, Younkin L, Ebeling C, Turner SK, Westaway D, Younkin S, Ashe KH, Carlson GA, Iadecola C. (2000) Abeta 1-40-related reduction in functional hyperemia in mouse neocortex during somatosensory activation. *Proc Natl Acad Sci U S A* 97:9735-9740

O'Hara JA, Hou H, Demidenko E, Springett RJ, Khan N, Swartz HM. (2005) Simultaneous measurement of rat brain cortex PtO2 using EPR oximetry and a fluorescence fiber-optic sensor during normoxia and hyperoxia. *Physiol Meas* 26:203-213

Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M. (2009) Uniquely hominid features of adult human astrocytes. *J Neurosci* 29:3276-3287

Oberheim NA, Wang X, Goldman S, Nedergaard M. (2006) Astrocytic complexity distinguishes the human brain. *Trends Neurosci* 29:547-553

Ogata K, Kosaka T. (2002) Structural and quantitative analysis of astrocytes in the mouse hippocampus. *Neuroscience* 113:221-233

Ohashi H, Maruyama T, Higashi-Matsumoto H, Nomoto T, Nishimura S, Takeuchi Y. (2002) A novel binding assay for metabotropic glutamate receptors using [3H] L-quisqualic acid and recombinant receptors. *Z Naturforsch C* 57:348-355

Ohnuma T, Sakai Y, Maeshima H, Hatano T, Hanzawa R, Abe S, Kida S, Shibata N, Suzuki T, Arai H. (2008) Changes in plasma glycine, L-serine, and D-serine levels in patients with schizophrenia as their clinical symptoms improve: results from the Juntendo University Schizophrenia Projects (JUSP). *Prog Neuropsychopharmacol Biol Psychiatry* 32:1905-1912

Palygin O, Lalo U, Pankratov Y. (2011) Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes. *Br J Pharmacol* 163:1755-1766

Palygin O, Lalo U, Verkhratsky A, Pankratov Y. (2010) Ionotropic NMDA and P2X1/5 receptors mediate synaptically induced Ca2+ signalling in cortical astrocytes. *Cell Calcium* 48:225-231

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH. (2006) Glia-derived D-serine controls NMDA receptor activity and synaptic memory. *Cell* 125:775-784

Paoletti P, Ascher P, Neyton J. (1997) High-affinity zinc inhibition of NMDA NR1-

NR2A receptors. *J Neurosci* 17:5711-5725

Parfenova H, Fedinec A, Leffler CW. (2003) Ionotropic glutamate receptors in cerebral microvascular endothelium are functionally linked to heme oxygenase. *J Cereb Blood Flow Metab* 23:190-197

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747

Parpura V, Fang Y, Basarsky T, Jahn R, Haydon PG. (1995) Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. *FEBS Lett* 377:489-492

Pasquier F, Boulogne A, Leys D, Fontaine P. (2006) Diabetes mellitus and dementia. *Diabetes Metab* 32:403-414

Patton AJ, Genever PG, Birch MA, Suva LJ, Skerry TM. (1998) Expression of an N-methyl-D-aspartate-type receptor by human and rat osteoblasts and osteoclasts suggests a novel glutamate signaling pathway in bone. *Bone* 22:645-649

Paulson OB, Newman EA. (1987) Does the release of potassium from astrocyte endfeet regulate cerebral blood flow? *Science* 237:896-898

Pellegri G, Rossier C, Magistretti PJ, Martin JL. (1996) Cloning, localization and induction of mouse brain glycogen synthase. *Brain Res Mol Brain Res* 38:191-199

Pellerin L, Magistretti PJ. (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91:10625-10629

Pellerin L, Pellegri G, Bittar PG, Charnay Y, Bouras C, Martin JL, Stella N, Magistretti PJ. (1998) Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev Neurosci* 20:291-299

Pelligrino DA, Gay RL, 3rd, Baughman VL, Wang Q. (1996) NO synthase inhibition modulates NMDA-induced changes in cerebral blood flow and EEG activity. *Am*

J Physiol 271:H990-995

Pelligrino DA, Vetri F, Xu HL. (2011) Purinergic mechanisms in gliovascular coupling. *Semin Cell Dev Biol* 22:229-236

Pelligrino DA, Wang Q, Koenig HM, Albrecht RF. (1995) Role of nitric oxide, adenosine, N-methyl-D-aspartate receptors, and neuronal activation in hypoxia-induced pial arteriolar dilation in rats. *Brain Res* 704:61-70

Pelligrino DA, Xu HL, Vetri F. (2010) Caffeine and the control of cerebral hemodynamics. *J Alzheimers Dis* 20 Suppl 1:S51-62

Peng X, Carhuapoma JR, Bhardwaj A, Alkayed NJ, Falck JR, Harder DR, Traystman RJ, Koehler RC. (2002) Suppression of cortical functional hyperemia to vibrissal stimulation in the rat by epoxygenase inhibitors. *Am J Physiol Heart Circ Physiol* 283:H2029-2037

Peppiatt CM, Howarth C, Mobbs P, Attwell D. (2006) Bidirectional control of CNS capillary diameter by pericytes. *Nature* 443:700-704

Pereira de Vasconcelos A, Riban V, Wasterlain C, Nehlig A. (2006) Role of endothelial nitric oxide synthase in cerebral blood flow changes during kainate seizures: a genetic approach using knockout mice. *Neurobiol Dis* 23:219-227

Peters S, Koh J, Choi DW. (1987) Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons. *Science* 236:589-593

Phelps CH. (1972) Barbiturate-induced glycogen accumulation in brain. An electron microscopic study. *Brain Res* 39:225-234

Philip S, Armstead WM. (2004) NMDA dilates pial arteries by KATP and Kca channel activation. *Brain Res Bull* 63:127-131

Phillis JW, Ren J, O'Regan MH. (2000) Transporter reversal as a mechanism of glutamate release from the ischemic rat cerebral cortex: studies with DL-threo-beta-benzyloxyaspartate. *Brain Res* 868:105-112

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-

Mathisen J, Seeberg E, Kanner BI. (1992) Cloning and expression of a rat brain L-glutamate transporter. *Nature* 360:464-467

Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G. (2007) Physiological functions of D-amino acid oxidases: from yeast to humans. *Cell Mol Life Sci* 64:1373-1394

Pollegioni L, Sacchi S. (2010) Metabolism of the neuromodulator D-serine. *Cell Mol Life Sci* 67:2387-2404

Poloyac SM, Zhang Y, Bies RR, Kochanek PM, Graham SH. (2006) Protective effect of the 20-HETE inhibitor HET0016 on brain damage after temporary focal ischemia. *J Cereb Blood Flow Metab* 26:1551-1561

Preston SD, Steart PV, Wilkinson A, Nicoll JA, Weller RO. (2003) Capillary and arterial cerebral amyloid angiopathy in Alzheimer's disease: defining the perivascular route for the elimination of amyloid beta from the human brain. *Neuropathol Appl Neurobiol* 29:106-117

Prichard J, Rothman D, Novotny E, Petroff O, Kuwabara T, Avison M, Howseman A, Hanstock C, Shulman R. (1991) Lactate rise detected by 1H NMR in human visual cortex during physiologic stimulation. *Proc Natl Acad Sci U S A* 88:5829-5831

Proper EA, Hoogland G, Kappen SM, Jansen GH, Rensen MG, Schrama LH, van Veelen CW, van Rijen PC, van Nieuwenhuizen O, Gispen WH, de Graan PN. (2002) Distribution of glutamate transporters in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. *Brain* 125:32-43

Pryazhnikov E, Khiroug L. (2008) Sub-micromolar increase in [Ca(2+)](i) triggers delayed exocytosis of ATP in cultured astrocytes. *Glia* 56:38-49

Puro DG. (2007) Physiology and pathobiology of the pericyte-containing retinal microvasculature: new developments. *Microcirculation* 14:1-10

Rana S, Dringen R. (2007) Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. *Neurosci Lett* 415:45-48

Ransom RW, Deschenes NL. (1990) Polyamines regulate glycine interaction with the N-

methyl-D-aspartate receptor. *Synapse* 5:294-298

Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. (1990) Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 101:746-752

Reijerkerk A, Kooij G, van der Pol SM, Leyen T, Lakeman K, van Het Hof B, Vivien D, de Vries HE. (2010) The NR1 subunit of NMDA receptor regulates monocyte transmigration through the brain endothelial cell barrier. *J Neurochem* 113:447-453

Reilly JF, Maher PA, Kumari VG. (1998) Regulation of astrocyte GFAP expression by TGF-beta1 and FGF-2. *Glia* 22:202-210

Renic M, Klaus JA, Omura T, Kawashima N, Onishi M, Miyata N, Koehler RC, Harder DR, Roman RJ. (2009) Effect of 20-HETE inhibition on infarct volume and cerebral blood flow after transient middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 29:629-639

Ribeiro CS, Reis M, Panizzutti R, de Miranda J, Wolosker H. (2002) Glial transport of the neuromodulator D-serine. *Brain Res* 929:202-209

Robinson JS, Fedinec AL, Leffler CW. (2002) Role of carbon monoxide in glutamate receptor-induced dilation of newborn pig pial arterioles. *Am J Physiol Heart Circ Physiol* 282:H2371-2376

Rock DM, MacDonald RL. (1992) Spermine and related polyamines produce a voltage-dependent reduction of N-methyl-D-aspartate receptor single-channel conductance. *Mol Pharmacol* 42:157-164

Rodriguez JJ, Olabarria M, Chvatal A, Verkhratsky A. (2009) Astroglia in dementia and Alzheimer's disease. *Cell Death Differ* 16:378-385

Roman LJ, Martasek P, Miller RT, Harris DE, de La Garza MA, Shea TM, Kim JJ, Masters BS. (2000) The C termini of constitutive nitric-oxide synthases control electron flow through the flavin and heme domains and affect modulation by calmodulin. *J Biol Chem* 275:29225-29232

Rosenberg D, Kartvelishvily E, Shleper M, Klinker CM, Bowser MT, Wolosker H.

(2010) Neuronal release of D-serine: a physiological pathway controlling extracellular D-serine concentration. *Faseb J* 24:2951-2961

Rossi DJ, Brady JD, Mohr C. (2007) Astrocyte metabolism and signaling during brain ischemia. *Nat Neurosci* 10:1377-1386

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13:713-725

Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C. (2002) Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell* 94:457-475

Rouach N, Koulakoff A, Abudara V, Willecke K, Giaume C. (2008) Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science* 322:1551-1555

Roy CS, Sherrington CS. (1890) On the Regulation of the Blood-supply of the Brain. *J Physiol* 11:85-158 117

Rubart M, Patlak JB, Nelson MT. (1996) Ca2+ currents in cerebral artery smooth muscle cells of rat at physiological Ca2+ concentrations. *J Gen Physiol* 107:459-472

Rungger-Brandle E, Dosso AA, Leuenberger PM. (2000) Glial reactivity, an early feature of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 41:1971-1980

Sagar SM, Sharp FR, Swanson RA. (1987) The regional distribution of glycogen in rat brain fixed by microwave irradiation. *Brain Res* 417:172-174

Said SI, Berisha HI, Pakbaz H. (1996) Excitotoxicity in the lung: N-methyl-D-aspartate-induced, nitric oxide-dependent, pulmonary edema is attenuated by vasoactive intestinal peptide and by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci U S A* 93:4688-4692

Salter MG, Fern R. (2005) NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature* 438:1167-1171

Sandor P. (1999) Nervous control of the cerebrovascular system: doubts and facts.

Neurochem Int 35:237-259

Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S. (2007) D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. *EMBO J* 26:4149-4159

Scemes E, Dermietzel R, Spray DC. (1998) Calcium waves between astrocytes from Cx43 knockout mice. *Glia* 24:65-73

Scemes E, Giaume C. (2006) Astrocyte calcium waves: what they are and what they do. *Glia* 54:716-725

Scharfman HE. (2007) The neurobiology of epilepsy. *Curr Neurol Neurosci Rep* 7:348-354

Schell MJ, Molliver ME, Snyder SH. (1995) D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. *Proc Natl Acad Sci U S A* 92:3948-3952

Schild L, Huppelsberg J, Kahlert S, Keilhoff G, Reiser G. (2003) Brain mitochondria are primed by moderate Ca2+ rise upon hypoxia/reoxygenation for functional breakdown and morphological disintegration. *J Biol Chem* 278:25454-25460

Schneider U, Poole RC, Halestrap AP, Grafe P. (1993) Lactate-proton co-transport and its contribution to interstitial acidification during hypoxia in isolated rat spinal roots. *Neuroscience* 53:1153-1162

Schurr A, Miller JJ, Payne RS, Rigor BM. (1999) An increase in lactate output by brain tissue serves to meet the energy needs of glutamate-activated neurons. *J Neurosci* 19:34-39

Schurr A, Payne RS, Miller JJ, Rigor BM. (1997) Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: an in vitro study. *Brain Res* 744:105-111

Schurr A, Payne RS, Miller JJ, Tseng MT, Rigor BM. (2001) Blockade of lactate transport exacerbates delayed neuronal damage in a rat model of cerebral

ischemia. *Brain Res* 895:268-272

Schurr A, West CA, Rigor BM. (1988) Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science* 240:1326-1328

Scimemi A, Fine A, Kullmann DM, Rusakov DA. (2004) NR2B-containing receptors mediate cross talk among hippocampal synapses. *J Neurosci* 24:4767-4777

Sciotti VM, Park TS, Berne RM, Van Wylen DG. (1993) Changes in extracellular adenosine during chemical or electrical brain stimulation. *Brain Res* 613:16-20

Scott GS, Bowman SR, Smith T, Flower RJ, Bolton C. (2007) Glutamate-stimulated peroxynitrite production in a brain-derived endothelial cell line is dependent on N-methyl-D-aspartate (NMDA) receptor activation. *Biochem Pharmacol* 73:228-236

Selkoe DJ, Schenk D. (2003) Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Annu Rev Pharmacol Toxicol* 43:545-584

Senitz D, Reichenbach A, Smith TG, Jr. (1995) Surface complexity of human neocortical astrocytic cells: changes with development, aging, and dementia. *J Hirnforsch* 36:531-537

Shank RP, Bennett GS, Freytag SO, Campbell GL. (1985) Pyruvate carboxylase: an astrocyte-specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Res* 329:364-367

Shao Z, Kamboj A, Anderson CM. (2009) Functional and immunocytochemical characterization of D-serine transporters in cortical neuron and astrocyte cultures. *J Neurosci Res* 87:2520-2530

Sharp CD, Hines, I, Houghton, J, Warren, A, Jackson, T H, Jawahar, A, Nanda, A, Elrod, J W, Long, A, Chi, A, Minagar, A, and Alexander, J S. (2003) Glutamate causes a loss in human cerebral endothelial barrier integrity through activation of NMDA receptor. *Am J Physiol Heart Circ Physiol* 285:H2592–H2598

Sharp CD, Houghton J, Elrod JW, Warren A, Jackson THt, Jawahar A, Nanda A, Minagar A, Alexander JS. (2005) N-methyl-D-aspartate receptor activation in human cerebral endothelium promotes intracellular oxidant stress. *Am J Physiol*

Heart Circ Physiol 288:H1893-1899

Shashidharan P, Wittenberg I, Plaitakis A. (1994) Molecular cloning of human brain glutamate/aspartate transporter II. *Biochim Biophys Acta* 1191:393-396

Sheppard CA, Simpson PB, Sharp AH, Nucifora FC, Ross CA, Lange GD, Russell JT. (1997) Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca2+ release sites that support Ca2+ waves in cultured astrocytes. *J Neurochem* 68:2317-2327

Shepro D, Morel NM. (1993) Pericyte physiology. *FASEB J* 7:1031-1038

Shi Y, Liu X, Gebremedhin D, Falck JR, Harder DR, Koehler RC. (2007) Interaction of mechanisms involving epoxyeicosatrienoic acids, adenosine receptors, and metabotropic glutamate receptors in neurovascular coupling in rat whisker barrel cortex. *J Cereb Blood Flow Metab*

Shigetomi E, Kracun S, Sofroniew MV, Khakh BS. (2010) A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. *Nat Neurosci* 13:759-766

Shimizu H, Watanabe E, Hiyama TY, Nagakura A, Fujikawa A, Okado H, Yanagawa Y, Obata K, Noda M. (2007) Glial Nax channels control lactate signaling to neurons for brain [Na+] sensing. *Neuron* 54:59-72

Shleper M, Kartvelishvily E, Wolosker H. (2005) D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. *J Neurosci* 25:9413-9417

Shoji K, Mariotto S, Ciampa AR, Suzuki H. (2006a) Mutual regulation between serine and nitric oxide metabolism in human glioblastoma cells. *Neurosci Lett* 394:163-167

Shoji K, Mariotto S, Ciampa AR, Suzuki H. (2006b) Regulation of serine racemase activity by D-serine and nitric oxide in human glioblastoma cells. *Neurosci Lett* 392:75-78

Shulman RG, Hyder F, Rothman DL. (2001) Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. *Proc Natl Acad Sci U S A* 98:6417-

6422

Sickmann HM, Walls AB, Schousboe A, Bouman SD, Waagepetersen HS. (2009) Functional significance of brain glycogen in sustaining glutamatergic neurotransmission. *J Neurochem* 109 Suppl 1:80-86

Silver IA, Deas J, Erecinska M. (1997) Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. *Neuroscience* 78:589-601

Simandle SA, Kerr BA, Lacza Z, Eckman DM, Busija DW, Bari F. (2005) Piglet pial arteries respond to N-methyl-D-aspartate in vivo but not in vitro. *Microvasc Res* 70:76-83

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. (2003) Signaling at the gliovascular interface. *J Neurosci* 23:9254-9262

Simpkins AN, Rudic RD, Schreihofer DA, Roy S, Manhiani M, Tsai HJ, Hammock BD, Imig JD. (2009) Soluble epoxide inhibition is protective against cerebral ischemia via vascular and neural protection. *Am J Pathol* 174:2086-2095

Simpson IA, Carruthers A, Vannucci SJ. (2007) Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J Cereb Blood Flow Metab* 27:1766-1791

Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, Savva G, Brayne C, Wharton SB. (2010) Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain. *Neurobiol Aging* 31:578-590

Sontheimer H. (1994) Voltage-dependent ion channels in glial cells. *Glia* 11:156-172

Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF. (2003) D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proc Natl Acad Sci U S A* 100:6789-6794

Storck T, Schulte S, Hofmann K, Stoffel W. (1992) Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. *Proc*

Natl Acad Sci U S A 89:10955-10959

Straub SV, Bonev AD, Wilkerson MK, Nelson MT. (2006) Dynamic inositol trisphosphate-mediated calcium signals within astrocytic endfeet underlie vasodilation of cerebral arterioles. *J Gen Physiol* 128:659-669

Suadicani SO, Brosnan CF, Scemes E. (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. *J Neurosci* 26:1378-1385

Suh M, Ma H, Zhao M, Sharif S, Schwartz TH. (2006) Neurovascular coupling and oximetry during epileptic events. *Mol Neurobiol* 33:181-197

Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, Swanson RA. (2007) Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316,819 ([R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmet hyl)propyl]-1H-indole-2-carboxamide). *J Pharmacol Exp Ther* 321:45-50

Sun CW, Falck JR, Okamoto H, Harder DR, Roman RJ. (2000) Role of cGMP versus 20-HETE in the vasodilator response to nitric oxide in rat cerebral arteries. *Am J Physiol Heart Circ Physiol* 279:H339-350

Sun GY, Xu J, Jensen MD, Yu S, Wood WG, Gonzalez FA, Simonyi A, Sun AY, Weisman GA. (2005) Phospholipase A2 in astrocytes: responses to oxidative stress, inflammation, and G protein-coupled receptor agonists. *Mol Neurobiol* 31:27-41

Sun XL, Hu G. (2010) ATP-sensitive potassium channels: a promising target for protecting neurovascular unit function in stroke. *Clin Exp Pharmacol Physiol* 37:243-252

Swanson RA, Choi DW. (1993) Glial glycogen stores affect neuronal survival during glucose deprivation in vitro. *J Cereb Blood Flow Metab* 13:162-169

Tabernero A, Medina JM, Giaume C. (2006) Glucose metabolism and proliferation in glia: role of astrocytic gap junctions. *J Neurochem* 99:1049-1061

Takai H, Katayama K, Uetsuka K, Nakayama H, Doi K. (2003) Distribution of N-methyl-D-aspartate receptors (NMDARs) in the developing rat brain. *Exp Mol Pathol*

75:89-94

Takano T, Han X, Deane R, Zlokovic B, Nedergaard M. (2007) Two-photon imaging of astrocytic Ca2+ signaling and the microvasculature in experimental mice models of Alzheimer's disease. *Ann N Y Acad Sci* 1097:40-50

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. (2006) Astrocyte-mediated control of cerebral blood flow. *Nat Neurosci* 9:260-267

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 276:1699-1702

Tanaka Y, Omura T, Fukasawa M, Horiuchi N, Miyata N, Minagawa T, Yoshida S, Nakaike S. (2007) Continuous inhibition of 20-HETE synthesis by TS-011 improves neurological and functional outcomes after transient focal cerebral ischemia in rats. *Neurosci Res* 59:475-480

Tang LH, Aizenman E. (1993) Allosteric modulation of the NMDA receptor by dihydrolipoic and lipoic acid in rat cortical neurons in vitro. *Neuron* 11:857-863

Tekkok SB, Brown AM, Westenbroek R, Pellerin L, Ransom BR. (2005) Transfer of glycogen-derived lactate from astrocytes to axons via specific monocarboxylate transporters supports mouse optic nerve activity. *J Neurosci Res* 81:644-652

Thomas T, Thomas G, McLendon C, Sutton T, Mullan M. (1996) beta-Amyloid-mediated vasoactivity and vascular endothelial damage. *Nature* 380:168-171

Tian GF, Azmi H, Takano T, Xu Q, Peng W, Lin J, Oberheim N, Lou N, Wang X, Zielke HR, Kang J, Nedergaard M. (2005) An astrocytic basis of epilepsy. *Nat Med* 11:973-981

Tian R, Vogel P, Lassen NA, Mulvany MJ, Andreasen F, Aalkjaer C. (1995) Role of extracellular and intracellular acidosis for hypercapnia-induced inhibition of tension of isolated rat cerebral arteries. *Circ Res* 76:269-275

Tong XK, Hamel E. (2000) Basal forebrain nitric oxide synthase (NOS)-containing neurons project to microvessels and NOS neurons in the rat neocortex: cellular

basis for cortical blood flow regulation. *Eur J Neurosci* 12:2769-2780

Traynelis SF, Burgess MF, Zheng F, Lyuboslavsky P, Powers JL. (1998) Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. *J Neurosci* 18:6163-6175

Traynelis SF, Hartley M, Heinemann SF. (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science* 268:873-876

Trombley PQ, Shepherd GM. (1996) Differential modulation by zinc and copper of amino acid receptors from rat olfactory bulb neurons. *J Neurophysiol* 76:2536-2546

Tsacopoulos M, Magistretti PJ. (1996) Metabolic coupling between glia and neurons. *J Neurosci* 16:877-885

Udosen IT, Jiang H, Hercule HC, Oyekan AO. (2003) Nitric oxide-epoxygenase interactions and arachidonate-induced dilation of rat renal microvessels. *Am J Physiol Heart Circ Physiol* 285:H2054-2063

Uhlhaas PJ, Singer W. (2006) Neural synchrony in brain disorders: relevance for cognitive dysfunctions and pathophysiology. *Neuron* 52:155-168

Ventura R, Harris KM. (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. *J Neurosci* 19:6897-6906

Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L. (2003) Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron* 37:275-286

Wallraff A, Kohling R, Heinemann U, Theis M, Willecke K, Steinhauser C. (2006) The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. *J Neurosci* 26:5438-5447

Walls AB, Heimburger CM, Bouman SD, Schousboe A, Waagepetersen HS. (2009) Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and

adrenergic agents. *Neuroscience* 158:284-292

Walz W, Mukerji S. (1990) Simulation of aspects of ischemia in cell culture: changes in lactate compartmentation. *Glia* 3:522-528

Wang Y, Bukoski RD. (1999) Use of acute phenolic denervation to show the neuronal dependence of Ca2+-induced relaxation of isolated arteries. *Life Sci* 64:887-894

Ward MM, Jobling AI, Kalloniatis M, Fletcher EL. (2005) Glutamate uptake in retinal glial cells during diabetes. *Diabetologia* 48:351-360

Warkentin S, Passant U. (1997) Functional imaging of the frontal lobes in organic dementia. Regional cerebral blood flow findings in normals, in patients with frontotemporal dementia and in patients with Alzheimer's disease, performing a word fluency test. *Dement Geriatr Cogn Disord* 8:105-109

Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K. (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. *Eur J Neurosci* 10:976-988

Wender R, Brown AM, Fern R, Swanson RA, Farrell K, Ransom BR. (2000) Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J Neurosci* 20:6804-6810

Wendling WW, Chen D, Daniels FB, Monteforte MR, Fischer MB, Harakal C, Carlsson C. (1996) The effects of N-methyl-D-aspartate agonists and antagonists on isolated bovine cerebral arteries. *Anesth Analg* 82:264-268

Wiencken AE, Casagrande VA. (1999) Endothelial nitric oxide synthetase (eNOS) in astrocytes: another source of nitric oxide in neocortex. *Glia* 26:280-290

Wilhelmus MM, Otte-Holler I, van Triel JJ, Veerhuis R, Maat-Schieman ML, Bu G, de Waal RM, Verbeek MM. (2007) Lipoprotein receptor-related protein-1 mediates amyloid-beta-mediated cell death of cerebrovascular cells. *Am J Pathol* 171:1989-1999

Wolburg H, Neuhaus J, Kniesel U, Krauss B, Schmid EM, Ocalan M, Farrell C, Risau W. (1994) Modulation of tight junction structure in blood-brain barrier endothelial

cells. Effects of tissue culture, second messengers and cocultured astrocytes. *J Cell Sci* 107 (Pt 5):1347-1357

Wolff JR, Stuke K, Missler M, Tytko H, Schwarz P, Rohlmann A, Chao TI. (1998) Autocellular coupling by gap junctions in cultured astrocytes: a new view on cellular autoregulation during process formation. *Glia* 24:121-140

Wolosker H, Blackshaw S, Snyder SH. (1999a) Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc Natl Acad Sci U S A* 96:13409-13414

Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO, Jr., Ferris CD, Snyder SH. (1999b) Purification of serine racemase: biosynthesis of the neuromodulator D-serine. *Proc Natl Acad Sci U S A* 96:721-725

Wu L, Cao K, Lu Y, Wang R. (2002) Different mechanisms underlying the stimulation of K(Ca) channels by nitric oxide and carbon monoxide. *J Clin Invest* 110:691-700

Wu SZ, Bodles AM, Porter MM, Griffin WS, Basile AS, Barger SW. (2004) Induction of serine racemase expression and D-serine release from microglia by amyloid beta-peptide. *J Neuroinflammation* 1:2

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM, Sheng M. (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* 385:439-442

Xi Q, Tcheranova D, Basuroy S, Parfenova H, Jaggar JH, Leffler CW. (2011) Glutamate-induced calcium signals stimulate CO production in piglet astrocytes. *Am J Physiol Heart Circ Physiol* 301:H428-433

Xi Q, Umstot E, Zhao G, Narayanan D, Leffler CW, Jaggar JH. (2010) Glutamate regulates Ca2+ signals in smooth muscle cells of newborn piglet brain slice arterioles through astrocyte- and heme oxygenase-dependent mechanisms. *Am J Physiol Heart Circ Physiol* 298:H562-569

Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP. (2004) Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 118:687-698

Yamamoto T, Nishizaki I, Furuya S, Hirabayashi Y, Takahashi K, Okuyama S, Yamamoto H. (2003) Characterization of rapid and high-affinity uptake of L-serine in neurons and astrocytes in primary culture. *FEBS Lett* 548:69-73

Yang SN, Tang YG, Zucker RS. (1999) Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. *J Neurophysiol* 81:781-787

Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S. (2003) Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. *Proc Natl Acad Sci U S A* 100:15194-15199

Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR. (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. *J Neurosci* 23:3588-3596

Yemisci M, Gursoy-Ozdemir Y, Vural A, Can A, Topalkara K, Dalkara T. (2009) Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. *Nat Med* 15:1031-1037

Yu AC, Drejer J, Hertz L, Schousboe A. (1983) Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. *J Neurochem* 41:1484-1487

Zhang W, Koerner IP, Noppens R, Grafe M, Tsai HJ, Morisseau C, Luria A, Hammock BD, Falck JR, Alkayed NJ. (2007) Soluble epoxide hydrolase: a novel therapeutic target in stroke. *J Cereb Blood Flow Metab* 27:1931-1940

Zhang W, Otsuka T, Sugo N, Ardeshiri A, Alhadid YK, Iliff JJ, DeBarber AE, Koop DR, Alkayed NJ. (2008a) Soluble epoxide hydrolase gene deletion is protective against experimental cerebral ischemia. *Stroke* 39:2073-2078

Zhang Y, Pertens E, Janssen LJ. (2005) 8-isoprostaglandin E(2) activates Ca(2+)-dependent K(+) current via cyclic AMP signaling pathway in murine renal artery. *Eur J Pharmacol* 520:22-28

Zhang Z, Gong N, Wang W, Xu L, Xu TL. (2008b) Bell-shaped D-serine actions on hippocampal long-term depression and spatial memory retrieval. *Cereb Cortex* 18:2391-2401

Zheng F, Gingrich MB, Traynelis SF, Conn PJ. (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nat Neurosci* 1:185-

Zhu HJ, Liu GQ. (2004) Glutamate up-regulates P-glycoprotein expression in rat brain microvessel endothelial cells by an NMDA receptor-mediated mechanism. *Life Sci* 75:1313-1322

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G. (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6:43-50