Nitric oxide in astrocyte-neuron signaling

Nianzhen Li
Iowa State University

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Nitric oxide in astrocyte-neuron signaling

by

Nianzhen Li

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular, and Developmental Biology

Program of Study Committee:
Philip Haydon, Major Professor
Michael McCloskey
Alan Myers
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Iowa State University
Ames, Iowa
2002
Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Nianzhen Li

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program
To my parents and my husband

For their unconditional love and support
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<tr>
<td>AMPA</td>
<td>(S)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid</td>
</tr>
<tr>
<td>2-APB</td>
<td>2-aminoethoxy-diphenylborate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine trisphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic ADP-ribose</td>
</tr>
<tr>
<td>Caged NO</td>
<td>caged nitric oxide I (potassium nitrosyl-pentachlororuthenate)</td>
</tr>
<tr>
<td>CCE</td>
<td>capacitative Ca(^{2+}) entry</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAF-2</td>
<td>4,5-diaminofluorescein</td>
</tr>
<tr>
<td>DAF-FM</td>
<td>4-amino-5-methylamino-2',7'-difluorofluorescein</td>
</tr>
<tr>
<td>DAF-FM DA</td>
<td>4-amino-5-methylamino-2',7'-difluorofluorescein diacetate</td>
</tr>
<tr>
<td>D-AP5</td>
<td>D-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino butyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>I\textsubscript{crac}</td>
<td>Ca(^{2+})-release-activated current</td>
</tr>
<tr>
<td>MAP-2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>(N^G)-monomethyl-L-arginine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>D-NMMA</td>
<td>N&lt;sup&gt;G&lt;/sup&gt;-monomethyl-D-arginine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PPADS</td>
<td>pyridoxalphosphate-6-azophenyl-2,4-disulfonic acid, tetrasodium</td>
</tr>
<tr>
<td>PSCs</td>
<td>postsynaptic currents</td>
</tr>
<tr>
<td>PTIO</td>
<td>2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
</tr>
<tr>
<td>SIC</td>
<td>slow inward current</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitrosol-N-acetylpenicillamine</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide attachment protein receptor</td>
</tr>
<tr>
<td>SOC</td>
<td>store-operated Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
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<td>TRP</td>
<td>transient receptor potential</td>
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Astrocytes, a subtype of glial cells, have recently been shown to exhibit Ca\textsuperscript{2+} elevations in response to neurotransmitters. A Ca\textsuperscript{2+} elevation can propagate to adjacent astrocytes as a Ca\textsuperscript{2+} wave, which allows an astrocyte to communicate with its neighbors. Additionally, glutamate can be released from astrocytes via a Ca\textsuperscript{2+}-dependent mechanism, thus modulating neuronal activity and synaptic transmission.

In this dissertation, I investigated the roles of another endogenous signal, nitric oxide (NO), in astrocyte-neuron signaling. First I tested if NO is generated during astrocytic Ca\textsuperscript{2+} signaling by imaging NO in purified murine cortical astrocyte cultures. Physiological concentrations of a natural messenger, ATP, caused a Ca\textsuperscript{2+}-dependent NO production. To test the roles of NO in astrocytic Ca\textsuperscript{2+} signaling, I applied NO to astrocyte cultures via addition of a NO donor, S-nitrosol-N-acetylpenicillamine (SNAP). NO induced an influx of external Ca\textsuperscript{2+}, possibly through store-operated Ca\textsuperscript{2+} channels. The NO-induced Ca\textsuperscript{2+} signaling is cGMP-independent since 8-Br-cGMP, an agonistic analog of cGMP, did not induce a detectable Ca\textsuperscript{2+} change. The consequence of this NO-induced Ca\textsuperscript{2+} influx was assessed by simultaneously monitoring of cytosolic and internal store Ca\textsuperscript{2+} using fluorescent Ca\textsuperscript{2+} indicators x-rhod-1 and mag-fluo-4. Blockade of NO signaling with the NO scavenger PTIO significantly reduced the refilling percentage of internal stores following ATP-induced Ca\textsuperscript{2+} release, suggesting that NO modulates internal store refilling. Furthermore, locally photo-release of NO to a single astrocyte led to a Ca\textsuperscript{2+} elevation in the stimulated astrocyte and a subsequent Ca\textsuperscript{2+} wave to neighbors. Finally, I tested the role of NO in glutamate-mediated astrocyte-neuron signaling by recording the astrocyte-evoked glutamate-dependent
neuronal slow inward current (SIC). Although NO is not required for the SIC, PTIO reduced SIC amplitude, suggesting that NO modulates glutamate release from astrocytes or glutamate receptor sensitivity of neurons.

Taken together, these results demonstrate that NO is actively involved in astrocyte-neuron signaling. NO has been implicated in many nervous system functions including synaptic plasticity and neurotoxicity, but its exact role and functioning mechanisms are still unclear. By investigating the roles of NO in astrocyte-neuron signaling, this study could provide new insights into development, modulation and pathology of the nervous system.
CHAPTER 1. INTRODUCTION

The objective of this dissertation is to investigate the roles of nitric oxide (NO), an endogenous gaseous messenger, in astrocyte physiology and astrocyte-neuron signaling. I conceived the idea of investigating the roles of nitric oxide in astrocyte-neuron signaling and designed the experiments with close discussion with Dr. Philip Haydon. Except for the photolysis experiments, which I collaborated with Dr. Jai-Yoon Sul, I performed all the experiments independently.

Astrocytes: the Unacknowledged Partner

The impetus of this study is based on our recently revised understanding of the physiology of astrocytes and the interactions between astrocytes and neurons.

For many years, astrocytes, a type of neuroglial cell, were considered to be only the supporting cells in the central nervous system. The word “Glia” comes from the Greek word “glue”, and is descriptive of the bridge that astrocytes form between neuronal membrane and the border of the CNS (the blood system or the ventricular walls). The importance of astrocytes in neuronal function is suggested by the fact that they are the most abundant cell type in the mammalian brain. However, early electrophysiological studies revealed that astrocytes do not have action potentials as neurons do, and therefore they were considered as a "quiet" cell type. This oversight of the dynamic nature of astrocytes was predicted by the great neuroanatomist Ramón y Cajal who one hundred years ago wrote in “The neuron and the glial cell” (Cajal, 1899): “This is truly a prejudgment, in that the neuroglial cells in the same manner as the collagenous bundles of the connective tissue act in relation to the
muscular or granular cell. We accept this relationship as though it was a solid established fact that these fibrils form a passive support to simply fill out and bind the tissue in a matrix that is swelled with nutritive substance. Every investigator who wishes to form a rational opinion concerning the activity of neuroglial cells should abandon this manner of thinking which predisposes this judgment.” But the dogma of astrocytes being only subservient to neurons was still dominant until very recently.

Only until the development of fluorescent Ca\(^{2+}\) indicators (Gryniewicz et al., 1985; Minta et al., 1989), were astrocytes shown to be “internally Ca\(^{2+}\) excitable” (Verkhratsky et al., 1998). Astrocytes can respond to various neurochemical, electrical and mechanical stimuli by releasing Ca\(^{2+}\) from internal Ca\(^{2+}\) stores or by influx of extracellular Ca\(^{2+}\); and Ca\(^{2+}\) elevation in one astrocyte can spread as a Ca\(^{2+}\) wave to neighboring astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Dani et al., 1992; Finkbeiner, 1992; Salter and Hicks, 1994; Duffy and MacVicar, 1995; Porter and McCarthy, 1996; Newman and Zahs, 1997; Bečzi et al., 1998; Harris-White et al., 1998; Guthrie et al., 1999; Shelton and McCarthy, 2000). Additionally, Ca\(^{2+}\) elevation is necessary and sufficient for the release of glutamate, the most common excitatory neurotransmitter, from astrocytes (Parpura et al., 1994; Innocenti et al., 2000). This astrocyte-released glutamate can then lead to a delayed Ca\(^{2+}\) elevation in neurons (Charles, 1994; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998a; Bečzi et al., 1998), and modulates neuronal activity (Araque et al., 1998a; Newman and Zahs, 1998; Araque et al., 1999; Parpura and Haydon, 2000) and synaptic transmission (Araque et al., 1998b; Kang et al., 1998).
These exciting findings challenged the old view of astrocytes as being quiet and submissive; instead, the roles of astrocytes as the active partner in a "tripartite synapse" gained more and more appreciation (Araque et al., 1999). However, the mechanisms and physiological and pathological consequences of astrocyte-neuron signaling are far from being understood. The work presented here described new findings that show that nitric oxide (NO) is an endogenous modulator of astrocyte-neuron signaling.

**Nitric Oxide: an Unconventional Neurotransmitter**

NO is a short-lived, endogenously produced gas which acts as a signaling molecule in the body. NO was first discovered as a physiological messenger in vascular smooth muscle relaxation (Furchgott and Zawadzki, 1980; Furchgott et al., 1987; Ignarro et al., 1987; Palmer et al., 1987; Palmer et al., 1988). The concept of a freely-diffusible gaseous molecule being an endogenous messenger was a breakthrough in the research of signal transduction. The pioneering work by Furgott, Ignarro and Murad lead to their award of the 1998 Nobel Prize in physiology and medicine. NO was proposed to be the "endothelium-derived relaxing factor (EDRF)" by Furchgott and Ignarro in 1986, and a variety of biological and pharmacological studies confirmed the role of NO in the cardiovascular system.

Further studies have shown that NO also carries out diverse tasks in various other tissues and organs and affects almost all cellular processes. NO has especially profound roles in the central nervous system. Unlike most neurotransmitters that are packaged in synaptic vesicles and released from specialized nerve endings, NO acts as an unconventional neurotransmitter in that it is membrane permeant and diffuses from its site of production in the absence of any specialized release machinery. Hence NO can signal across greater
distances than the synaptic cleft and in doing so can act as a signal molecule between distant synapses and cells. NO signaling has now been implicated in neuronal development, synaptic transmission, and synaptic plasticity, as well as both neuroprotection and neurotoxicity (Lipton et al., 1994; Schuman and Madison, 1994; Jaffrey and Snyder, 1995; Hoischer, 1997; Crepel, 1998; Hawkins et al., 1998).

Endogenous NO is produced by NO synthases (NOS). There are three isoforms of NOSs, which are named after the tissues in which they were first cloned and characterized: neuronal NOS (nNOS or type 1 NOS), immune or inducible NOS (iNOS or type 2 NOS), and endothelial NOS (eNOS or type 3 NOS) (Bredt and Snyder, 1990; Dawson et al., 1991; Lamas et al., 1992; Sessa et al., 1992; Xie et al., 1992). Generally, nNOS and eNOS are constitutively expressed whereas expression of iNOS is induced during the immune response. All three isoforms of NOS contain a calmodulin-binding site and calmodulin binding can activate the enzyme. But iNOS binds calmodulin with very high affinity so that calmodulin forms a constitutively active subunit of iNOS. Therefore, nNOS and eNOS but not iNOS are activated in a dose-dependent manner by Ca\(^{2+}\) and calmodulin. All three isoforms of NOS have been shown to be expressed in other tissues. For example, all three isoforms have been found in astrocytes (Galea et al., 1992; Bo et al., 1994; Endoh et al., 1994; Galea et al., 1994; Arbones et al., 1996; Kugler and Drenckhahn, 1996; Togashi et al., 1997; Colasanti et al., 1998; Loihl et al., 1999; Wiencken and Casagrande, 1999).

**Nitric Oxide in Astrocytes**

Although many studies on NO in the nervous system have been conducted, there are very few about the roles of astrocyte-derived NO and effects of NO in astrocyte-neuron
signaling. **My hypothesis is that NO is actively involved in astrocyte-neuron signaling.**

The rationale behind this hypothesis is: All three isoforms of NOS have been found in astrocytes *in vitro* and *in vivo*. Because the activity of nNOS and eNOS is Ca^{2+}-dependent, the stimulation-induced Ca^{2+} elevation in astrocytes will likely cause NO to be produced. Once produced, NO would likely act on ryanodine receptors (Xu et al., 1998; Eu et al., 2000; Sun et al., 2001), L-type Ca^{2+} channels (Campbell et al., 1996; Poteser et al., 2001) or store-operated Ca^{2+} channels (Favre et al., 1998; Ma et al., 1999; van Rossum et al., 2000), which are all known to be modulated by S-nitrosylation. NO has been demonstrated in some other cell types to induce Ca^{2+} mobilization by cGMP-G Kinase-dependent activation of ADP-ribosyl cyclase which leads to cyclic ADP ribose (cADPR)-mediated ryanodine receptors activation (Willmott et al., 1995; Willmott et al., 1996; Lee, 1997; Looms et al., 2001). The cGMP-G Kinase can also result in phosphorylation of IP_3 receptors (Guihard et al., 1996; Rooney et al., 1996). NO has also been demonstrated to liberate Ca^{2+} by a cGMP independent pathway (Volk et al., 1997; Watson et al., 1999; Berkels et al., 2000; Loitto et al., 2000). All these findings raise the possibility that NO may also induce Ca^{2+} mobilization in astrocytes, and in turn modulate Ca^{2+}-dependent glutamate release from astrocyte to neuron.

This dissertation examined the Ca^{2+}-dependent formation of NO in astrocytes and the roles of NO in astrocytic Ca^{2+} signaling and astrocyte-neuron interaction. NO is a non-conventional neurotransmitter implicated in many aspects of nervous system functions, but its exact role and functional mechanisms are still unclear. Astrocyte-neuron signaling is a relatively new but very exciting field. Astrocyte signaling and modulation of neuronal
activity could have critical effects on physiological and pathological processes (Araque et al., 2001; Mazzanti et al., 2001).
CHAPTER 2. BACKGROUND

What Is an Astrocyte?

There are four major types of glial cells, which include astrocytes, oligodendrocytes and microglia in the central nervous system and Schwann cells in the peripheral nervous system. Each type of glial cell has different histological characteristics and different functions. Among these, astrocytes are the primary focus of my graduate study.

Astrocytes are of ectoderm origin, and develop from immature cells of the subventricular zone (Levison and Goldman. 1993: Luskin and McDermott. 1994). In cultures of perinatal rat optic nerve cells there are two separate types of glial precursors: a "type 1" astrocyte precursor that generate only type 1 astrocytes and an "O-2A" progenitor can give rise to either oligodendrocytes or "type 2" astrocytes (Raff et al., 1983: Raff, 1989).

Morphologically, astrocytes can be further divided into three subgroups: radial astrocytes, fibrous astrocytes, and protoplasmic astrocytes with transitional forms between these populations. Bergmann glial cells of the cerebellum are an example of radial glial cells. Fibrous astrocytes have a large number of processes in all directions, whereas protoplasmic astrocytes, mainly located in the gray matter, have short ramified and crimped processes.

Astrocytes are the most numerous and diverse population of glia cells. What is the definition of an astrocyte? In fact there is still no definite answer to this question. Expression of intermediate filament protein glial fibrillary acidic protein (GFAP) and S100 have been used as specific markers of astrocytes. However, not all astrocytes express GFAP and the expression of GFAP can vary among astrocytes and during development and particularly pathological conditions (Eng and Lee, 1995). In fact Müller cells in the retina do
not express GFAP under normal conditions, but express GFAP under pathological conditions (). “Reactive astrocytes” is a term used to describe astrocytes after injury, and these cells exhibit a characteristic increase in GFAP level. Cultured astrocytes resemble reactive astrocytes in that they express increased GFAP (Eng and Lee, 1995).

Although astrocytes are a very divergent population in terms of location, shape, and protein expression, most astrocytes share a common characteristic: they have two contact sites. One is the neuronal membrane (synaptic regions in the gray matter and axons in the white matter), and the other is the border of the CNS, either the blood system or the ventricular walls. This can be regarded as the definition of an astrocyte, and closely related to the functions of astrocytes.

**Functions of Astrocytes**

Prior to the discovery of Ca$^{2+}$ signaling in astrocytes, these cells were recognized for their many important functions in supporting neurons. These include:

**Mechanical support:** The word “glia” came from Greek “glue”. Glial cells were looked upon by their discoverer, Rudolph Virchow, as the “glue” (nervenkitt) that holds the neurons together (Virchow, 1846; Somjen, 1988). Astrocytes are the most abundant cells in the brain and form a mechanical scaffold for neurons. One astrocyte makes contacts with many neurons. 57% of synapses are surrounded by astrocyte membranes in the hippocampus (Ventura and Harris, 1999).

**Nutritional support:** Based on the observation that astrocytes are often positioned between neurons and blood vessels, Golgi first speculated that astrocytes could provide nutritional support to neurons (Kuffler and Nicholls, 1966; Somjen, 1988). Later astrocytes
were found to be the only cells in the brain containing a substantial amount of glycogen. During times of glucose deprivation (such as after brain injury), glycogen can be broken down to provide lactate to neurons (Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996; Ransom and Fern, 1997). Thus astrocytes play a critical role by providing nutritional support to neurons. Astrocytes can also release various growth factors which have trophic effects on neurons (Labourdette and Sensenbrenner, 1995; Muller et al., 1995).

**Maintenance of extracellular K⁺ homeostasis:** When neurons are excited, K⁺ can accumulate in the extracellular space. Astrocytes can take up the K⁺ by using metabolic energy and a variety of voltage-gated ion channels (including a depolarization-inhibited K⁺ channel) (Orkand et al., 1966; Largo et al., 1996b). Thus astrocytes regulate extracellular K⁺ concentration and maintain a stable environment for neurons and other glia cells.

**Clearance of extracellular neurotransmitters:** Astrocytes processes enwrap central synapses where a variety of transporters clear the extracellular space of neurotransmitters (e.g. glutamate and GABA) that have been released from synaptic terminals (Kimelberg et al., 1990; Mennerick and Zorumski, 1994; Largo et al., 1996a). The specialized glutamine synthetase that converts glutamate to glutamine is only present in astrocytes. Glutamine can then be taken up by nerve endings for resynthesizing glutamate and GABA.

**Axon guidance:** During CNS development, radial glia cells serve to guide axons to their target destinations (Rakic, 1990). Astrocytes can also inhibit, as well as stimulate, axon outgrowth during CNS development, and in this way provide boundaries to ensure proper axon targeting (Vickland and Silver, 1996).
Recently astrocytes have been found to play more active roles in the nervous system. Theses newly discovered functions include:

**Generation of new cells in adult brain:** The discovery of adult stem cells in the brain was a breakthrough in the field of neural development (Eriksson et al., 1998; Johansson et al., 1999b; Johansson et al., 1999a). Contrary to long-held belief, new brain cells (neurons and glia cells) can be generated in the adult, which provided hope for repair of brain injury and cure of neurodegenerative diseases. Recent findings suggest that astrocytes can serve as stem cells (Barres, 1999; Doetsch et al., 1999).

**Synaptogenesis:** The critical role of astrocytes in brain development was brought to our attention in 1997 when Pfrieger and Barres found that astrocytes can enhance the synaptic efficacy of cultured retinal ganglion cells by 10 fold (Pfrieger and Barres, 1997). In 2001 the same group discovered that astrocytes increased the number of mature functional synapses by 7 fold and that astrocytes are required for the maintenance of mature synapses *in vitro* (Ullian et al., 2001). Later it was demonstrated that glia-derived cholesterol is necessary for the formation of mature synapses in CNS (Mauch et al., 2001). Astrocytes can also selectively enhance the formation of excitatory synapse (Doyle et al., 2001) and N-type Ca\(^{2+}\) current in rat hippocampal cultures (Mazzanti and Haydon, 2001).

**Modulation of neuronal activity and neurotransmission:** While astrocytes have long been considered only as supportive cells in the nervous system, recently they have been shown to directly communicate with neurons and thereby actively modulate neuronal activity and synaptic transmission. Because my work focuses on how NO plays a role in this aspect of astrocyte function, it will be the focus of the reminder of this chapter.
Astrocytes are "Calcium Excitable"

Astrocytes were believed to be "quiet" cells because they do not generate action potentials under normal conditions. Neurons, on the other hand, can generate action potentials and propagate electrical excitability. But ultimately, chemical neurotransmitters are the signals in synapses that control information exchange.

Astrocytes express functional neurotransmitter receptors: One of the most surprising developments in glial research was the discovery that glial cells (including astrocytes) also express functional receptors to a variety of classical neurotransmitters, neuromodulators and hormones previously known to affect neurons. The growing list includes glutamate, ATP, epinephrine, norepinephrine, GABA, acetylcholine, histamine, bradykinin, prostaglandins, endothelin, serotonin, substance P, oxytocin, vasopressin, neuropeptide Y, and complement fragments (Porter and McCarthy, 1997; Verkhratsky et al., 1998). The expression patterns of these receptors are heterogeneous in vitro and in situ (Dave et al., 1991; Shao et al., 1994), and show plasticity depending on development stage and environment factors (Shao and McCarthy, 1993, 1994; Bernstein et al., 1996). Furthermore, it appears that glial cells express a set of receptors similar to those of closely apposed neurons (Verkhratsky and Kettenmann, 1996). These observations led to the following questions: why do glial cells express neurotransmitter receptors? How do glial cells respond to the activation of these receptors?

Astrocytes respond to neurotransmitters with a Ca\(^{2+}\) elevation and intercellular Ca\(^{2+}\) waves: The development of fluorescent Ca\(^{2+}\) indicators together with quantitative imaging techniques provided a great tool in answering these questions (Gryniewicz et al., 1985; Minta et al., 1989). Ca\(^{2+}\) is a ubiquitous second messenger in cells. It triggers new life
at fertilization, controls many developmental processes, controls diverse tasks in
differentiated cells such as gene expression, metabolism, proliferation, contraction, secretion,
and plasticity (Berridge et al., 2000). Because of the many pathways that it initiates, Ca$^{2+}$ is
considered to be one of the most important molecules inside a cell. A breakthrough in glial
physiology came in 1990, when it was discovered that cultured astrocytes can respond to
glutamate by increasing intracellular Ca$^{2+}$ (Cornell-Bell et al., 1990). In addition to a
response to a neuronal glutamate signal, it was shown that astrocytes also communicate with
each other. The rise in Ca$^{2+}$ levels in one astrocyte can trigger similar Ca$^{2+}$ increases in
neighboring astrocytes, and cause a propagating wave of rising Ca$^{2+}$ levels in a network of
astrocytes (Cornell-Bell et al., 1990; Cornell-Bell and Finkbeiner, 1991). Later it was found
that in addition to glutamate, a variety of other chemical, mechanical and electrical stimuli
can also induce Ca$^{2+}$ elevations and Ca$^{2+}$ waves in astrocytes (Charles et al., 1991;
Finkbeiner, 1992; Salter and Hicks, 1994; Duffy and MacVicar, 1995; Bezzi et al., 1998;
Guthrie et al., 1999; Shelton and McCarthy, 2000). The astrocytic Ca$^{2+}$ elevation and Ca$^{2+}$
wave is not an artifact of culture systems, because several studies using brain slices and slice
cultures have now confirmed that this Ca$^{2+}$ signaling pathway is present in astrocytes in situ
(Dani et al., 1992; Duffy and MacVicar, 1995; Porter and McCarthy, 1996; Bezzi et al., 1998;
Harris-White et al., 1998; Sul and Haydon, 2001). Ca$^{2+}$ waves have also been observed in
the intact retina (Newman and Zahs, 1997).

**Sources of Ca$^{2+}$ elevation:** The source of Ca$^{2+}$ for elevations in cytosolic Ca$^{2+}$ can
either be extracellular Ca$^{2+}$ or Ca$^{2+}$ released from internal stores. Neurotransmitters (e.g.
glutamate, ATP)-induced Ca$^{2+}$ signaling in astrocytes mostly utilizes inositol (1, 4, 5)-
trisphosphate (IP$_3$)-sensitive internal stores (Cornell-Bell et al., 1990; Charles et al., 1993;
Salter and Hicks, 1995; Venance et al., 1997). Metabotropic receptors are coupled through Gq-proteins which in turn couple with phospholipase C to generate IP₃. IP₃ activates IP₃ receptors located on the membrane of endoplasmic reticulum (ER) and induces the release of Ca²⁺. Another type of internal store is the ryanodine-sensitive store whose natural modulator is cyclic-ADP ribose (cADPR). Although there were reports supporting the existence of ryanodine-sensitive stores in astrocytes (Langley and Pearce, 1994; Golovina and Blaustein, 1997; Simpson et al., 1998), their existence and participation in astrocytic Ca²⁺ signaling are still controversial (Charles et al., 1993; Duffy and MacVicar, 1994). After release of Ca²⁺ from the internal store, the internal store has to be replenished. Store-operated Ca²⁺ channels (SOCs) mediate Ca²⁺ entry following agonist-induced internal store depletion, a process called “capacitative Ca²⁺ entry (CCE)” (Putney, 1977). CCE is suggested to be mediated by a family of transient receptor potential (TRP) channel gene products (Harteneck et al., 2000; Clapham et al., 2001) which were first cloned in Drosophila (Birnbaumer et al., 1996; Zhu et al., 1996). Electrophysiologically, the flow of Ca²⁺ through SOCs is defined as a Ca²⁺-release-activated current (Icrac) and has been well characterized in some cell types like mast cells (Lewis and Cahalan, 1989; Hoth and Penner, 1993), although very few studies on SOCs in astrocytes exist (Wu et al., 1999; Jung et al., 2000; Pizzo et al., 2001; Lo et al., 2002). Astrocytes also express L-type voltage-gated Ca²⁺ channels (MacVicar and Tse, 1988; Eriksson et al., 1993) and Ca²⁺-permeant AMPA receptors (Condorelli et al., 1993; Fan et al., 1999; Iino et al., 2001; Seifert and Steinhauser, 2001) which mediate an influx of extracellular Ca²⁺. Although an astrocyte can respond to a variety of stimuli with an elevation of cytosolic Ca²⁺, Ca²⁺ signaling exhibits the flexibility to ensure the stimulus-specific response.
**Mechanisms for Ca\(^{2+}\) wave propagation:** A Ca\(^{2+}\) elevation in one astrocyte can spread as an intercellular Ca\(^{2+}\) wave into neighboring astrocytes, both in vitro and in situ, which provides a means of information transfer between astrocytes. Both gap junctions and ATP are very important in the propagation of Ca\(^{2+}\) waves. The mechanism for propagation of Ca\(^{2+}\) waves was first thought to be IP\(_3\) traveling through gap-junctions, because astrocytes are tightly coupled with each other by gap junctions and some non-specific gap junction blockers could inhibit Ca\(^{2+}\) waves (Finkbeiner, 1992; Sneyd et al., 1994; Sneyd et al., 1995; Giaume and McCarthy, 1996; Venance et al., 1997; Leybaert et al., 1998). In addition, uncaging IP\(_3\) can cause a smaller Ca\(^{2+}\) wave in astrocytes (Leybaert et al., 1998). But later it was found that Ca\(^{2+}\) waves can propagate to astrocytes across cell-free areas, which indicates the existence of an diffusible extracellular messenger in mediating Ca\(^{2+}\) waves (Hassinger et al., 1996). This extracellular messenger was confirmed to be ATP in later studies (Guthrie et al., 1999). Pharmacologically blocking the extracellular ATP pathway by degrading ATP or by inhibiting purinergic receptors either reduced or abolished intercellular Ca\(^{2+}\) waves (Guthrie et al., 1999). Luciferin-luciferase chemiluminescence assays demonstrated the generation of ATP during Ca\(^{2+}\) waves and suggested that release of ATP is Ca\(^{2+}\)-independent (Wang et al., 2000). ATP was also found to be critical in mediating Ca\(^{2+}\) waves in astrocytes and Müller cells in the retina (Newman, 2001). Recently it was found in the hippocampal slice that ATP can facilitate the glutamate-evoked Ca\(^{2+}\) waves in astrocytes (Sul and Haydon, 2001). It appears that the propagation of the Ca\(^{2+}\) wave is a fine-tuned process and requires both gap junctions and extracellular messengers. Short-range Ca\(^{2+}\) signaling can be caused by IP\(_3\) diffusing to neighboring cells through gap junctions. Longer-range Ca\(^{2+}\) signaling
requires the release of ATP, which causes the regenerative production of IP$_3$ and further release of ATP from neighboring astrocytes (Haydon, 2001).

Since astrocytes are capable of responding to a variety of stimuli with an increase of intracellular Ca$^{2+}$ and are also capable of propagating Ca$^{2+}$ waves to neighboring cells, the classical view of astrocytes being "unexcitable" should be replaced by the concept that astrocytes are "calcium excitable" (Verkhratsky et al., 1998).

**Ca$^{2+}$-dependent Glutamate Release from Astrocytes Can Signal Back to Neurons**

Astrocytes can respond to signals from neurons, and also communicate with other astrocytes, but can they also signal back to neurons? The answer is yes.

**Astrocytes can release glutamate via a Ca$^{2+}$-dependent pathway:** In 1994, Parpura et al demonstrated that glutamate can be released from cultured astrocytes, leading to a rise in intracellular Ca$^{2+}$ in adjacent neurons (Parpura et al., 1994). Ca$^{2+}$ is both necessary and sufficient to evoke the release of glutamate from astrocytes (Parpura et al., 1994; Araque et al., 1998a). Astrocytic release of glutamate is SNARE protein-dependent, suggesting that it is mediated by exocytosis (Araque et al., 2000; Pasti et al., 2001). Astrocytes express a set of proteins normally associated with vesicle release and synaptic transmission: blocking the function of these proteins by injection of clostridial toxins into astrocytes (Araque et al., 2000; Pasti et al., 2001) or by selective expression of the synaptobrevin II SNARE domain in astrocytes (Zhang et al., 2001) can block the Ca$^{2+}$-dependent glutamate release. The release of glutamate during Ca$^{2+}$ waves has been visualized in cortical astrocyte cultures with a glutamate dehydrogenase-based enzyme-linked assay (Innocenti et al., 2000). Evidence also
suggests that astrocytes can release glutamate in brain slices (Pasti et al., 1997; Bezzi et al., 1998).

Glutamate released from astrocytes leads to Ca\textsuperscript{2+} elevations in adjacent neurons: Using a variety of stimuli, several groups have reported that a Ca\textsuperscript{2+} elevation in astrocytes leads to a Ca\textsuperscript{2+} elevation in adjacent neurons, both in culture (Charles, 1994; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995; Araque et al., 1998a) and in situ (Pasti et al., 1997; Bezzi et al., 1998). One group has shown that gap junction blockers can attenuate this astrocyte-neuron signaling (Nedergaard, 1994), but several other groups provided evidence that signaling is mediated by glutamate released from astrocytes acting on NMDA and non-NMDA receptors (Parpura et al., 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998a; Araque et al., 1998b; Bezzi et al., 1998).

Glutamate released from astrocytes can modulate neuronal electrical activity: Astrocytes evoke an NMDA and non-NMDA receptor-dependent slow inward current (SIC) in hippocampal neurons (Araque et al., 1998a; Parpura and Haydon, 2000). In current clamp mode, a subthreshold depolarization elicited by injecting an outward current can become superthreshold by summation with the astrocyte-induced slow depolarization (Araque et al., 1999). In the retina eyecup, changes in the light-evoked spike activity of neurons within the ganglion cell layer occurred when the glial Ca\textsuperscript{3+} wave reached the neuron (Newman and Zahs, 1998). The mechanism of the modulation is suggested to be the activation of inhibitory interneurons which are themselves stimulated by glutamate released from glia cells.

Glutamate released from astrocytes can modulate synaptic transmission: Astrocytes caused an NMDA receptor-dependent increase in the frequency of spontaneous miniature PSCs (Araque et al., 1998b) and a metabotropic glutamate receptor-dependent
inhibition of evoked PSCs in cultured hippocampal neuron (Araque et al., 1998a). In the hippocampal slice, repetitive firing of an interneuron decreased the probability of synaptic failures in spike-evoked inhibitory postsynaptic currents (unitary IPSCs) in CA1 pyramidal neurons. Astrocytes were suggested to be a necessary intermediary in this activity-dependent modulation of inhibitory synapses because direct stimulation of astrocytes, or application of the GABA_B-receptor agonist baclofen, potentiated miniature inhibitory postsynaptic currents (mIPSCs). Furthermore, the astrocyte-induced synaptic augmentation was blocked by the NMDA receptor antagonist D-AP5, which implies that glutamate released from astrocytes is responsible for the synaptic modulation (Kang et al., 1998).

Astrocytes make intimate contacts with synapses (Ventura and Harris, 1999). The interactions of neurons and astrocytes are bi-directional: astrocytes can respond to synaptically-released chemical transmitters with a Ca^{2+} increase; Ca^{2+} increase in astrocytes evokes glutamate release from astrocytes and this astrocyte-released glutamate can then modulate neuronal activity and synaptic transmission. Thus astrocytes are not passive members of CNS, instead the classical synapse can now be viewed as a "tripartite synapse" including pre- and post- synaptic terminals and also the astrocyte as an active partner (Araque et al., 1999).

It is worth noting that in addition to glutamate, astrocytes can also release other neurotransmitters and neuromodulators, which may also participate in astrocyte-neuron signaling. ATP acts as an excitatory neurotransmitter in addition to its function of mediating glial Ca^{2+} waves. D-serine, an endogenous modulator of NMDA receptors, can also be released from astrocytes (Schell et al., 1995; Wolosker et al., 1999; Mothet et al., 2000). Nitric oxide is also a diffusible chemical signal which can be released from astrocytes.
Nitric Oxide is an Endogenous Gaseous Second Messenger

Before the discovery of nitric oxide as the endogenous "endothelium-derived relaxing factor (EDRF)" in 1986 (Furchgott and Zawadzki, 1980; Furchgott et al., 1987; Ignarro et al., 1987; Palmer et al., 1987; Palmer et al., 1988), nitric oxide was only known as a toxic gas in the air. The discovery that a gas, especially a potentially toxic gas, can be an endogenous second messenger in the body was a breakthrough in signal transduction research. The importance was highlighted when the 1998 Nobel Prize in physiology or medicine was awarded to Furchgott, Ignarro and Murad for their pioneering work on nitric oxide. Today it is well recognized that nitric oxide (NO) is a short-lived, endogenously produced gas which freely diffuses across cell membranes, acts as a signaling molecule and regulates a vast number of biological processes.

**Formation of Nitric Oxide:** Endogenous NO is produced by Nitric Oxide Synthases (NOSs). They catalyze oxidation of L-arginine to NO and L-citrulline in the presence of NADPH and O₂, requiring FAD, FMN, heme, Ca²⁺, calmodulin and 6(R)-tretra-hydro-L-biopterin (BH₄) as cofactors. Three isoforms of the NOS enzyme, each the product of a unique gene, have been identified and characterized. They are named after the tissues in which they were first cloned and characterized: neuronal NOS (nNOS or type 1 NOS), immune or inducible NOS (iNOS or type 2 NOS), and endothelial NOS (eNOS or type 3 NOS) (Bredt and Snyder, 1990; Dawson et al., 1991; Lamas et al., 1992; Sessa et al., 1992; Xie et al., 1992), but all three isoforms of NOSs have been shown to be expressed in other tissues. For example, all three isoforms have been found in astrocytes (Galea et al., 1992; Bo et al., 1994; Endoh et al., 1994; Galea et al., 1994; Arbones et al., 1996; Kugler and
The human enzymes share 51-57% homology at the amino acid level. Generally, nNOS and eNOS are constitutively expressed whereas expression of iNOS is induced during the immune response. All three isoforms of NOSs contain a calmodulin-binding site such that calmodulin binding can activate the enzyme. But iNOS binds calmodulin with very high affinity so that calmodulin constitutively activates iNOS. Therefore, activities of nNOS and eNOS but not iNOS are Ca$^{2+}$ and calmodulin concentration-dependent (Griffith and Stuehr, 1995; Gross and Wolin, 1995).

**A new tool to image NO formation:** Only after the recent development of NO-specific fluorescence indicators, the real-time imaging of NO production became possible. The DAF family of NO sensitive fluorescent indicators was first developed by Kojima and collaborators (Kojima et al., 1998). These compounds are based on fluorescein but with the addition of aromatic vicinal diamine groups, they are essentially non-fluorescent until they react with NO in the presence of dioxygen to form a fluorescent benzotriazole. The diacetate form of DAF dyes are cell-permeant thus passively diffuses across cell membranes. Once inside cells, the acetate groups are cleaved by intracellular endogenous esterases. The fluorescence quantum yield of DAF dyes dramatically increases after reacting with NO (160-fold increase for DAF-FM, from ~0.005 to 0.81; figure 1). DAF-2 was the most successful fluorescence indicator of NO because it is highly selective to NO versus other reactive oxygen species and can detect NO levels as low as 5 nM. Later the same group developed DAF-FM (Kojima et al., 1999), a new fluorescent NO indicator, which has several advantages over DAF-2, such as improved sensitivity (NO detection limit: 3 nM), improved photostability, and pH-insensitivity.
**Targets of Nitric Oxide Action:** To understand the diversity of biological targets of NO, it is essential to understand that the cellular effects of NO can result from not only NO itself but an array of NO-related species in different redox-states such as N\textsubscript{2}O, NH\textsubscript{2}OH, NO\textsuperscript{-}, NO\textsuperscript{+}, NO\textsubscript{2}\textsuperscript{-}, N\textsubscript{2}O\textsubscript{3}\textsuperscript{-}, OONO\textsuperscript{-}. Thus the term "NO" is used in a generic or collective sense.

NO-stimulated soluble Guanylyl Cyclase(sGC)-cyclic GMP(cGMP) pathway was first demonstrated in the vascular system where it is of significance in controlling vessel dilation (Arnold et al., 1977; Rapoport et al., 1983; Bredt and Snyder, 1989). Soluble GC is a heme protein and because NO can interact with transition metals (Fe in this case) it can activate sGC and stimulate the cGMP-protein kinase G pathway.

The NO-cGMP pathway is just a small part of the NO story. Recently with the development of new techniques to detect nitrosylated proteins (Jaffrey et al., 2001; Jaffrey and Snyder, 2001), it has been established that NO can directly nitrosylate the regulatory thiol group of numerous proteins, including NMDA receptors (Lipton et al., 1993; Choi et al., 2000; Jaffrey et al., 2001), ryanodine receptors (Xu et al., 1998; Eu et al., 2000), L-type Ca\textsuperscript{2+} channels (Campbell et al., 1996; Poteser et al., 2001), store-operated Ca\textsuperscript{2+} channels (Favre et al., 1998; Ma et al., 1999; van Rossum et al., 2000), P21\textsuperscript{ras} (Lander et al., 1997), and many other proteins (Stamler et al., 2001). The majority of these proteins are regulated by S-nitrosylation of a single critical cysteine residue (Lander et al., 1997; Broillet, 2000; Choi et al., 2000; Hess et al., 2001; Sun et al., 2001). S-nitrosylation is emerging as a specific and fundamental post-translational protein modification, potentially as important as O-phosphorylation (Davis et al., 2001; Lane et al., 2001; Stamler et al., 2001).
NO can also induce nitrosation, oxidation or nitration of proteins through its interaction with either O$_2$ or O$_2$~. For example, numerous reactive nitrogen species and their CO$_2$~ or Cl~ adducts can oxidize, nitrate, or deaminate genomic DNA, resulting in strand breaking and mutations (Davis et al., 2001).

**Roles of Nitric Oxide:** To date NO has been shown to play a role in many biological processes including apoptosis, inflammation, penile erection, kidney function, diabetes, oxidative stress and aging (Burnett et al., 1992; Bredt and Snyder, 1994; Gross and Wolin, 1995; Nicotera et al., 1995; Lipton, 1996; Stamler and Meissner, 2001). The impetus of the present study is based on the finding that NO has profound roles in central nervous system. Under normal conditions, NO acts as a neurotransmitter. Unlike most neurotransmitters that are packaged in synaptic vesicles and released from specialized nerve endings, NO is an unconventional transmitter. NO is membrane permeant and diffuses from its site of production in the absence of any specialized release machinery. NO can modulate NMDA receptors by S-nitrosylation. Several different NO donors have been shown to either reduce (Manzoni et al., 1992; Omerovic et al., 1994) or enhance (Gbadegesin et al., 1999) NMDA currents. NO also interacts with n-secl-syntaxin/SNAP-25 complex, thus promoting synaptic vesicle docking (Meffert et al., 1996). Although there are some contradictory results, evidence suggests that NO plays important roles in synaptic plasticity (Bohme et al., 1993; Lipton et al., 1994; Schuman and Madison, 1994; Jaffrey and Snyder, 1995; Holscher, 1997; Crepel, 1998; Hawkins et al., 1998). NO inhibitors blocked hippocampal long-term potentiation and cerebellar long-term depression, both of which are models for learning and memory (Crepel and Jaillard, 1990; Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Bohme et al., 1993). NOS knockout and knockdown animals also showed
deficiencies in learning and memory (O'Dell et al., 1994; Linden et al., 1995; Kantor et al., 1996; Son et al., 1996). NO has neuroprotective effects, but excess NO production can lead to neurotoxicity, which may account for neural damage in vascular stroke and AIDS dementia (Lipton et al., 1993; Gross and Wolin, 1995; Jaffrey and Snyder, 1995; Heales et al., 1997).

**Nitric Oxide in Astrocytes**

Although there have been many studies concerning NO in the nervous system, few have focused on the roles of NO in astrocyte-neuron signaling. All three NOS isoforms are expressed in astrocytes (Murphy, 2000). Astrocytes in culture were observed to form NO-dependent cGMP in a Ca\(^{2+}\)-dependent manner in response to bradykinin, noradrenalin, glutamate receptor agonists (Agullo et al., 1995; Baltrons and Garcia, 1997), or endothelin (Saadoun and Garcia, 1999) in cultures. NO has been measured directly in response to adenosine receptor activation (Janigro et al., 1996). A recent study by Willmott et al. suggested that NO might induce astrocytic Ca\(^{2+}\) signaling via cGMP-G Kinase activation of ryanodine receptors (Willmott et al., 2000). However, they used bolus applications of aqueous NO and NO donors onto a mixed culture of astrocytes and neurons, and thus were unable to differentiate astrocyte-derived NO from neuron-derived NO. Nor could they differentiate between the direct effects of NO on astrocytes and secondary effects due to NO effects on neurons. After partial results of our work had been presented in two conference meetings, another group using cerebellar slices reported that stimulation of parallel fibers on the cerebellar cortex triggered a transient Ca\(^{2+}\) increase in Bergmann glial cells which could be blocked by NOS inhibitors and mimicked by NO donors (Matyash et al., 2001). Their
results suggest that Bergmann glia cells can sense neuronal-released NO and might be involved in NO-dependent cerebellar long-term depression.

In summary, astrocytes are no longer considered as only the quiet supportive cells in the CNS: they are Ca^{2+} excitable, and Ca^{2+} dependent-glutamate release from astrocytes can modulate neuronal activity and synaptic transmission, which could have critical effects on physiological processes such as learning and memory, and also pathological processes such as epilepsy and stroke. In addition to Ca^{2+} and glutamate, nitric oxide, a diffusible neurotransmitter which is implicated in many aspects of nervous system activities such as synaptic plasticity and neurotoxicity, might also be an important signal in astrocyte-neuron signaling, but its roles are largely undefined. Investigating the roles of nitric oxide in astrocyte-neuron signaling could provide significant new insights into development, modulation and pathology of the nervous system.
Figure 1. DAF-FM is a fluorescent indicator of nitric oxide. The diagram summarizes the mechanism of DAF-FM as a fluorescent indicator of nitric oxide. Cells are first loaded with non-fluorescent DAF-FM Diacetate. This diacetate form is cell membrane permeant. Once DAF-FM DA enters the cell, endogenous esterases then cleave the diacetate group, leaving weakly-fluorescent DAF-FM. When DAF-FM reacts with nitric oxide in the presence of oxygen, the newly formed benzotriazole derivative of DAF-FM (DAF-FM T) is highly fluorescent with an excitation/emission maxima of 495/515nm. The formation of DAF-FM T is essentially irreversible.
CHAPTER 3. MATERIALS AND METHODS

Purified Cortical Astrocyte Cultures

The majority of the studies were performed using purified cortical astrocyte cultures. Post-natal 0-2 day old mice were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ). After the brain was removed and placed into Ca\(^{2+}\)/Mg\(^{2+}\)-free Earle's balanced salt solution (EBSS, pH 7.35; Gibco, Carlsbad, CA), cortices were dissected and incubated for 1 hr at 37 °C in EBSS containing papain (20 U/ml), HEPES (10 mM), L-cysteine (0.2 mg/ml), glucose (20 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Tissue was washed once with fresh EBSS and then placed in EBSS containing HEPES (10 mM) and trypsin inhibitor (10 mg/ml) for 5 minutes to stop further digestion. After being rinsed, cortices were mechanically dissociated in culture medium by triturating through sterile serological glass pipettes. Culture medium consisted of minimum essential medium (Earle's salts, phenol-free; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and containing glucose (40 mM), NaHCO\(_3\) (14 mM), L-glutamine (2 mM), pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), pH 7.35. Cells were plated into 25 cm Falcon culture flasks (BD Biosciences, Bedford, MA) and grown to confluence at 37°C in a humidified 5% CO\(_2\)/95% air atmosphere. Cells were fed every 3-4 days by exchanging 50% of the medium with fresh medium. After 7-14 days the flasks were shaken twice on a horizontal orbital shaker at 260 rpm, first for 1.5 hr, and then, after replacement with cold medium, again for 18 hr. The remaining adherent cells were enzymatically detached with trypsin (0.1%), then pelleted (800 g, 10 min), resuspended in culture medium, and plated onto poly-L-Lysine (1 mg/ml, MW 40,000-100,000) coated
glass coverslips. The cells were used in experiments after 1-4 days by which time they had grown to confluence. All animal procedures were approved by the Iowa State University LAR committee and in accordance with PHS guideline on the use of vertebrate animal in research.

**Primary Cultures of Mixed Hippocampal Neurons and Astrocytes**

The roles of nitric oxide on glutamate-mediated astrocyte-neuron signaling were studied using primary cultures of mixed hippocampal neurons and astrocytes. Post-natal 0-2 day old mice were anesthetized with halothane. After the brain was removed and placed into Ca^{2+}/Mg^{2+}-free EBSS, hippocampi were dissected and incubated for 1 hr at 37 °C in EBSS containing papain (20 U/ml), HEPES (10 mM), L-cysteine (0.2 mg/ml), glucose (20 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Tissue was washed once with fresh EBSS and then placed in EBSS containing HEPES (10 mM) and trypsin inhibitor (10 mg/ml) for 5 minutes to stop further digestion. After being rinsed, hippocampi were mechanically dissociated in culture medium by triturating through a sterile serological glass pipette. Culture medium consisted of Earle’s minimum essential medium supplemented with 5% FBS and containing serum extender (mito+, 0.1%; Collaborative Biomedical Products, Bedford, MA), glucose (25 mM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were plated onto poly-L-Lysine (1 mg/ml, MW 40,000-100,000) coated glass coverslips. Mitotic inhibitor (5-fluoro-2'-deoxyuridine and uridine, 5 μM) was added after 3 days in culture. Cells were fed once a week by exchanging 30% of the medium with fresh medium and used after 8-18 days in culture.
Nitric Oxide Measurement

Relative changes in cytosolic NO concentration in astrocytes were monitored using the fluorescent NO probe DAF-FM. Cells were loaded with 10 μM of DAF-FM DA (Molecular Probes, Eugene, OR) for 45 minutes at room temperature (20-23 °C) in normal external saline containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose, and 6 mM sucrose (pH 7.35), and then de-acetylated for 45 minutes.

Calcium Measurement

Ca²⁺ levels in astrocytes were imaged by fluorescence microscopy. Unless otherwise specified, cultures were incubated at room temperature for 45 minutes in the presence of fluo-3 AM (10 μg/ml; Molecular Probes, Eugene, CA) and de-esterified for 45 minutes.

Simultaneous Measurement of Cytosolic and Internal Store Ca²⁺

In some experiments where we simultaneously monitored cytosolic and internal store Ca²⁺ levels, purified cortical astrocyte cultures were first loaded with mag-fluo-4 AM (10 μg/ml; Molecular Probes) at 37 °C for 45 minutes, then washed and loaded with x-Rhod-1 AM (2.5 μg/ml; Molecular Probes) at room temperature for 45 minutes, and then allowed to de-esterify for 45 minutes before imaging.

Image Acquisition and Processing

In single-dye imaging experiments, coverslips containing dye loaded cells were visualized using a cooled digital camera (ORCA; Hamamatsu, Hamamatsu City, Japan) attached to a Nikon 200 inverted microscope or a Noran Odyssey confocal microscope. For
experiments using either fluo-3 or DAF-FM. light from a xenon arc lamp (100 W) was filtered at 480 nm (480DF10; Omega Optical, Brattleboro, VT) and delivered to the sample through a 20× objective. Fluorescent emission was collected through a dichroic mirror (510DRLP; Omega Optical) and filtered with a 515EFLP filter (Omega Optical). Time-lapse images were acquired by either Automation software (Prairie Technologies, Middleton, WI) or Metamorph software (Universal Imaging Corp., West Chester, PA). Frame acquisition interval was 3s.

For two-wavelength imaging experiments, dye-loaded cells were excited with 488 nm and 568 nm laser lines, and fluorescence was imaged in two channels with a confocal microscope (Prairie Technologies) using Confocal-v1.47 software (Prairie Technologies). A 488/568 nm dual dichroic mirror was used as the primary dichroic, which can reflect both 488 nm and 568 nm excitation light. A 550DRLP dichroic mirror was used between channel 1 and 2. Emission filters for channel 1 and 2 were 610/75BP and 525/50 BP respectively.

All imaging experiments were performed at room temperature. We used an imaging chamber with a 150 µl volume. Normal external saline as described in Nitric Oxide Measurement was continuously perfused at a rate of 1~2 ml/min unless otherwise specified.

For quantitative studies, the temporal dynamics in fluorescence were expressed as background-subtracted dF/F₀ (%), where F₀ represents the fluorescence level of the optical field before cell stimulation, and dF represents the change in fluorescence occurring upon stimulation of the cell. Statistical differences were established using the Student's t-test at P<0.05 (*), P<0.01 (**) and P<0.001 (***)*. Data are expressed as mean ± SEM.
Photolysis of Caged Nitric Oxide

In some experiments flash photolysis was used to provide a fast localized NO stimulus. Caged-NO (potassium nitrosyl-pentachlororuthenate, 1 mM; Molecular Probes) was included in the external saline. We used a Noran Odyssey confocal upright microscope which contains a wide-field fluorescence illumination pathway together with an optical pathway for laser excitation for photolysis. A pulsed Nitrogen laser (337 nm, 3 ns pulse duration) launched into a UV transmitting optical fiber was used for photolysis excitation. A second 635 nm laser was coupled into the same optical fiber to allow the positioning of the UV beam. The illumination spot on the sample was approximately 6 μm in diameter. Stepping motors were used to move the position of the fiber in the image plane. Positioning and shuttering of the laser were all controlled by computer software.

Electrophysiology

Whole-cell patch clamp recordings were made from neurons using patch-pipettes made from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL), with a DC resistance in the range of 5-10 MΩ. Internal solution contained (in mM): 140 K-gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, and 10 HEPES, pH 7.35. External bath solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 10 glucose, and 6 sucrose, pH 7.35. The membrane potential was held at -70mV. The bath was constantly perfused with fresh external saline at a slow rate (~0.5 ml/min) throughout the recording. All experiments were performed at room temperature (20-23 °C). Data were sampled and analyzed with an Axopatch-1C amplifier and pClamp software (Axon Instruments, Union City, CA). Currents were filtered at 1-2 kHz and sampled above 1 kHz. The morphological
identification of neurons was confirmed electrophysiologically by their ability to generate TTX-sensitive Na⁺-mediated action potentials and by the presence of fast synaptic currents. Confluent astrocytes, ~25-150 μm from the soma of the neuron recorded from in patch clamp recordings, were stimulated mechanically using glass micropipettes filled with external saline. The incidence of astrocyte-induced slow inward currents (SICs) was defined as the proportion of responses relative to the total number of astrocytes on each experimental day. Therefore, for this variable, n values correspond to the number of experiments, whereas for the amplitude of the SICs, n represents the number of cells examined. Statistical differences were established using the Student's t-test at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) . Data are expressed as mean ± SEM.

**Immunocytochemistry**

The purity of astrocytes in the purified cortical astrocyte cultures was confirmed by immunocytochemistry. Co-cultures of cortical astrocytes and neurons were used as a positive control for neurons and astrocytes. Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.3) at room temperature for 30 minutes, and then rinsed with PBS and permeabilized with triton X-100 (0.25% in PBS) for 10 min. The cultures were then incubated in PBS containing 5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN₃ for 30 min to block non-specific binding. Cultures were incubated with mouse anti-Glial Fibrillary Acidic Protein (GFAP) monoclonal antibody (Sigma) and rabbit anti-Microtubule-Associated Protein 2 (MAP2) polyclonal antibody (Chemicon International, Temecula, CA)) for 12 hours at 4 °C. Primary antibodies were removed by washing with PBS containing 5% normal goat serum, 0.25% Triton X-100 and
0.02% Na\textsubscript{3}N, and then incubated with fluorescent dye-labeled secondary antibodies (Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti-rabbit IgG; Molecular Probes) for 2 hours. The coverslips were then mounted in n-propyl gallate in glycerol onto glass microscope slides, and imaged with a laser scanning confocal microscope.

**Chemicals**

Caged nitric oxide, DAF-FM AM, fluo-3 AM, mag-fluo-4 AM, x-Rhod-1 AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) AM and S-nitrosol-N-acetylpenicillamine (SNAP) were purchased from Molecular Probes: 2-aminoethoxydiphenylborate (2-APB), N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) and N\textsuperscript{G}-monomethyl-D-arginine (D-NMMA) were from CalBiochem; all other chemicals were from Sigma unless otherwise specified.
CHAPTER 4. RESULTS

For studying the roles of nitric oxide in astrocyte-neuron signaling, it's essential to differentiate the astrocytic-originated nitric oxide from neuronally-derived nitric oxide and study the direct effect of nitric oxide on astrocytes. The purified murine cortical astrocyte cultures used in this study provided a good system to specifically study astrocytic nitric oxide production and direct effects of nitric oxide on astrocytes. After the purification procedure as stated in Material and Method, the cultures consisted predominately of astrocytes. Morphologically astrocytes can be distinguished from neurons which generally have a more rounded cell body, bear longer processes, and appear much brighter under a phase-contrast microscope. Immuno-staining with antibodies against the astrocyte specific marker glial fibrillary acid protein (GFAP) and the neuron specific marker microtubule-associated protein 2 (MAP-2) confirmed that the ratio of astrocytes to neurons in the cultures were greater than 99:1 (Figure 2).

ATP Induces Nitric Oxide Production in Astrocytes

ATP is a critical natural extracellular messenger involved in Ca^{2+} signaling in astrocytes. It induces an astrocytic Ca^{2+} elevation, and mediates the propagation of Ca^{2+} waves between astrocytes (Guthrie et al., 1999). Activities of nNOS and eNOS are known to be Ca^{2+} dependent (Griffith and Stuehr, 1995; Gross and Wolin, 1995). Therefore, I tested if ATP can induce NO production using the new NO-sensitive fluorescence indicator DAF-FM. Astrocytes were loaded with DAF-FM as described in Methods. Application of 10 µM ATP induced a small but reliable increase of DAF-FM fluorescence (Figure 3). Control
experiments confirmed that the increase of DAF-FM fluorescence was due to NO production, since both the NO scavenger PTIO (100 μM, 5 min) (Figure 4A) and the NOS inhibitor L-NMMA (300 μM, 30 min), but not its inactive analog D-NMMA (300 nM, 30 min), blocked the ATP-induced DAF-FM fluorescence increase (Figure 3B, 4B).

In experiments using PTIO, the same cells were first stimulated with ATP, then after washing with normal saline for 10 minutes, perfused with PTIO for 5 minutes and stimulated again with ATP in the presence of PTIO. After washing for another 15 minutes, the same cells were stimulated a third time with ATP. The first and third ATP applications were used as internal controls for each cell. When NO was scavenged by PTIO, the ATP-induced DAF-FM fluorescence increase was significantly reduced (student's t-test, p < 0.001). The effect of PTIO is reversible. The average change in fluorescence intensity of DAF-FM in response to the three ATP applications was 3.15 ± 0.09%, 0.76 ± 0.09% (ATP in PTIO), and 2.97 ± 0.07%, respectively (n = 171 cells, 4 experiments).

When NOS activity was inhibited by L-NMMA, the average ATP-induced change in fluorescence of DAF-FM was significantly reduced to 0.11 ± 0.05% (n = 244; p < 0.001), compared to 1.99 ± 0.10% (n = 188) in control conditions (Figure 4B). Parallel experiments demonstrated that L-NMMA had no effect on Ca^{2+} signaling (Figure 3D). But D-NMMA, the inactive analog of L-NMMA did not have such effect (DAF-FM dF/F_0: 1.28 ± 0.09%; n = 197: no significant difference from control condition), which confirmed the specificity of NOS inhibition by L-NMMA.

The time course of the ATP-induced Ca^{2+} elevation was different with that of the DAF-FM fluorescence increase. In response to ATP, intracellular Ca^{2+} rose to a peak level within a few seconds and then gradually returned to the baseline, whereas DAF-FM
fluorescence increased to its peak more slowly in the presence of ATP. However, the peak of the first derivative of the change in DAF-FM fluorescence, i.e. the maximal rate of DAF-FM fluorescence increase, occurred at the same time with the Ca\(^{2+}\) peak (Figure 3C), suggesting that new NO is instantly (within 3 sec) generated during Ca\(^{2+}\) signaling, and that the rate of NO generation is correlated with the magnitude of Ca\(^{2+}\) elevation. This is the first evidence showing an endogenous Ca\(^{2+}\) mobilizing agent in its physiological concentration is able to quickly induce NO production, which may have profound implications in astrocyte-neuron signaling.

**Ca\(^{2+}\) is Necessary for the ATP-induced NO Production**

Since the activity of NOSs are Ca\(^{2+}\)-dependent, and ATP induces a Ca\(^{2+}\) elevation in astrocytes, it is logical to test whether a Ca\(^{2+}\) elevation is required for the ATP-induced NO production. In order to test this hypothesis, I chelated the cytosolic Ca\(^{2+}\) by incubating astrocytes in BAPTA AM (30 μM, 45 min). In the presence of BAPTA, ATP no longer induced an increase in DAF-FM fluorescence (DAF-FM dF/dF\(_{0}\): 0.03 ± 0.03% in BAPTA (n = 240) vs. 2.97 ± 0.08% in control (n = 174), 4 experiments each; p < 0.001; Figure 4D). Separate experiments of imaging with the Ca\(^{2+}\) indicator fluo-3 confirmed that BAPTA blocked the Ca\(^{2+}\) increase caused by ATP (fluo-3 dF/dF\(_{0}\): 14.0 ± 0.3% in BAPTA (n = 242) vs. 487.8 ± 7.1% in control (n = 314), 3 experiments each; p < 0.001). These results demonstrate that an increase of internal Ca\(^{2+}\) is necessary for the ATP-induced NO production.

In addition, when astrocytes were treated with PPADS (50 μM, 5 min), a P\(_{2}\) receptor antagonist, the ATP-induced NO production was also inhibited. The three ATP application
protocol was also used with the first and third ATP applications being administrated in normal saline whereas the second ATP application was carried out after PPADS treatment. The average DAF-FM dF/F₀ values after the three ATP applications were: 5.83 ± 0.37%, 1.25 ± 0.07% and 5.99 ± 0.40% (n = 150, 4 experiments), respectively (Figure 4C). The effectiveness of PPADS in blocking astrocytic Ca²⁺ elevation was confirmed with Ca²⁺ imaging experiments, in which PPADS reversibly blocked ATP-induced cytosolic Ca²⁺ elevation in astrocytes (data not shown). This is consistent with a P₂ receptor-mediated Ca²⁺ elevation stimulating NO production.

**Nitric Oxide Induces a Ca²⁺ Influx into Astrocytes**

The observation that nitric oxide can be produced in astrocytes via a Ca²⁺-dependent mechanism led to a logical question: what are the roles of nitric oxide in astrocytes? Since Ca²⁺ is a critical second messenger in cells, Ca²⁺ excitability is an essential characteristic of astrocytes, and Ca²⁺ waves represent the communication pathway between astrocytes, I determined whether nitric oxide can modulate Ca²⁺ signaling in astrocytes. Nitric oxide has been shown to modulate Ca²⁺ signaling in other cell types via a cGMP-dependent pathway or direct nitrosylation of proteins (Willmott et al., 1995; Guihard et al., 1996; Rooney et al., 1996; Willmott et al., 1996; Lee, 1997; Volk et al., 1997; Berkels et al., 2000; Loitto et al., 2000; Looms et al., 2001), but its roles in astrocytes are largely unknown.

In order to test the effects of NO on astrocyte Ca²⁺ signaling, I first used NO donors, which are compounds which can spontaneously release NO. I applied the NO donor S-nitrosol-N-acetylpenicillamine (SNAP) to astrocytes and asked if NO can induce a Ca²⁺ elevation in astrocytes. Figure 5A shows that perfusion with 100 μM SNAP increased Ca²⁺
levels in the majority of astrocytes. The increase of Ca\(^{2+}\) by SNAP was reversibly blocked by addition of the NO scavenger PTIO (100 \(\mu\)M; \(n = 114\) cells, 3 experiments; \(p < 0.001\); Figure 5B), which confirmed this Ca\(^{2+}\) increase is due to the action of NO, not to SNAP itself or other byproducts.

While the NO donor SNAP can induce a Ca\(^{2+}\) increase in astrocytes, the cGMP pathway agonist 8-Br-cGMP (up to 3 mM) did not induce any detectable change in fluo-3 fluorescence (\(n = 4\) experiments; data not shown), suggesting that the cGMP pathway is not involved in the NO-induced Ca\(^{2+}\) signaling in astrocytes.

Interestingly, when 100 \(\mu\)M SNAP was applied to the same cells in zero-Ca\(^{2+}\) external saline, it no longer induced an increase of intracellular Ca\(^{2+}\); however when reapplied in the presence of external Ca\(^{2+}\), SNAP could again induce cytosolic Ca\(^{2+}\) elevations (Figure 5C and D), suggesting that NO stimulates a Ca\(^{2+}\) influx pathway in astrocytes.

Cadmium (Cd\(^{2+}\), a non-selective Ca\(^{2+}\) channel blocker, 100 \(\mu\)M) and 2-aminoethoxydiphenylboratae (2-APB, an IP\(_3\) receptor antagonist and capacitative Ca\(^{2+}\) entry blocker, 75 \(\mu\)M) also reversibly blocked SNAP-induced Ca\(^{2+}\) elevations (Figure 6). Cd\(^{2+}\) reduced the average fluo-3 dF/F\(_0\) by ATP to 2.9 \(\pm\) 0.5%, compared with 70.6 \(\pm\) 3.4% (first ATP application) and 82.7 \(\pm\) 2.5% (third ATP application after washing out Cd\(^{2+}\)) of the same cells in the normal saline (\(n = 205\) cells, 3 experiments). The average fluo-3 dF/F\(_0\) induced by ATP in 2-APB treated cells was 2.9 \(\pm\) 0.5%, compared with responses of 138.7 \(\pm\) 5.2% (first ATP application) and 112.9 \(\pm\) 4.6% (third ATP application) in normal saline and after washing out 2-APB (\(n = 96\) cells, 3 experiments). In conclusion, these results show that NO raises astrocytic Ca\(^{2+}\) by inducing an influx of extracellular Ca\(^{2+}\), and possibly by activating a capacitative Ca\(^{2+}\) entry pathway (\textit{store-operated Ca}\(^{2+}\) \textit{channels}).
Nitric Oxide Modulates the Refilling of Internal Ca\textsuperscript{2+} Stores.

It has been shown that many neurotransmitters (ATP, glutamate, norepinephrine) induce an elevation of intracellular Ca\textsuperscript{2+} in astrocytes by causing IP\textsubscript{3}-mediated release of Ca\textsuperscript{2+} from internal Ca\textsuperscript{2+} stores (Cornell-Bell et al., 1990; Charles et al., 1993; Salter and Hicks, 1995; Venance et al., 1997), but the mechanism of refilling these internal Ca\textsuperscript{2+} stores is largely undetermined. Capacitative Ca\textsuperscript{2+} entry, or store depletion–induced store refilling, has been intensively investigated in several other cell types (Putney, 1977; Lewis and Cahalan, 1989; Hoth and Penner, 1993; Clapham, 1995; Zhang and McCloskey, 1995; Birnbaumer et al., 1996; Parekh and Penner, 1997; Putney and McKay, 1999; van Rossum et al., 2000; Clapham et al., 2001), but has not been widely studied in astrocytes. Since an ATP-induced Ca\textsuperscript{2+} elevation can induce NO production in astrocytes, and NO can induce external Ca\textsuperscript{2+} influx, it is possible that NO production is responsible for refilling internal Ca\textsuperscript{2+} stores.

Blockade of NO signaling reduces Ca\textsuperscript{2+} responses to subsequent stimulations of ATP: The magnitude of the increase in cytosolic Ca\textsuperscript{2+} induced by ATP is correlated with the availability of Ca\textsuperscript{2+} from internal stores. Thus if nitric oxide modulates the refilling of internal Ca\textsuperscript{2+} stores, then blocking NO accumulation with the NO scavenger PTIO could reduce the Ca\textsuperscript{2+} response to subsequent applications of ATP. Consequently I applied ATP three times to astrocytes with the first application as an internal control. Then I applied the NO scavenger PTIO during the second and third application of ATP to determine if blockade of NO signaling during ATP application led to a reduction in subsequent ATP-induced Ca\textsuperscript{2+} response.
Figure 7A shows the average change in fluorescence of astrocytes in response to the three applications of ATP. In the first group of cells the amplitude of three Ca\(^{2+}\) responses is shown in control condition as a comparison base to show that ATP is able to reliably increase cytosolic Ca\(^{2+}\) levels. In the second group, maximal depletion of internal store was promoted by perfusion in zero-Ca\(^{2+}\) external saline together with 1 mM EGTA during the second and third application of ATP. This control and zero-Ca\(^{2+}\) EGTA group can be considered as the extremes of maximal and minimal refilling of internal stores against which NO treatment can be compared. Treatment with PTIO did not immediately affect the amplitude of ATP-induced Ca\(^{2+}\) signal. However, by the third ATP stimulus, the mobilization of Ca\(^{2+}\) was modestly reduced in amplitude. This result suggests that PTIO reduced the refilling of internal stores following the ATP-induced Ca\(^{2+}\) release. The magnitude of average normalized Ca\(^{2+}\) responses is presented in Figure 7B, where the increase in fluo-3 fluorescence to the first ATP application is normalized to 100% in each cell. PTIO significantly reduced the response to the third ATP application from 87.3 ± 0.9% in normal conditions to 78.3 ± 0.8% (n = 3 experiments each. 176 and 259 cells for control and PTIO respectively; p < 0.001). Note that response of a cell to subsequent ATP application was always slightly smaller in magnitude, probably resulting from the desensitization of ATP receptors. Thus the responses in the PTIO-treated group are compared in parallel to control conditions.

**Simultaneous monitoring of cytosolic and internal store Ca\(^{2+}\):** To further test for a role for NO signaling in regulating the Ca\(^{2+}\) levels of internal stores, I directly imaged relative Ca\(^{2+}\) levels of internal stores using a low-Ca\(^{2+}\) affinity fluorescent indicator mag-fluo-4 (Ex/Em maxima: 493/517nm). When cells were incubated with mag-fluo-4 AM at 37
\[ ^\circ C \text{, loading of this dye into intracellular compartments was facilitated. Because the } K_d,_{Ca} \text{ of mag-fluo-4 is } 22 \mu M \text{ and Ca}^{2+}\text{ levels in stores are up to about } 200 \mu M \text{ (Golovina and Blaustein, 2000) whereas cytosolic Ca}^{2+}\text{ concentration is about } 100 \text{ nM, this indicator preferentially reports store Ca}^{2+}\text{ levels. After loaded with mag-fluo-4, astrocytes were then loaded with a high affinity Ca}^{2+}\text{ indicator x-Rhod-1 (Ex/Em maxima: } 580/602 \text{ nm; } K_d,_{Ca} = 700 \text{ nM) at room temperature. Since the } K_d,_{Ca} \text{ of this indicator is in the operating range of Ca}^{2+}\text{ in the cytosol, it preferentially reports cytosolic Ca}^{2+}\text{ levels. As illustrated in Figure 8A, using two laser lines for excitation and carefully-selected filter sets, I could spectrally separate the two fluorescent signals from mag-fluo-4 and x-rhod-1, and monitor in real-time the cytosolic and internal store Ca}^{2+}\text{ simultaneously.}

Two examples of astrocytes co-loaded with mag-fluo-4 and x-rhod-1 viewed with a 60x objective are shown in Figure 8B. Note that x-rhod-1 staining is more homogenous, while mag-fluo-4 staining shows clear punctate patterns in the periphery of cells and devoid of fluorescence in nuclear regions (reflecting the cytoplasmic), which is consistent with x-rhod-1 and mag-fluo-4 loading into cytosol and internal stores respectively. To test whether these indicators do indeed report the Ca}^{2+}\text{ levels in the two different compartments, I applied thapsigargin, an irreversible ER Ca}^{2+}\text{-ATPase inhibitor. In the presence of thapsigargin, Ca}^{2+}\text{ leaks from ER internal stores, causing cytosolic Ca}^{2+}\text{ to increase, and causing internal stores to become gradually depleted. As shown in Figure 9A, thapsigargin reduced mag-fluo-4 fluorescence, and increased x-rhod-1 fluorescence at the same time (} n = 4 \text{ experiments). The results confirm that we were simultaneously monitoring cytosolic and internal store (mostly ER) Ca}^{2+}\text{ with x-rhod-1 and mag-fluo-4. Although mag-fluo-4 might be loading into other} \]
stores and organelles, we will refer to the mag-fluo-4 fluorescence as an indicator of internal Ca\textsuperscript{2+} stores.

ATP is a neurotransmitter which is known to induce Ca\textsuperscript{2+} release from the ER via the IP\textsubscript{3} pathway. Therefore, to further confirm the validity of the two-indicator approach in reporting changes in cytosolic and internal store Ca\textsuperscript{2+}, I applied ATP to astrocytes. In response to ATP, x-rhod-1 fluorescence exhibited a biphasic increase with a short latency peak followed by a plateau, consistent with the known biphasic cytosolic Ca\textsuperscript{2+} increase to ATP. Simultaneous with the increase in cytosolic Ca\textsuperscript{2+} was a reduction of the fluorescence of mag-fluo-4, in agreement with the reduction of internal store Ca\textsuperscript{2+} caused by Ca\textsuperscript{2+} release from stores (Figure 9B left). The same cells were then treated with 30 µM BAPTA AM for 20 minutes so that cytosolic Ca\textsuperscript{2+} would be chelated without affecting the release and refilling of store Ca\textsuperscript{2+}. The increase in x-rhod-1 fluorescence was blocked, but the reduction of mag-fluo-4 fluorescence was still the same (Figure 9B right). These results are all consistent with x-rhod-1 and mag-fluo-4 respectively monitoring cytosolic and internal store Ca\textsuperscript{2+}.

Occasionally a small transient increase in mag-fluo-4 fluorescence was observed in response to ATP. This small increase was abolished after cells were treated with BAPTA (n = 3 experiments; Figure 9B), suggesting that this small increase resulted from highly-elevated cytosolic Ca\textsuperscript{2+} induced by ATP. Thus sometimes a small amount of mag-fluo-4 might be present in cytosol at resting Ca\textsuperscript{2+} levels (~100 nM), the fluorescence from cytosolic mag-fluo-4 only consists a negligible fraction of total mag-fluo-4 fluorescence (remember K\textsubscript{d, ca} of mag-fluo-4 is 22 µM), but when the cytosolic Ca\textsuperscript{2+} level is highly elevated by stimulation (up to 1-2 µM), the total mag-fluo-4 fluorescence can be mildly contaminated
with cytosolic Ca$^{2+}$. Since I’m mainly interested in the magnitude of store depletion and refilling after this transient, mag-fluo-4 can still be used as a good indicator of internal store Ca$^{2+}$.

Further evidence supporting the notion that these two fluorescent indicators preferentially report cytosolic and internal store Ca$^{2+}$ came from experiments in which I stimulated Ca$^{2+}$ release from internal stores by application of ATP in the presence of zero-Ca$^{2+}$ EGTA external saline (Figure 9C right graph). When ATP (20 μM) was applied to cells in the presence of zero-Ca$^{2+}$ EGTA, x-rhod-1 fluorescence increased, but this increase lacked an elevated plateau phase; and mag-fluo-4 fluorescence decreased in a larger magnitude than in Ca$^{2+}$-containing saline (Figure 9C left graph). After washout of ATP, mag-fluo-4 fluorescence did not recover until Ca$^{2+}$ was added back into the saline, consistent with the elimination of Ca$^{2+}$ influx and refilling of internal store by zero-Ca$^{2+}$ EGTA.

Taken together, these results support the notion that the two indicators, x-rhod-1 and mag-fluo-4, preferentially report changes in cytosolic and internal store Ca$^{2+}$ respectively, and that they can be used for investigating the role of NO in the regulation of Ca$^{2+}$ store refilling.

**PTIO reduced the refilling percentage of internal stores:** To probe the role of NO in store refilling, I directly visualized ATP-induced store depletion and subsequent store refilling and asked whether NO modulates store refilling. Figure 10 demonstrates the results from one such experiment using ATP. Both the images and the trace show the ATP (20 μM, 60 sec)-induced cytosolic Ca$^{2+}$ elevation resulting from release of Ca$^{2+}$ from internal stores. Note that the internal stores were able to be fully refilled 2 minutes after removal of ATP (Figure 10B, data point d).
The effect of nitric oxide on the refilling of internal stores was then studied by blockade of NO signaling with the NO scavenger PTIO. Figure 11A and 11C shows that when cells were pretreated with PTIO (100 μM, 10 min), internal stores were no longer able to fully refill, as mag-fluo-4 fluorescence did not recover to the pre-stimulation fluorescence intensity. If I define the refilling percentage of internal stores as the percent fluorescence recovery: in the presence of PTIO, Ca\(^{2+}\) of internal stores only refilled to 71.2 ± 5.0 % after 2 minutes after removal of ATP, as opposed to 104.5 ± 7.7% in control conditions (n = 9 experiments; p < 0.01; Figure 11B). This finding directly supports the hypothesis that nitric oxide modulates the refilling of internal stores.

**Nitric Oxide Induces a Ca\(^{2+}\) Wave between Astrocytes**

Intercellular Ca\(^{2+}\) waves provide an important mechanism for astrocytes to communicate with each other. Ca\(^{2+}\) waves were first discovered in culture and later confirmed in situ and shown to be induced by a variety of stimuli including natural neuro-messengers (Cornell-Bell et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Dani et al., 1992; Hassinger et al., 1996; Newman and Zahs, 1997; Harris-White et al., 1998; Guthrie et al., 1999; Sul and Haydon, 2001). We asked whether NO can induce a Ca\(^{2+}\) wave between astrocytes by using a localized NO source. Caged nitric oxide I (potassium nitrosyl-pentachlororuthenate) that releases nitric oxide upon UV illumination was used. Our photolysis instrumentation provided an effective, fast and localized NO source to directly stimulate a single astrocyte. The instrumentation set-up is illustrated in Figure 12A. The illumination spot on the sample is approximately 6 μm in diameter, which is smaller than a single astrocyte.
Photolysis (1 UV pulse, 3 ns) of caged-NO (1 mM) first induced a Ca\textsuperscript{2+} elevation in the directly stimulated astrocyte (fluor-3 \( \Delta F/F_0 \): 691.4 ± 260.2%; \( n = 6 \); Figure 12B). The increase in intracellular Ca\textsuperscript{2+} was not due to UV exposure, because when the same UV pulse was delivered to astrocytes in the absence of the caged compound, astrocytes didn't show a Ca\textsuperscript{2+} elevation (fluor-3 \( \Delta F/F_0 \): 1.8 ± 1.2%; \( n = 4 \); Figure 12C). In another set of control experiments, the NO scavenger PTIO (100 \( \mu \)M) prevented flash photolysis of caged NO from inducing a Ca\textsuperscript{2+} elevation (Figure 12C), confirming that flash photolysis normally induces a Ca\textsuperscript{2+} signal as a result of the liberation of NO.

Photo-release of nitric oxide not only induced a Ca\textsuperscript{2+} elevation in the directly stimulated astrocyte, but also initiated a propagating intercellular Ca\textsuperscript{2+} wave into the neighbors. Figure 13A shows an example of photoliberated NO-induced Ca\textsuperscript{2+} waves. The magnitude of Ca\textsuperscript{2+} rise, the rate of Ca\textsuperscript{2+} rise, and the propagation rate of NO-induced Ca\textsuperscript{2+} waves are all similar to those induced by mechanical stimulation. To determine whether the Ca\textsuperscript{2+} increase in the neighboring cells resulted from regenerative Ca\textsuperscript{2+} waves rather than due to diffusion of photo-released NO, we uncaged NO in a cell free area, adjacent to a cell. When we photo-released NO as close as 18 \( \mu \)m away from an astrocyte, we did not detect a Ca\textsuperscript{2+} response in this cell (Figure 13B). Thus when we photo-released NO onto an astrocyte and induced a Ca\textsuperscript{2+} wave which propagated for at least 50 \( \mu \)m (Figure 13A), this wave resulted from intrinsic properties of those cells rather than the diffusion of NO from the source of photo-release.
Nitric Oxide Does Not Mediate the Propagation of Ca\(^{2+}\) Waves

Since NO can be produced when the Ca\(^{2+}\) level is increased, as seen by DAF-FM imaging (Figure 3), the newly synthesized NO might be initiating the Ca\(^{2+}\) elevation in neighboring cells and in turn causing regenerative Ca\(^{2+}\) and NO production. To ask whether newly synthesized NO is required for the Ca\(^{2+}\) wave, we incubated cells in the NOS inhibitor L-NMMA (300 μM, 30 min). Flash photolysis of NO in the presence of L-NMMA still induced a similar Ca\(^{2+}\) wave (Figure 13C), indicating that NO synthesis is not necessary for the propagation of the NO-initiated Ca\(^{2+}\) wave. Consistent with this data, neither the NO scavenger PTIO (100 μM, 5 min) nor the NOS inhibitor L-NMMA (300 μM, 30 min) affected a mechanical stimulus-induced Ca\(^{2+}\) wave (Figure 14). Both the percentage of astrocytes involved in the Ca\(^{2+}\) wave and the average increase in Ca\(^{2+}\) were unchanged with the blockade of NO signaling, suggesting that NO is not required for the propagation of the mechanical stimulation-induced astrocyte Ca\(^{2+}\) wave.

Nitric Oxide Modulates Glutamate-mediated Astrocyte-Neuron Signaling

Previously our laboratory has shown that when an astrocyte is mechanically stimulated, a slow-inward current (SIC) is evoked in adjacent neurons, which results from the Ca\(^{2+}\)-dependent release of glutamate from astrocytes (Araque et al., 1998a). I have asked whether NO modulates this SIC.

Figure 15 shows that although the slow inward current can still be evoked when cells were treated with the NO scavenger PTIO and the NOS inhibitor L-NMMA, the average amplitude of SIC was smaller in PTIO though not L-NMMA-treated cells compared to the control condition (Figure 15C). These results demonstrate that while nitric oxide is neither
required for the Ca\(^{2+}\) wave nor the SIC, the NO scavenger PTIO significantly reduced the amplitude of the SIC, suggesting that nitric oxide modulates either the glutamate release pathway or glutamate receptor sensitivity in neurons. However, because this is a small effect, it was not investigated further.
**Figure 2.** Immunocytochemistry confirmed that >99% of the cells in the purified astrocytes cultures are astrocytes. Cultures were labeled with antibodies against the astrocyte-specific protein GFAP (left image) and the neuron-specific protein MAP-2 (right image). Both images are taken from the same area.
Figure 3. ATP induces NO production in astrocytes. (A) An example fluorescence image of purified astrocytes loaded with NO sensitive dye DAF-FM. (B) Typical traces of 10 μM ATP-induced increase of DAF-FM fluorescence in control conditions (solid line) and after treatment with the NOS inhibitor L-NMMA (300 μM, 30 minutes; dashed line). The data points are averages of all cells in the imaging field in one experiment. Error bars are S.E.M.s. (C) The plot of first derivative (over time) of ATP-induced DAF-FM fluorescence change, showing the rate of DAF-FM fluorescence increase. (D) Ca^{2+} elevation by 10 μM ATP as imaged with Ca^{2+} indicator fluo-3. Data in (D) is from a separate experiment than that shown in (A)-(C), and is shown to indicate the time course of changes in cytosolic Ca^{2+} in relation to NO production.
A.

B. ATP (10μM)

C.

D.
**Figure 4.** ATP-induced NO production is Ca\(^{2+}\)-dependent and P\(_2\) receptor-mediated.

(A) Summary graph showing that the nitric oxide scavenger PTIO significantly reduced the amplitude of ATP-induced DAF-FM fluorescence increase in astrocytes (n = 171 cells, 4 experiments). Cells were first stimulated with 10 μM ATP for 30 seconds, then washed for 10 minutes in normal saline and 5 minutes in 100 μM PTIO, stimulated again with ATP in PTIO, then after washing in normal saline for 15 minutes, stimulated with ATP for a third time. 

(B) Pretreatment with the NOS inhibitor L-NMMA (300 μM, 30 minutes; n = 244 cells, 4 experiments) but not its inactive analog D-NMMA (300 μM, 30 minutes; n = 197 cells, 4 experiments) prevented the DAF-FM fluorescence increase caused by ATP (control untreated: n = 188 cells, 4 experiments). 

(C) Treatment with the P\(_2\) receptor antagonist PPADS significantly reduced the amplitude of ATP-induced DAF-FM fluorescence increase (n = 150 cells, 4 experiments). 

(D) After cells were incubated with the Ca\(^{2+}\) chelator BAPTA (BAPTA AM 30 μM, 45 min), ATP induced little change in DAF-FM fluorescence (n = 240 cells in BAPTA and 174 cells in control conditions, 4 experiments each). Statistical differences were established using the Student's t-test at P< 0.05 (*), P < 0.01 (**), and P <0.001 (***)}. Error bars indicate S.E.M.s.
A.  

![Graph A](image)

B.  

![Graph B](image)

C.  

![Graph C](image)

D.  

![Graph D](image)
**Figure 5.** The NO donor SNAP induces an influx of external Ca\(^{2+}\) into astrocytes. Astrocytes were loaded with fluorescent Ca\(^{2+}\) indicator fluo-3. Cells were stimulated by perfusion with S-nitroso-N-acetylpenicillamine (SNAP, 100 \(\mu\text{M}\)), a nitric oxide donor. (A) A typical astrocytic Ca\(^{2+}\) increase in response to SNAP. The trace is the average of all the cells in the imaging field (\(n = 31\) cells). Error bars are S.E.M.s. The inserted graph shows the SNAP-induced DAF-FM fluorescence increase, confirming that SNAP was releasing nitric oxide. (B) Nitric oxide is responsible for the SNAP-induced Ca\(^{2+}\) increase as the nitric oxide scavenger PTIO inhibited fluo-3 fluorescence increase by SNAP. Three application protocols were used. The first and third SNAP applications were used as internal controls for each cell (\(n = 114\) cells, 3 experiments). (C) After washing with normal saline for 10 minutes, the same cells as shown in (A) were treated with zero-Ca\(^{2+}\) saline for 30 seconds and stimulated with SNAP again. This time little Ca\(^{2+}\) change was induced by SNAP. (D) The summary histogram shows that the second SNAP application in zero-Ca\(^{2+}\) saline induced a smaller astrocytic Ca\(^{2+}\) increase than the first (control) and third (recovery) applications in normal saline (\(n = 84\) cells, 4 experiments). Statistical differences were established using the Student's t-test at \(P < 0.05\) (*), \(P < 0.01\) (**) and \(P < 0.001\) (***)}. Error bars indicate S.E.M.s.
A. SNAP (100 μM)

B. SNAP (100 μM)

C. SNAP (100 μM)

D. SNAP (100 μM)
Figure 6. External Cd\textsuperscript{2+} and 2-APB block the SNAP-induced Ca\textsuperscript{2+} elevation in astrocytes, suggesting that NO induces a Ca\textsuperscript{2+} influx through store-operated channels. (A) An example experiment trace showing cadmium (Cd\textsuperscript{2+}, a non-specific Ca\textsuperscript{2+} channel blocker, 100 μM) blocked SNAP-induced Ca\textsuperscript{2+} influx. The first and third SNAP applications were performed in normal saline, while the second SNAP application was performed in the presence of Cd\textsuperscript{2+}. (B) Summary histogram of the effect of Cd\textsuperscript{2+} on SNAP-induced Ca\textsuperscript{2+} influx (n = 205 cells, 3 experiments). (C) 2-aminoethoxy-diphenylborate (2-APB; 75 μM, 10 min), an IP\textsubscript{3} receptor antagonist and capacitive Ca\textsuperscript{2+} entry inhibitor, reduced SNAP-induced Ca\textsuperscript{2+} increase significantly. Similar protocols were used as in (A). (D) Summary histogram of the effect of 2-APB on SNAP-induced Ca\textsuperscript{2+} influx (n = 96 cells, 3 experiments). Statistical differences were established using the Student's t-test at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***)). Error bars indicate S.E.M.s.
**Figure 7.** Treatment with PTIO during ATP application reduces astrocytic Ca\(^{2+}\) responses to subsequent ATP stimulations. (A) Average traces of the intracellular Ca\(^{2+}\) responses to three consecutive ATP applications, showing that after treatment with PTIO (100 µM) during ATP application, astrocytes responded to the subsequent ATP stimulation with a smaller increase in Ca\(^{2+}\). The first ATP application in normal saline was used as an internal control for each cell. For the PTIO-treated cells, PTIO was applied 10 minutes before the second ATP application and was then present throughout the remainder of the experiment. Zero-Ca\(^{2+}\)EGTA external saline was used in separate experiments to completely exclude the refilling of internal Ca\(^{2+}\) stores (n = 3 experiments each, 176, 259 and 160 cells for control, PTIO-, and zero-CaEGTA-treated group, respectively). (B) Summary of normalized Ca\(^{2+}\) responses showing the significance of PTIO in reducing Ca\(^{2+}\) signaling in response to repeated ATP applications. Note that in the ATP-PTIO group, the responses to the second ATP applications were similar as in control conditions, but the responses to the third ATP applications were significantly smaller, suggesting PTIO partially inhibited the refilling of internal Ca\(^{2+}\) stores. Ca\(^{2+}\) responses were normalized in each cell by defining the increase of fluo-3 fluorescence to the first ATP application as 100%. Statistical differences were established using the Student’s t-test at P< 0.05 (*), P < 0.01 (**) and P <0.001 (**). Error bars indicate S.E.M.s.
A. ATP (10 μM) or PTIO or Ca²⁺EGTA

B. ATP Application

Normalized Ca²⁺ Response (%)
Figure 8. Co-loading of astrocytes with two fluorescence Ca^{2+} indicators, x-rhod-1 and mag-fluo-4. (A) Simple illustration of our experimental set-up. Cells were first loaded with the low-affinity fluorescent Ca^{2+} indicator mag-fluo-4 ($K_d, C_a = 22 \mu M$, Ex/Em maxima: 493/517 nm) at 37 °C for 45 minutes, then loaded with the high-affinity Ca^{2+} indicator x-rhod-1 ($K_d, C_a = 700 \text{nM}$, Ex/Em maxima: 580/602 nm) at room temperature. Using two excitation lasers and carefully-selected emission filters, fluorescent signals from mag-fluo-4 and x-rhod-1 can be spectrally separated. (B) Two example sets of images showing astrocytes co-loaded with mag-fluo-4 and x-rhod-1 and viewed with 60x objective. X-rhod-1 and mag-fluo-4 images are shown in red and green, respectively, and the third column shows the overlay of these two channels. X-rhod-1 staining is more homogeneous, while there are clear punctate patterns in mag-fluo-4 staining, which are consistent with x-rhod-1 and mag-fluo-4 loading into cytosolic and internal store compartment respectively.
Figure 9. X-rhod-1 and mag-fluo-4 simultaneously report Ca\(^{2+}\) levels in cytosol and internal stores respectively. (A) Thapsigargin, an irreversible ER Ca\(^{2+}\)-ATPase inhibitor which can increase cytosolic Ca\(^{2+}\) by depleting ER internal Ca\(^{2+}\) stores, led to a decrease of mag-fluo-4 fluorescence and an increase in x-rhod-1 fluorescence at the same time. The results confirm that we were simultaneously monitoring cytosolic and internal store (mostly ER) Ca\(^{2+}\) with x-Rhod-1 and mag-fluo-4. (B) ATP (20 μM), known to induce cytosolic Ca elevation by releasing of Ca\(^{2+}\) from ER stores, caused a reduction in mag-fluo-4 fluorescence and a biphasic increase of x-rhod-1 fluorescence, supporting that the two fluorescence signals respectively indicate cytosolic and internal store Ca\(^{2+}\). But occasionally the fluorescence signal of mag-fluo-4 may be mildly contaminated when cytosolic Ca\(^{2+}\) becomes highly elevated, thus an initial small rise in mag-fluo-4 fluorescence in response to ATP was sometimes observed (left graph), which could be abolished by chelating cytosolic Ca\(^{2+}\) with BAPTA (right graph, same cells as in left). (C) Experiments using zero-Ca\(^{2+}\) EGTA external saline with ATP further validate the two-indicator approach for simultaneous monitoring of cytosolic and internal store Ca\(^{2+}\). In the presence of zero-Ca\(^{2+}\) EGTA, the ATP-induced reduction in mag-fluo-4 fluorescence could not recover after the removal of ATP until Ca\(^{2+}\) was reintroduced into the bath, and the increase in x-rhod-1 fluorescence lacked an elevated plateau phase, both consistent with effects of an elimination of Ca\(^{2+}\) influx.
Figure 10. ATP induces release of Ca\textsuperscript{2+} from internal stores and subsequent store refilling. (A) An example experiment showing that the ATP-evoked increase in cytosolic Ca\textsuperscript{2+} results from the release of Ca\textsuperscript{2+} from internal stores. These images are pseudocolor images of mag-fluo-4 and x-rhod-1 fluorescence corresponding to times labeled in the graph in (B) (a: before ATP application; b: 10 sec after starting ATP; c: 40 sec after starting ATP; d: 2 minutes after removal of ATP showing full recovery). Note that mag-fluo-4 fluorescence decreased and x-rhod-1 fluorescence increased in response to ATP, but 2 minutes after the removal of ATP, both signals went back to their base levels. (B) Traces showing the average changes in mag-fluo-4 and x-rhod-1 fluorescence of the cells in (A).
Figure 11. The NO scavenger PTIO reduced the refilling of internal Ca\(^{2+}\) stores. (A) Example traces of one experiment, showing the ATP-induced elevation of cytosolic Ca\(^{2+}\) and depletion of internal store Ca\(^{2+}\) and subsequent store refilling in PTIO-treated cells. Note that unlike control conditions (figure 9), internal Ca\(^{2+}\) store only partially refilled in the presence of PTIO. The horizontal lines in the graph are artificial lines drawn to indicate the baseline level (b; average of 3 frames immediately before ATP application), maxima decrease (v) and recovered level (r; average of 3 frames before 2 minutes post-ATP removal) of mag-fluo-4 dF/F0. \(\Delta J_1\) and \(\Delta J_2\) are the differences of (b and v) and (r and v), respectively. (B) Summary of the effect of PTIO on internal Ca\(^{2+}\) store refilling. The refilling percentage is defined as \(\Delta J_2 / \Delta J_1 \times 100\%\). In PTIO-treated cells, the refilling percentage was significantly reduced (n = 9 experiments each). Statistical differences were established using the Student’s t-test at P< 0.05 (*), P < 0.01 (**) and P <0.001 (***) (C) The time-course of the changes in internal store Ca\(^{2+}\) level in control conditions and in PTIO-treated cells, demonstrating that internal stores did not fully refill in the presence of PTIO compared to the control conditions. The data points represent the average of all the cells in all experiments for each group. The normalized store depletion percentage is calculated in each cell based on 0% depletion at rest before ATP application and -100% as maximum depletion after each ATP application. Error bars indicate S.E.M.s.
Figure 12. Photo-release of nitric oxide induces a $\text{Ca}^{2+}$ elevation in the stimulated astrocyte. (A) Our photolysis set-up provides a fast and localized NO source. It consists of a 337 nm photolysis UV laser coupled with an optical fiber together with a 635 nm positioning laser. The photolysis spot is approximately 6 $\mu$m in diameter. Changes in $\text{Ca}^{2+}$ level were monitored in purified astrocyte cultures with fluo-3. Caged NO (1 mM) was introduced into the bath. (B) An example of the $\text{Ca}^{2+}$ elevation in an astrocyte directly stimulated with NO photo-released by 1 UV pulse (3 ns). The arrow indicates the time of photolysis. (C) The summary graph showing that photo-liberation of NO caused an increase in $\text{Ca}^{2+}$ (column 1, n = 6) which is prevented by the NO scavenger PTIO (column 3, n = 3). UV illumination without the presence of caged compound did not induce a change in fluo-3 fluorescence (column 2, n = 4). Statistical differences were established using the Student’s t-test at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (**). Error bars indicate S.E.M.s.
A. Confocal Microscope

B. UV

caged nitric oxide

Caged NO  +  -  +
PTIO  -  -  +
Figure 13. Photo-release of NO initiates an intercellular Ca$^{2+}$ wave between astrocytes.

(A) A representative example of NO-induced Ca$^{2+}$ waves. The images are sequential pseudocolor images from immediately before photolysis to 12 seconds after stimulation. The arrow and white circle inside the second image indicates the timing and position of photolysis. Fluo-3 fluorescence first increased in the stimulated astrocyte, and subsequently propagated to the neighbors. (B) Lateral diffusion of nitric oxide does not cause the Ca$^{2+}$ wave. When the photolysis site (represented by the white circle) was adjacent to but not on the cell (B1), lateral diffusion of photo-released nitric oxide did not cause a Ca$^{2+}$ elevation in this astrocyte. In (B2), the same astrocyte in (B1) was then moved into the photolysis spot, and photo-release of nitric oxide induced a Ca$^{2+}$ elevation in the flashed astrocyte and subsequently in neighbors. (C) New nitric oxide synthesis is not required for the Ca$^{2+}$ wave initiated by photo-released nitric oxide. Astrocytes were incubated with the nitric oxide synthases inhibitor L-NMMA (300µM) for 30 minutes before Ca$^{2+}$ imaging. Photo-release of nitric oxide still induced a similar astrocytic Ca$^{2+}$ wave.
Figure 14. Incubation in NOS inhibitor or NO scavenger does not prevent mechanical-stimulation-induced Ca\(^{2+}\) signaling. (A) An example of mechanical stimulation-induced Ca\(^{2+}\) wave. Purified astrocyte cultures were loaded with Ca\(^{2+}\) indicator Fluo-3. Mechanical stimulations were performed by gently touch an astrocyte with a glass micropipette filled with external saline. The leftmost is the bright-field image. We can clearly see the micropipette tip position. The right images are sequential images from immediately before mechanical stimulation to 12 seconds after stimulation. The arrow inside the second image indicates the time when the mechanical stimulus was applied. Fluo-3 fluorescence first increased in the touched cell, and then in the neighboring cells. (B) PTIO (nitric oxide scavenger, 100 µM, 5 min) and L-NMMA (nitric oxide synthases inhibitor, 300 µM, 30 min) did not reduce mechanical stimulation-induced astrocyte Ca\(^{2+}\) wave. Neither the percentage of cells exhibiting a Ca\(^{2+}\) increase nor the amplitude of average increase in fluorescence intensity was affected by PTIO (n = 25 experiments) or L-NMMA (n = 8) treatment (Control, n = 21). Bars represent S.E.M.s. Statistical differences were established using the Student’s t-test at P<0.05.
Figure 15. Nitric oxide is not required for the astrocyte-evoked glutamate-dependent slow inward current. (A) Schematic diagram representing a confluent layer of astrocytes (thin lines) and two neurons (thick lines). Astrocytes were mechanically stimulated with a glass micropipette, and whole cell currents were recorded from adjacent neurons. (B) A typical slow inward current (SIC) evoked by astrocyte stimulation. The asterisk indicates the timing of the stimulus. (C) Summary of the effects of the NO scavenger PTIO and the NOS inhibitor L-NMMA on SIC incidence and amplitude. SICs could still be evoked after the treatment of PTIO (100 µM) or L-NMMA (300 µM), but were evoked in fewer cells (n = 10, 5 and 4 experiments for control, PTIO and L-NMMA, respectively), with a smaller amplitude (n = 67, 33 and 32 responded neurons for control, PTIO and L-NMMA, respectively). The NMDA receptor antagonist D-AP5 (50 µM) and the non-NMDA receptor antagonist CNQX (20 µM) were used to confirm the glutamate-dependency of SICs (n = 3 experiments, 5 responded cells). Only currents ≥ 10 pA were considered to be astrocyte-evoked SIC, i.e. at least three times higher than the standard deviation of the baseline noise. Statistical differences were established using the Student’s t-test at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (**). Error bars represents S.E.M.s.
CHAPTER 5. DISCUSSION AND CONCLUSION

Ca²⁺-dependent Nitric Oxide Production

Using the NO-sensitive fluorescent indicator DAF-FM, I observed that ATP, an important messenger in astrocytic Ca²⁺ waves, induced a cellular production of NO in purified astrocyte cultures. This NO production is Ca²⁺-dependent and P₂ receptor-mediated, since the Ca²⁺-chelator BAPTA and the P₂ receptor antagonist PPADS both blocked the ATP-induced NO production. To my knowledge, this is the first report directly visualizing NO production in astrocytes induced by a natural cellular messenger such as ATP. The ATP concentration we used is in the physiological range (10 μM), which implies that this NO production can happen under physiological conditions. The ATP-induced generation of NO was also blocked by a broad-spectrum NOS inhibitor L-NMMA, in agreement with the function of NOSs in forming NO. Although I couldn’t differentiate the specific isoforms of NOSs with my pharmacological studies, it is likely that the Ca²⁺-dependent constitutive NOSs (eNOSs and nNOSs) are responsible, based on the Ca²⁺-dependence of this ATP-induced NO production. NO has profound roles in the central nervous system, and is considered as a “double-edged sword”, referring to its neuromodulating and neuroprotective roles at low concentrations and neurotoxic roles at high concentrations which usually occur due to the activation of iNOSs during pathological conditions. Although DAF-FM does not quantitatively measure NO levels, it is reasonable to speculate that this astrocytic Ca²⁺-dependent NO synthesis would be in the range of normal physiological concentrations, and therefore might be involved in modulating normal information processing.
Nitric Oxide Induces $Ca^{2+}$ Influx into Astrocytes and Modulates Internal $Ca^{2+}$ Store Refilling

When a NO donor, SNAP, was applied to purified astrocyte cultures, it induced a NO-dependent $Ca^{2+}$ increase in astrocytes. This increase in $Ca^{2+}$ was due to an influx of $Ca^{2+}$ from the extracellular space, since both zero-$Ca^{2+}$ external saline and the non-specific $Ca^{2+}$ channel blocker Cd$^{2+}$ both abolished this $Ca^{2+}$ increase. In agreement with my finding is the recent report by Matyash et al., in which the authors reported that NO-triggered $Ca^{2+}$ influx into Bergmann glial cells (Matyash et al., 2001). 8-Br-cGMP, an agonistic analog of cGMP, did not induce a detectable $Ca^{2+}$ change, suggesting that the NO-induced $Ca^{2+}$ increase is via a cGMP-independent pathway. Willmott et al. reported that NO induced $Ca^{2+}$ mobilization via cGMP-G Kinase-dependent activation of ryanodine receptors (Willmott et al., 2000). However, because they used mixed cultures of astrocytes and neurons, they were unable to confirm that the NO effect that they observed in astrocytes was due to NO stimulation of astrocytes rather than to NO stimulation of adjacent neurons. The result that the cGMP pathway is not involved in the NO-induced $Ca^{2+}$ influx in astrocytes is not surprising, since this pathway is now known to be only one of the numerous NO targets. Recently it was also reported that NO can induce a cGMP-independent $Ca^{2+}$ influx in endothelial cells (Berkels et al., 2000). Further experiments will be needed to dissect out the components involved in the NO-induced $Ca^{2+}$ influx in astrocytes.

The NO-induced $Ca^{2+}$ influx in astrocytes can be blocked by 2-APB, an antagonist of the IP$_3$ receptor and the capacitative $Ca^{2+}$ entry pathway, suggesting that this NO-induced $Ca^{2+}$ influx is possibly through store-operated $Ca^{2+}$ channels. S-nitrosylation is emerging as an important specific post-translational modification of numerous proteins. Store-operated
Ca\textsuperscript{2+} channels are known targets of S-nitrosylation (Favre et al., 1998; Ma et al., 1999; van Rossum et al., 2000). NO could directly modulate store operated channels and thus induce an influx of Ca\textsuperscript{2+}. Store-operated channels and the CCE pathway have not been well characterized in astrocytes. It will be interesting to test whether I\textsubscript{cric} can be recorded from astrocytes, and whether NO can modulate this current.

To further understand the consequences of NO-induced Ca\textsuperscript{2+} influx in astrocytes, I determined if NO could modulate the refilling of internal Ca\textsuperscript{2+} stores. Results from the three ATP application experiments provide indirect evidence that NO might modulate the refilling of internal Ca\textsuperscript{2+} stores. Treatment with the NO scavenger, PTIO, during ATP application reduced the magnitude of the Ca\textsuperscript{2+} response to a subsequent ATP application, suggesting that internal Ca\textsuperscript{2+} stores are unable to fully replenish when NO signaling is blocked.

In order to more directly address this question, I directly imaged changes in internal store Ca\textsuperscript{2+}, and simultaneously monitored the changes in cytosolic Ca\textsuperscript{2+}. By loading the same astrocytes with two fluorescent Ca\textsuperscript{2+} indicators with different spectral profiles and with different Ca\textsuperscript{2+}-affinities, I managed to separate the internal store Ca\textsuperscript{2+} signal from the cytosolic Ca\textsuperscript{2+} signal. The separation of the two signals was first supported by the observation of the different fluorescent patterns generated by the two dyes in a single cell. X-rhod-1 staining is more homogenous, while mag-fluo-4 staining shows clear punctate patterns in the periphery of cells and devoid of fluorescence in nuclear regions (reflecting the cytoplasmic), which is consistent with x-rhod-1 and mag-fluo-4 loading into cytosol and internal stores respectively. Then the series of experiments using the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin and the Ca\textsuperscript{2+} mobilizing messenger ATP confirmed that these two fluorescence indicators report Ca\textsuperscript{2+} changes in the two different compartments. Thapsigargin reduced
mag-fluo-4 fluorescence and simultaneously increased x-rhod-1 fluorescence, consistent with thapsigargin induced Ca\(^{2+}\) leak from internal stores; ATP induced a biphasic increase of x-rhod-1 fluorescence and a simultaneous reduction of the fluorescence of mag-fluo-4, in agreement with ATP-induced Ca\(^{2+}\) release from internal stores; and mag-fluo-4 fluorescence could not return to the base level in the presence of zero-Ca\(^{2+}\)EGTA, consistent with zero-Ca\(^{2+}\)EGTA preventing Ca\(^{2+}\) influx and store refilling. Taken together, these results supported the notion that x-rhod-1 and mag-fluo-4 report changes in cytosolic and internal Ca\(^{2+}\) respectively.

However, occasionally mag-fluo-4 fluorescence can be contaminated with highly elevated cytosolic Ca\(^{2+}\), as a small transient rise in mag-fluo-4 fluorescence signal was sometimes observed in response to ATP, which was abolished after chelating cytosolic Ca\(^{2+}\) with BAPTA. Since my main interest is in the magnitude of store depletion and refilling at a time when cytosolic Ca\(^{2+}\) is already lowered from it peak, these two fluorescence signals were appropriate indicators for cytosolic and internal store Ca\(^{2+}\). An additional consideration is that mag-fluo-4 also becomes fluorescent upon Mg\(^{2+}\) binding (K\(_{d,mg}\) = 4.7 mM). In internal stores the free Ca\(^{2+}\) concentration is estimated to be ~110-150 μM in astrocytes (Golovina and Blaustein, 1997, 2000), about 5-7 times of K\(_{d,ca}\), and the Mg\(^{2+}\) concentration is estimated to be ~1 mM, only 1/5 of K\(_{d,mg}\) (Sugiyama and Goldman, 1995), thus mag-fluo-4 preferentially bind to Ca\(^{2+}\) in internal stores.

The role of NO in refilling of internal stores was then examined by pre-treating astrocytes with the NO scavenger PTIO and then applying ATP. ATP induced Ca\(^{2+}\) release from internal stores and internal stores were refilled after the removal of ATP. After blocking NO signaling, the refilling percentage of internal stores following ATP stimulation
was significantly reduced to about 70%, compared to a little over 100% refilling in control conditions.

Many neurotransmitters such as glutamate and ATP cause an IP₃-receptor-mediated Ca²⁺ release from ER Ca²⁺ stores in astrocytes (Cornell-Bell et al., 1990; Charles et al., 1993; Salter and Hicks, 1995; Venance et al., 1997). However, little is known about the mechanism of store refilling in astrocytes. Although the capacitative Ca²⁺ entry (CCE) pathway has been suggested in this store-depletion-induced store refilling, the existence and characteristics of CCE pathway in astrocytes has not been well investigated. Collectively our findings suggest that the Ca²⁺-dependent NO production has a role in modulating the refilling of internal Ca²⁺ stores in astrocytes, which is a very important link in the chain of Ca²⁺ signaling events.

There appears also to be NO-independent components in Ca²⁺ store refilling, since PTIO only blocked about 30% of store refilling. This might result from existence of both NO-dependent and NO-independent pathways in the store refilling process. Alternatively, the NO modulation effect could be fine-tuned in which case NO levels would be tightly linked to the ratio of Ca²⁺ entry and release. The store refilling is a very complicated process. In addition to capacitative Ca²⁺ entry, it may also involve Ca²⁺ recycling from cytosol and Ca²⁺ shuffling from other organelles like mitochondria. We found that NO can modulate the refilling of internal stores, but the exact mechanism of NO regulation will need further examination.
Nitric Oxide Initiates but Does Not Propagate the $\text{Ca}^{2+}$ Wave between Astrocytes

Taking advantage of our photolysis set-up, we directly photo-released NO onto a single astrocyte, and found that NO not only induced a $\text{Ca}^{2+}$ elevation in the directly stimulated astrocyte, but also initiated an intercellular propagating $\text{Ca}^{2+}$ wave in neighboring astrocytes, that was similar in time course and magnitude to the previously characterized mechanical stimulation-induced $\text{Ca}^{2+}$ wave. We performed careful control experiments and confirmed that these $\text{Ca}^{2+}$ responses were only due to photo-released NO directly on the stimulated astrocytes but not due to photodamage or diffusion of photoliberated NO. This finding adds NO to the list of neurotransmitters which can induce glial $\text{Ca}^{2+}$ waves, and thus reveal a new pathway for initiation of a $\text{Ca}^{2+}$ wave.

The propagation of $\text{Ca}^{2+}$ waves does not require NO, since neither the NO-induced $\text{Ca}^{2+}$ wave nor the mechanical stimulation-induced $\text{Ca}^{2+}$ wave was affected by the inhibition of NOSs. This finding is in agreement with our current knowledge about the $\text{Ca}^{2+}$ wave mechanisms (Guthrie et al., 1999; Haydon, 2001). Although NO can be generated from the directly stimulated astrocytes, the concentration of newly-synthesized NO is probably not sufficient to mediate a regenerative wave of $\text{Ca}^{2+}$ rise in neighboring cells. Other messengers should be responsible for propagating of the NO-induced $\text{Ca}^{2+}$ waves. ATP and possibly glutamate are good candidates. Additional experiments using antagonists of ATP or glutamate signaling pathway could confirm this speculation. Another interesting experiment would be to image the ATP production (if any) in astrocytes during NO-induced $\text{Ca}^{2+}$ waves. The pathway for ATP release in astrocytes is not understood. It is only known that the ATP release is not $\text{Ca}^{2+}$-dependent (Wang et al., 2000), possibly PLC-dependent (Guthrie et al., 1999), and unlikely to be SNARE-dependent (Araque et al., 2000). Since ATP can cause
Ca\textsuperscript{2+}-dependent NO production in astrocytes, how NO induces the release of ATP would be a very interesting question concerning the complicated web of Ca\textsuperscript{2+} signaling in astrocytes.

\textit{Nitric Oxide in Glutamate-mediated Astrocyte-Neuron Signaling}

A Ca\textsuperscript{2+} elevation in an astrocyte is necessary and sufficient to induce the release of glutamate (Parpura et al., 1994; Araque et al., 1998a). Glutamate from astrocytes is able to modulate neuronal activity and neurotransmission (Araque et al., 1998a; Araque et al., 1998b; Kang et al., 1998; Newman and Zahs, 1998; Parpura and Haydon, 2000). The astrocyte-evoked neuronal slow inward current is an example of this modulation. We found that NO is not required for the induction of slow-inward current, but PTIO reduced the magnitude of SIC, suggesting that NO has modulatory roles in this glutamate-dependent event. The effect of NO may result from a combination of its roles in different steps of SIC generation. Because NO can modulate Ca\textsuperscript{2+} signaling in astrocytes, we would expect NO might modulate Ca\textsuperscript{2+}-dependent glutamate release from astrocytes. Because astrocytic release of glutamate is also known to be SNARE protein-dependent (Araque et al., 2000; Pasti et al., 2001), NO might directly modulate the release machinery by S-nitrosylation of specific SNARE proteins. NO can also directly S-nitrosylate NMDA receptors of neurons and thus may modulate the glutamate-sensitivity of receptors.

We selected the SIC as a model to investigate the roles of NO in astrocyte-neuron signaling, but it is worth noting that the effect of NO in astrocyte-neuron signaling need not be limited to glutamate-mediated signaling. The astrocyte-originated NO could directly act on neurons and modulate numerous processes in synaptic transmission.
Concluding Remarks

In this dissertation, I demonstrated the Ca\(^{2+}\)-dependent production of nitric oxide in astrocytes, explored the roles of NO in astrocyte Ca\(^{2+}\) signaling and astrocyte-neuron interaction. A schematic model of the generation and effects of NO in astrocytes is presented in Figure 15, which shows that ATP induces Ca\(^{2+}\) release from IP\(_3\) receptor-mediated internal stores, and thus induces a Ca\(^{2+}\)-dependent production of NO. NO can then modulate the refilling of internal Ca\(^{2+}\) stores by inducing an influx of Ca\(^{2+}\) through store-operated Ca\(^{2+}\) channels, and also modulate the Ca\(^{2+}\)-dependent release of glutamate from astrocytes. In addition, NO diffuses through cell membranes, and thereby can directly act on other neighboring cells.

*In vitro* culture systems were used in this study because of their simplicity and more importantly because they allow us to selectively investigate the astrocytic generation of NO and responses to NO. This NO signaling is unlikely the artifact of culture system. Because astrocytic Ca\(^{2+}\) signaling and Ca\(^{2+}\)-dependent release of glutamate has been demonstrated *in situ*, and physiological concentrations of ATP can induce the production of NO, thus Ca\(^{2+}\)-dependent NO production and NO signaling is highly likely present in intact nervous systems. But future studies should be expanded to *in situ* and *in vivo* system so that we will understand more about astrocyte NO signaling in more physiological conditions.

During the progress of this research, I became more and more fascinated about the diverse roles of this gaseous messenger, NO. In the CNS, astrocytes make contacts with both neuronal membranes and blood vessels. Astrocytes could receive NO from adjacent neuronal synapses, neighboring astrocytes or adjacent endothelial cells. NO from all origins can modulate Ca\(^{2+}\) signaling in astrocytes and in turn modulate release of glutamate from
additional, astrocytes can generate NO following a Ca^{2+} increase induced by various neurotransmitters (including glutamate, ATP and NO). This astrocyte-derived NO can also signal to neurons and modulate synaptic plasticity, or signal to endothelial cells of blood vessels and thereby controlling the relationship (normal or pathological) between neural activity and blood flow/energy supply in the CNS.
Figure 16. A schematic diagram showing a model of the generation and functions of NO in astrocytes. ATP induces Ca$^{2+}$ release from IP$_3$ receptor-mediated internal stores, and thus induces a Ca$^{2+}$-dependent production of NO. NO can then modulate the refilling of internal Ca$^{2+}$ stores by inducing an influx of Ca$^{2+}$ through store-operated Ca$^{2+}$ channels, and also modulate the Ca$^{2+}$-dependent release of glutamate from astrocytes. In addition, NO diffuses through cell membranes, and thereby can directly act on other neighboring cells.
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"Aside from increased feelings of self-esteem and the approval of our own conscience, the conquest of a new truth is undoubtedly the greatest adventure to which a man can aspire." This is an advice that the great neuroanatomist Santiago Ramón y Cajal once given to a young investigator. I guess it holds true for most scientists and scholars. From that over-curious little girl who always asked too many questions to the young student now looking forward to getting her Ph.D. degree and starting a professional career in science, a lot of those question are still intriguing me and unanswered. Life had its up and downs along the way. There were times when I thought I can conquer the world, also were times when I felt myself so useless and feared nothing would ever change, but what constantly pulled me back to the road is still a child's curiosity to discover the truths about nature and ourselves.

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