ASTROCYTIC Gq-GPCR-LINKED IP_3R-DEPENDENT Ca^{2+} SIGNALING DOES NOT MEDIATE NEUROVASCULAR COUPLING IN MOUSE VISUAL CORTEX IN VIVO

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ABSTRACT

Daniel E. Bonder: Astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling does not mediate neurovascular coupling in mouse visual cortex in vivo
(Under the direction of Ken D. McCarthy)

Local blood flow is modulated in response to changing patterns of neuronal activity (Roy and Sherrington, 1890), a process termed neurovascular coupling. It has been proposed that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling drives this process, though in vivo tests of this hypothesis are largely lacking. We examined the impact of astrocytic Gq-GPCR and IP_3R-dependent Ca^{2+} signaling on cortical blood flow in awake, responsive mice using multiphoton laser-scanning microscopy and novel genetic tools that enable the selective manipulation of astrocytic signaling pathways in vivo. Selective stimulation of astrocytic Gq-GPCR cascades and downstream Ca^{2+} signaling with the hM3Dq DREADD designer receptor system was insufficient to modulate basal cortical blood flow. We found no evidence of observable astrocyte endfeet Ca^{2+} elevations following physiological visual stimulation despite robust dilations of adjacent arterioles using cyto-GCaMP3 and Lck-GCaMP6s, the most sensitive Ca^{2+} indicator available. Astrocytic Ca^{2+} elevations could be evoked when inducing the startle response with unexpected air puffs. However, startle-induced astrocytic Ca^{2+} signals did not precede corresponding startle-induced hemodynamic changes. Further, neurovascular coupling was intact in awake, responsive mice genetically lacking astrocytic IP_3R-dependent Ca^{2+} signaling (IP_3R2 KO). These data establish that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling does not mediate neurovascular coupling in visual cortex of awake, responsive mice.

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PREFACE

My beliefs about science and its ideal, my personal journey through graduate school, and why I decided on the career and life path I aim to follow are summarized, or more appropriately can be “deduced”, from the following quotes from a literary character who became one of my muses.

“From long habit the train of thoughts ran so swiftly through my mind that I arrived at the conclusion without being conscious of the intermediate steps.”

“It was easier to know it than to explain why I know it. If you were asked to prove that two and two made four, you might find some difficulty, and yet you are quite sure of the fact.”

“One’s ideas must be as broad as Nature if they are to interpret Nature.”

“From a drop of water a logician could infer the possibility of an Atlantic or a Niagara without having seen or heard of one or the other.”

Sherlock Holmes, *A Study in Scarlet*

“You see, but you do not observe. The distinction is clear.”

“It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts.”

Sherlock Holmes*, A Scandal in Bohemia*

“The world is full of obvious things which nobody by any chance ever observes.”

Sherlock Holmes, *The Hound of the Baskervilles*

“There is nothing more deceptive than an obvious fact.”

Sherlock Holmes, *The Bascombe Valley Mystery*

“No, it is not selfishness or conceit...If I claim full justice for my art, it is because it is an impersonal thing – a thing beyond myself. Crime is common. Logic is rare. Therefore it is upon the logic rather than the crime that you should dwell. You have degraded what should have been a course of lectures into a series of tales.”

Sherlock Holmes, *The Adventure of the Copper Beeches*

“I confess that I have been as blind as a mole, but it is better to learn wisdom late than never to learn it at all.”

Sherlock Holmes, *The Man with the Twisted Lip*

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

CHAPTER 2: THESIS RESEARCH

CHAPTER 3: FUTURE DIRECTIONS IN NEUROVASCULAR COUPLING

LIST OF ABBREVIATIONS AND SYMBOLS

AAV	Adeno-associated virus
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
cKO	Conditional knockout
CNO	Clozapine-*N* Oxide
Gq-GPCR	Gq G protein-coupled receptor
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
K^+	Potassium ion
KO	Knockout
Na^+	Sodium ion
Δ	Fold or percentage change

CHAPTER 1: INTRODUCTION

THE WHAT AND THE WHY

What is neurovascular coupling?

Neurovascular coupling is a well-documented phenomenon in which local blood flow to active regions of the brain is increased, similar to how blood flow to skeletal muscles increases during exercise. First described by researchers Roy and Sherrington in the year 1890, neurovascular coupling has garnered interest in the basic research and medical communities for its contribution to the Blood Oxygen Level-Dependent (BOLD) signal detected by functional magnetic resonance imaging (fMRI). Functional MRI is an incredibly powerful technique that permits noninvasive imaging of human brain activity in real time with millimeter spatial resolution.

While offering great utility and promise as a research and diagnostic technique, fMRI is limited in that the BOLD signal is not a direct measure of neuronal activation. Rather, the BOLD signal is an aggregation of blood volume, blood oxygen, and various metabolic rate changes within the tissue (Kim and Ogawa, 2012). In other words, BOLD is merely a correlate for neuronal activity. The cellular mechanisms responsible for each component of the evoked BOLD response have been debated for years, and are still being investigated today.

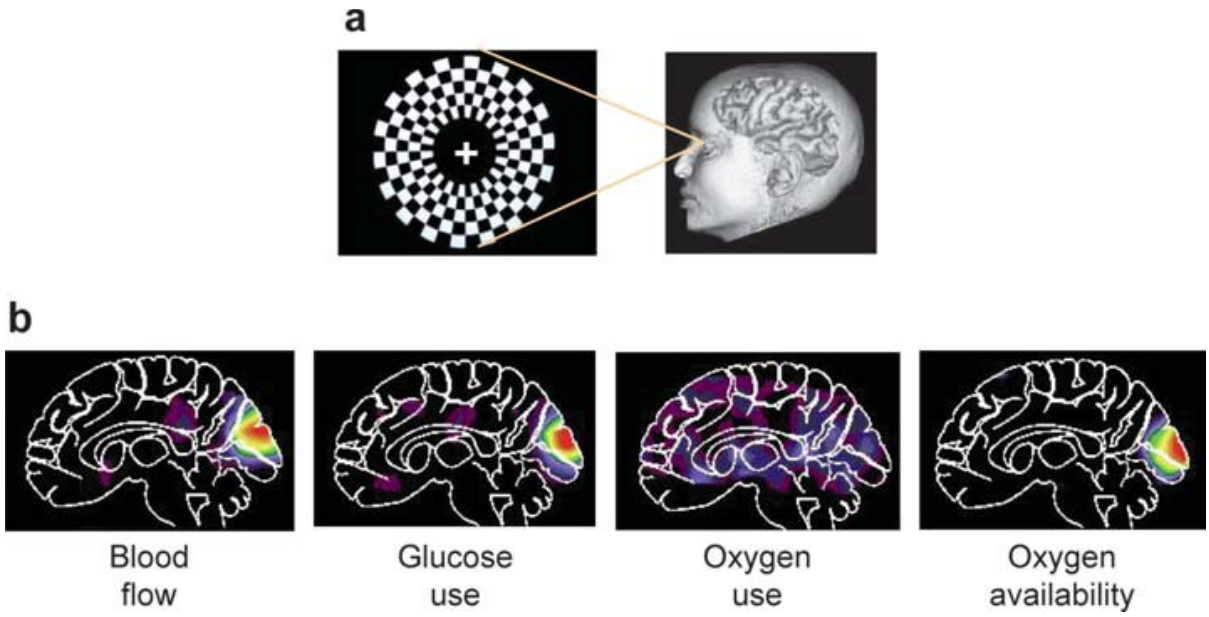

Figure 1 (Raichle and Mintun, 2006). *Measuring human brain activity using fMRI.* Presentation of a visual stimulus to an individual (a) results in increased blood flow to active regions of the brain, wherein neurons consume glucose and oxygen (b). Blood flow changes and metabolic rates underlie the BOLD signal measured in fMRI.

It is widely believed that neurovascular coupling underlying BOLD signals takes place so that delivery of metabolic substrates, such as glucose and oxygen, keeps pace with local metabolic demand within the tissue (refer to Figure 1), implying that local metabolic demand ultimately drives functional hemodynamic changes. Interestingly though, this assertion has yet to be proven. On the one hand, some studies indicate a relationship between rates of glycolysis or mechanisms specifically involving tissue NADH/NAD^+ ratio and neurovascular coupling (Ido et al., 2004; Vlassenko et al., 2006). Glucose metabolism, which occurs primarily in astrocytes (Kasischke et al., 2004), trends linearly with blood flow (Paulson et al., 2010), suggesting a potential causal link between anaerobic glycolysis and functional hemodynamics. On the other hand, tissue metabolic demand appears to be readily met by oxidative metabolism as opposed to anaerobic glycolysis (Lin et al., 2010)

and hyperoxia in vivo does not alter evoked blood flow increases (Lindauer et al., 2010; Mishra et al., 2011). The magnitude of blood flow increase and oxygen delivery far exceeds oxygen usage (Lin et al., 2010). These data suggest that metabolic demand, more accurately neuronal metabolic demand, in itself is not driving neurovascular coupling in vivo.

Instead, it is possible that neurotransmitter-related signaling events are responsible for hemodynamic changes in brain (Attwell and Iadecola, 2002). This idea does not necessarily conflict with the hypothesis that astrocytic glycolysis drives neurovascular coupling (Paulson et al., 2010), as it is plausible that these cellular processes could be interdependent.

Why do we suspect astrocytes are involved?

The linear coupling of blood flow increases and rates of glucose metabolism provides one indication that astrocytes might be central to mediating functional hemodynamic changes in the brain. There are important structural considerations as well. 19th century scientists working with relatively primitive imaging techniques first noted the close association of glial structural processes with cerebral vasculature (Somjen, 1988). Fluorescent and electron microscopic imaging studies have determined that over 99% of cerebral vascular surface is completely covered by astrocyte structures called “endfeet” (Mathiisen et al., 2010; McCaslin et al., 2011). Neuronal network dynamics and particular interneuronal subtypes (Cauli et al., 2004) can modulate vascular dynamics through a number of molecular mediators like nitric oxide (NO), cyclooxygenase derivatives such as prostaglandins, neurotransmitters, and peptides (Cauli and Hamel, 2010). Given the near-complete coverage of blood vessels by astrocytes, any signal originating from neurons, save

for gaseous signaling compounds like nitric oxide (NO), must be transported/transmitted/translated through an astrocytic compartment.

Within the last decade, a wealth of direct evidence has accumulated indicating that astrocytes are capable of modulating vascular diameter by means of intracellular Ca^{2+} elevations stimulated by the activation of G protein-coupled receptors linked to Gq signaling cascades (Gq-GPCRs), resulting in Ca^{2+}-dependent release of various vasoactive molecules from endfeet onto cerebral arterioles, most notably cyclooxygenase derivatives (Attwell et al., 2010; Petzold and Murthy, 2011; Newman, 2013; Howarth, 2014). This hypothesis (which serves as the basis for the present thesis project, described in detail in Chapter 2) is attractive because it provides a plausible and relatively straightforward, direct mechanism linking neuronal activity to local blood flow modulation. A further implication is that BOLD signals are a decent proxy for neuronal activity (assuming that astrocytic Ca^{2+} elevations translate neuronal activity "faithfully", as most data appeared to indicate) – excellent news for fMRI researchers and diagnosticians. Unfortunately, the results of more recent in vivo work, including the data and conclusions described in detail in Chapter 2, strongly indicate that this hypothesis is oversimplified or perhaps flatly incorrect.

This field of research is relatively young – the first article directly reporting astrocytic control of blood flow through Ca^{2+} signaling was published in 2003 (Zonta et al.). It would not be unreasonable to argue that missteps occur in the early phases of any new endeavor. But I believe this view is limited and does not encourage genuine scrutiny of potential underlying issues. The individual successes and missteps of any one piece are inextricably conditioned on and interwoven with those of the whole. While the research project (Chapter 2) and future research directions (Chapter 3) outlined in this thesis is focused in scope, other sections will consider not only the field of neurovascular coupling but glial biology as a whole, involving historical overview (Chapter 1) and philosophical examination (Chapter 4).

ASTROCYTE BIOLOGY: A HISTORICAL PERSPECTIVE

A brief overview of the beginnings of astrocyte biology

Astrocytes belong to a class of non-neuronal support cells in the central nervous system (CNS) termed “glia”, “glial cells”, or “neuroglia”. The name originates from the work of Rudolf Carl Virchow, a 19th century doctor, scientist, and politician in Germany, in which Virchow argued for the existence of sheets of connective tissue lying underneath the ventricular ependymal layer of the brain, penetrating into and filling all areas of the brain and separating neural tissue from vascular elements (Somjen, 1988). He named this connective tissue “Nervenkitt”, roughly translating to “nerve-glue” from which the term “neuroglia” (or simply “glia”) is derived (Somjen, 1988). While Virchow was not the first to actually identify non-neuronal cell types of the brain, his work did appear to bring about awareness of the potential importance of the non-neuronal components of the brain in possibly regulating brain function or structure (Somjen, 1988).

Early studies on glia focused primarily on distinguishing the various subtypes within this family of cells – protoplasmic and fibrous astrocytes, oligodendrocytes, and microglia – on the basis of histological techniques and light microscopy. For this purpose, the silver staining method of Camillo Golgi proved to be a highly valuable development. This technique allowed researchers to fully and clearly identify the cellular structures branching off of individual cells, a vast improvement over earlier tissue staining methods. Glia could clearly be differentiated from neurons based on the absence of an axon or axonal-like projections, and unique attributes of each cell subtype could be further characterized – for instance, the close association of protoplasmic astrocytic projections and blood vessels (Somjen, 1988). These early imaging studies directly revealed important functions of the various glial subtypes. For example, Pio del Rio-Hortega, a Spanish neuroscientist,

demonstrated the ability of microglial cells to phagocytose dying cells (del Rio-Hortega, 1933).

Sufficient circumstantial evidence was provided to additionally begin formulating plausible inferences about other functions of glia aside from structural support. Santiago Ramon y Cajal postulated that glia “insulate” nerve fibers (Ramon y Cajal, 1920), preempting our eventual knowledge that myelin is produced by oligodendrocytes or that many synapses are fully or partially ensheathed by astrocytic compartments. Golgi argued that glial cells “feed” neurons (Golgi, 1885-1886), a fore-runner to our current understanding of astrocyte-to-neuron metabolic relationships. Even by the year 1910, the notion that glial cells could secrete substances towards the purposes of modulating brain function, a highly-studied phenomenon we now term “gliotransmission”, was entering the mainstream (Somjen, 1988). The inventive Italian neuropsychiatrist Ernesto Lugaro offered a number of (at the time) wild claims that later proved to be true – that embryonic glial cells act as guides for migrating neurons during development, that glia regulate the extracellular environment through blood-brain barrier function or removing neuronal metabolic waste products, and that glia form close associations with “nervous articulations” (synapses) in order to take up neuroactive substances released by neurons as they signal to one another (Berlucchi, 2002).

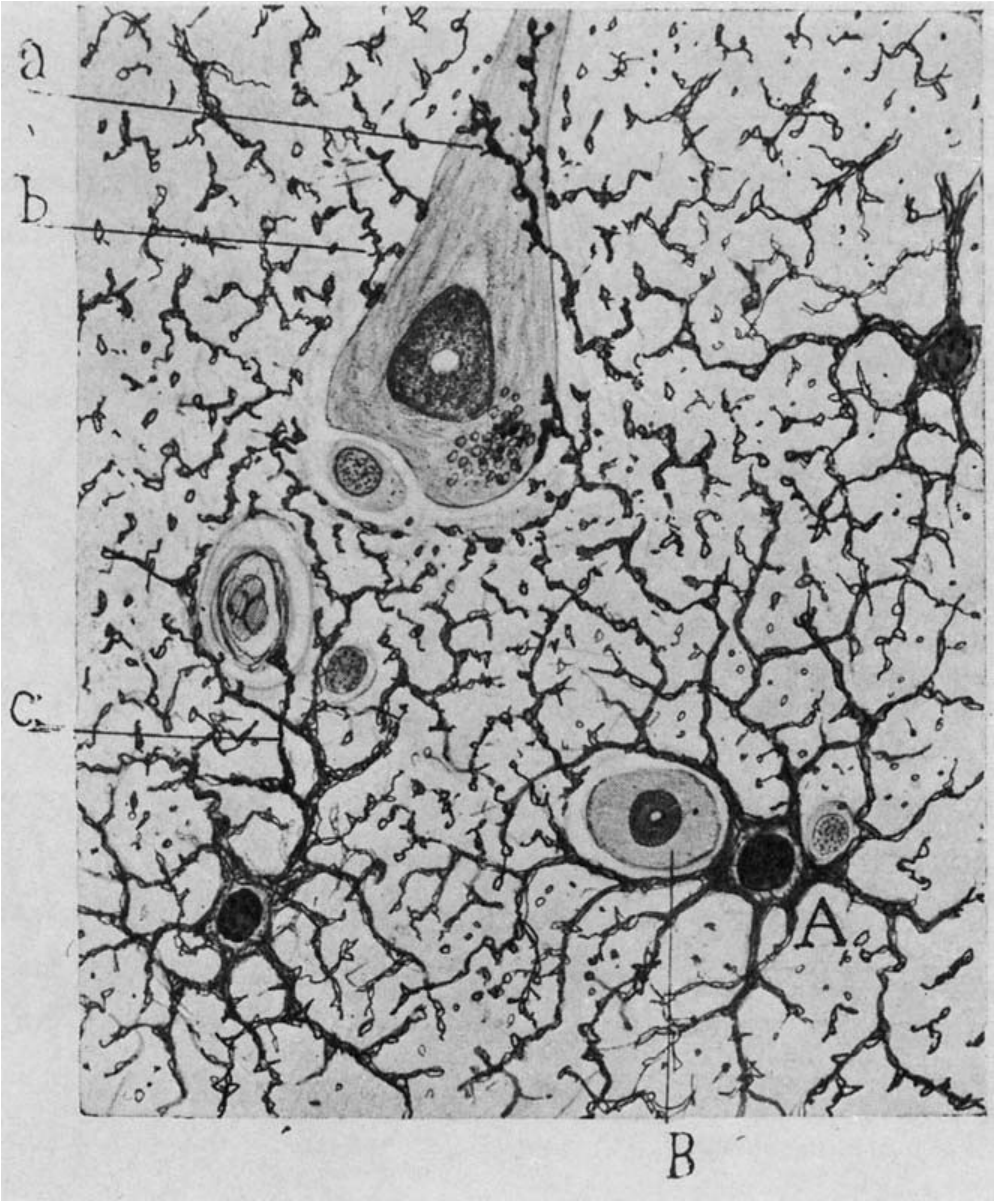

Figure 2 (Ramon y Cajal, 1913). *Astrocytes in the CNS*. Drawing by Ramon y Cajal showing protoplasmic astrocytes (A) with pericellular processes (a, b) and perivascular processes (c), and neurons (B).

On an interesting note, Lugaro's mentor, Eugenio Tanzi, believed that memory could have its basis in the strengthening of connections between neurons, perhaps by growth of the neuronal projections that decreased the space in between neuronal contact points

(Berlucchi, 2002), structures that would only later be characterized in more detail and be named “synapses”. Tanzi’s belief predicted modern-day studies on spine growth and dynamics during learning tasks.

In terms of imaging experimental technique, the field has advanced significantly, though this a relatively recent occurrence. In place of the Golgi stain we have powerful microscopic and fluorescent imaging tools, and have added the ability to manipulate astrocytic function through pharmacology and emerging genetic capabilities. It is fascinating though that conceptually, astrocyte biologists are only recently investigating (or perhaps more accurately, able to investigate) phenomena inferred by others over a century ago.

What seems perfectly clear, though, is that despite a paucity of solid evidence to justify their inferences the majority of early researchers in the field have always believed or assumed that glia are more than simply brain glue. Objectively, given what was known in the late 1800s and early 1900s, the alternative “brain glue” hypothesis was equally supportable. The assumption that astrocytes are more than simply passive or purely supportive brain elements has served as a guiding foundational principle for the field of glial biology ever since, despite its being rooted in unsubstantiated inference rather than hard facts. This idea will be investigated more thoroughly later in this chapter.

Astrocyte biology in the era of electrophysiology and biochemistry

Work primarily with insect nervous systems during the 1950s emphasized a metabolic relationship between glial cells and neurons. Histological analysis of central ganglia of *Rhodnius prolixus* revealed that, while there is no nutrient circulation into the depths of the ganglia, different types of glial cells “have extensive cytoplasm which surrounds the axons and dendrites” or “send deep tongue-like invaginations into the large

ganglion cells" (Wigglesworth, 1959). "Classical" glial cells and specialized perineurium cells within the insect nervous system were found to express high levels of metabolism-related enzymes such as esterases (Wigglesworth, 1958) and succinic dehydrogenase (Wigglesworth, 1956). Glycogen and triglycerides were also found in abundance in perineurium and glial cells, respectively (Wigglesworth, 1960). Interestingly, satellite cells of mammalian peripheral nervous system ganglia displayed a similar metabolic and enzymatic activity profile (Schmitt, 1958). This led researchers to believe that nutrients were stored in glial compartments and supplied to neurons via glial cells.

The advent of electrophysiological techniques in the middle part of the 20th century provided a novel method for studying the properties and functions of glial cells in the nervous system. It became appreciated that an important component of inter-neuronal communication is the action potential, a specialized cellular process driven by electrical and chemical gradients across neuronal membranes (Hodgkin and Huxley, 1952). Electrophysiology made it possible to study similar phenomena in glial cells. Do astrocytes communicate by electrical impulses similar to neurons? Might astrocytes influence neuronal activity through electrical activity?

Unlike neurons, astrocytes do not display rapid, impulse-like electrical signals. Instead, depolarizing stimuli result in graded, slow alterations in astrocytic membrane potential (Hild et al., 1958; Hild and Tasaki, 1962). These types of "glial responses" were evoked experimentally with electrical stimulus strengths exceeding those that would be produced by action potentials or other neuronal membrane potential changes (Hild and Tasaki, 1962). Direct neuronal stimulation, as opposed to field stimulation, proved insufficient to elicit impulse-like or slow electrical "glial responses" (Kuffler and Potter, 1964). Furthermore, in tissue cultures neuronal conduction was unimpaired in the absence of glial cells (Kuffler and Potter, 1964), demonstrating that astrocytes are not necessary for

successful neuronal conduction. These studies suggested a limited, supportive role for astrocytes in brain function.

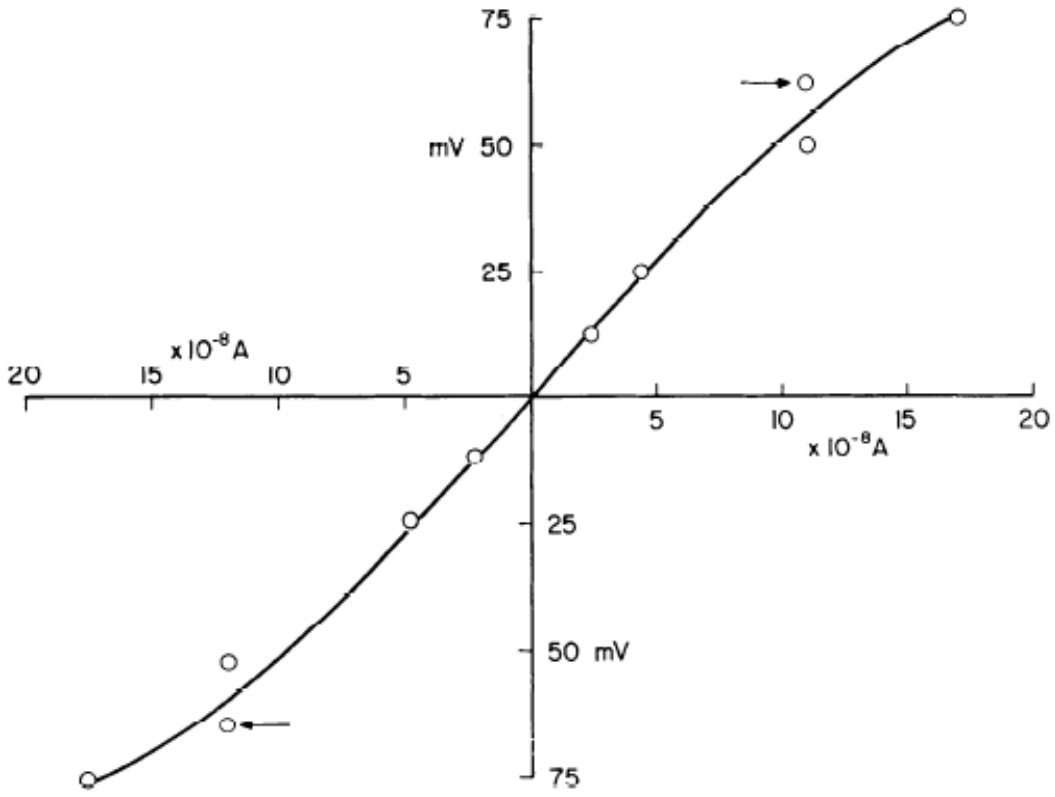

Figure 3 (Kuffler and Potter, 1964). *Passive electrical properties of astrocytes*. Connective glial cells in the leech CNS display a passive response to changes in membrane potential, indicated by a near-linear input resistance over a range of electrical potentials.

In hindsight, it would appear that electrical passivity was initially mistakenly interpreted as a lack of function. Numerous researchers referred to astrocytes as “idle”, “silent”, or “unresponsive” cells (Dennis and Gerschenfeld, 1969). At the very least, the idea that passive electrical properties of astrocytes are important for neuronal activity under normal conditions was dismissed. Instead, a connection was drawn between astrocytic

function and slow electrical processes such as potentials measured from the surface of the brain (what is measured in an electroencephalogram or EEG) or spreading depression (Hild et al., 1958), an abnormal condition.

The potential importance of other electrophysiological aspects of astrocytes was not immediately recognized. Compared to neurons, astrocytes have a notably low membrane resistance such that electrical currents produced by neurons would flow through glial cytoplasm rather than the extracellular space in between neurons and glia (Hild and Tasaki, 1962). Additionally, astrocytes possess special low-resistance connections (what would later be identified as gap junctions) that allow for direct electrical communication between cells (Kuffler and Potter, 1964). Similar intercellular connections were not found between neurons and glia (Kuffler and Potter, 1964). Several years following these results, a potential utility for these specialized electrical connections was inferred based on the realization that glial depolarization is the result of extracellular K^+ accumulation during neuronal firing (Orkland et al., 1966). The authors of this work argued that connected astrocyte networks could act as K^+ "spatial buffers", transporting extracellular K^+ away from areas of high concentration to areas of low K^+ concentration (Orkland et al., 1966). At the time it was unclear how this phenomenon, if true, could contribute to greater brain function. Even its functional importance as a homeostatic mechanism was understood to be limited: "Available experiments do not support the idea that currents in glial cells influence neurons…" (Orkland et al., 1966, p. 805).

The reality became much more clear and exciting with the seminal work of Newman and colleagues nearly two decades later. Retinal Muller glial cells, which have properties in common with cerebral astrocytes, were found to have regional specification with the majority of K^+ conductance located at structures called endfeet, which contact the vitreous humour of the eye (Newman, 1984). As a result of this regional specification, most K^+ taken up by retinal glial cells would be extruded into the vitreous, producing a plausible ionic buffering

mechanism (Newman, 1984). This cellular phenomenon was demonstrated to be a more effective method for buffering retinal K^+ levels than simple ionic diffusion through the extracellular space (Newman et al., 1984). Analogously, contacts between blood vessels and astrocytic processes within the cerebrum are nearly ubiquitous, a fact recognized by previously mentioned 19th century pioneers. This raised the possibility that similar mechanisms could operate within the brain as well. This suspicion was supported with the finding that endfeet of cerebral astrocytes possess roughly tenfold higher K^+ conductance than other cellular regions (Newman, 1986).

It was with this in mind that researchers took a bold conceptual step forward, trying to link individual cellular processes to greater brain functions and activities. As early as the year 1890, functional alterations in the blood supply to the brain had been documented (Roy and Sherrington, 1890). Roy and Sherrington (1890) proposed that "the brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of functional activity" (p. 105). Could K^+ extruded by astrocytes onto blood vessels during bouts of neuronal firing be the mechanism coupling neuronal activity to functional blood flow dynamics? Early mathematical modeling suggested it could (Paulson and Newman, 1987).

Glial biology was progressing on other fronts as well. Advancements in chemical and cell culture techniques led to an incredibly diverse volume of work in the 1970s and 1980s cataloguing diverse previously unrecognized functions of astrocytes. In large part, these studies were made possible by the ability to obtain pure primary non-neuronal (McCarthy and Partlow, 1976) and pure astroglial cell cultures (McCarthy and de Vellis, 1980), which offered significant advantages over other cell separation and purification methods.

One branch of research during this time period examined the expression of various classes of neurotransmitter receptors in cultured astrocytes. The downstream consequences of stimulating these receptors was typically examined by monitoring ion currents using

electrophysiology, performing ligand binding studies, or observing receptor-activated generation of intracellular second messengers such as cAMP or IP_3 (Murphy and Pearce, 1987). By these methods, many groups began describing astrocytic responsiveness to noradrenaline through alpha and beta adrenoceptors, acetylcholine through muscarinic receptors, histamine, 5-hydroxytryptamine (5HT, serotonin), dopamine, amino acids (glutamate, GABA), adenosine, peptides, prostanoids, and drugs such as benzodiazepines (reviewed in Murphy and Pearce, 1987).

Data from culture experiments indicated that astrocytes express nearly the same profile of receptors as neurons. In addition to metabotropic receptors coupled to the breakdown of membrane lipids (IP_3 generation, known to stimulate Ca^{2+} mobilization) or activation of adenylate cyclase and cAMP generation (reviewed in Murphy and Pearce, 1987), ionotropic receptors coupled to ion current flux were also found in cultured astrocytes. For instance, GABA-induced depolarization of cultured astrocytes (Kettenmann et al., 1984) were attributed to the activation of ionotropic $GABA_A$ receptors (Kettenmann and Schachner, 1985), which are expressed in many neurons.

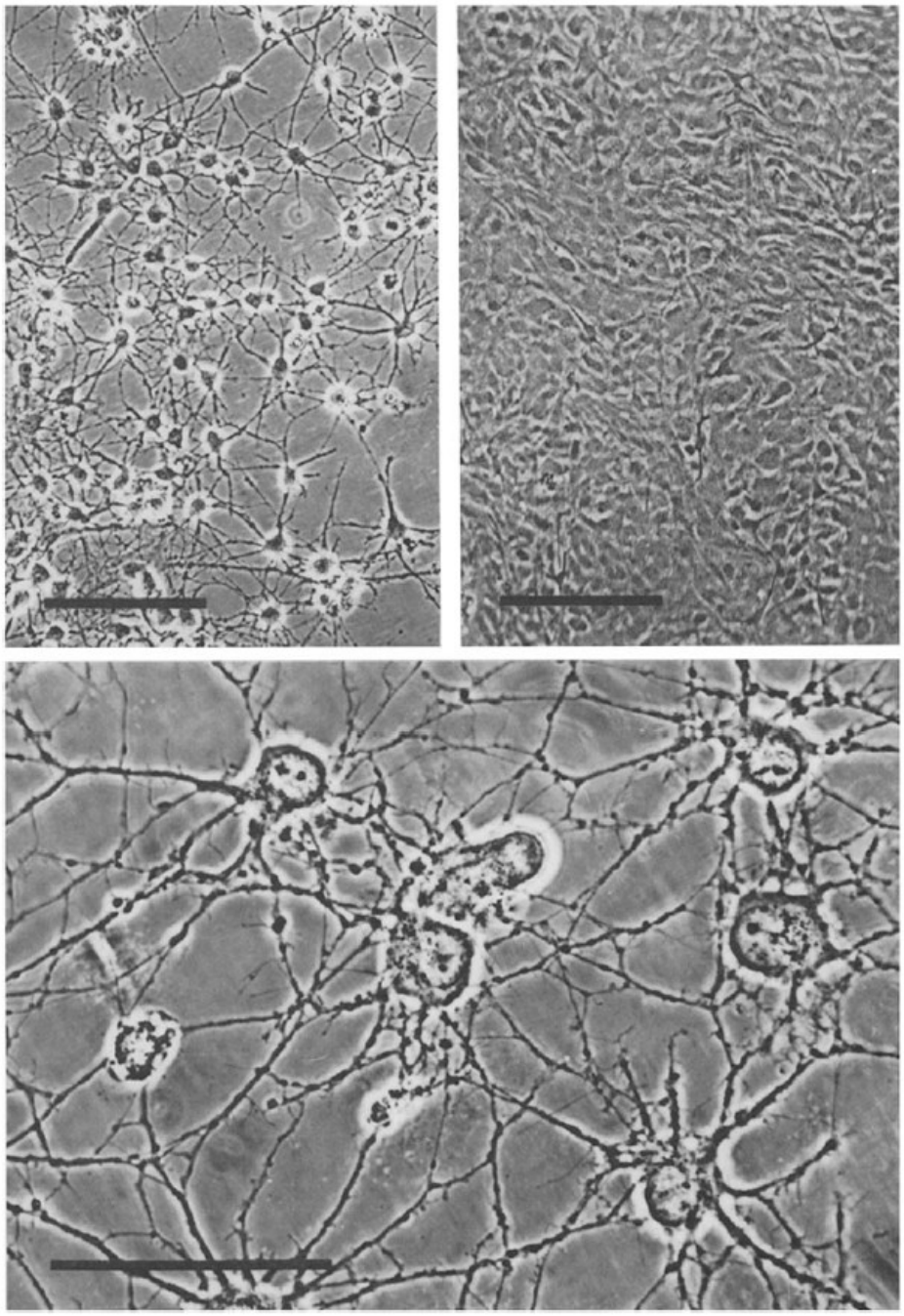

Figure 4 (McCarthy and de Vellis, 1980). *Primary glial cultures*. Phase contrast light micrographs of primary oligodendroglial cell (left) and astroglial cell cultures (right), with higher magnification image showing ramified oligodendroglial processes.

It was also recognized that astrocytes interacted with neurotransmitters in other ways besides receptor-mediated events. It was known that the brain has high affinity transport systems for the uptake of the primary neurotransmitters, glutamate and GABA, that were localized to synaptosomes (Logan and Snyder, 1972). Later work identified similar neurotransmitter uptake systems in glial cells (Henn, 1976; Schousboe, 1981). Over time, uptake mechanisms for a diverse range of neurotransmitters were detected within cultured astrocytes, including for serotonin (Katz and Kimelberg, 1985) and the catecholamines, noradrenaline and dopamine (Semenoff and Kimelberg, 1985). Glutamate-induced depolarization of cultured astrocytes (Kettenmann et al., 1984) were attributed to uptake mechanisms (Kettenmann and Schachner, 1985). More precise details on the electrogenic nature of glial neurotransmitter uptake mechanisms were characterized shortly thereafter (Brew and Attwell, 1987).

Despite the advantages of purified cultures and the experimental possibilities they opened up, it was also argued that isolated, cultured astrocytes should not necessarily be considered identical to their in vivo counterparts (Juurlink and Hertz, 1985). In particular, it was argued that cultured astrocytes express such a wide variety of receptors because they acquire de-differentiated properties or undergo arrested development due to the culture procedures or conditions, or isolation from other cells types (Murphy and Pearce, 1987). This concern appears to have been taken seriously. As other biochemical and imaging technologies became available in ensuing years, the use of primary astrocyte cultures gradually diminished. In the modern era most labs no longer make prominent use of the technique.

Regardless of the limitations of the primary methods used during this time period, great conceptual advances had been made throughout the field. No longer restricted to simple histology, researchers were exploring new facets of astrocyte biology, most notably astrocytic expression of neurotransmitter receptors. Additionally, there was further validation

of some previous suspicions, such as the idea that astrocytes store glycogen and maintain an important metabolic relationship with neurons that is governed in part by neuronal activity (Pentreath, 1982; Seal and Pentreath, 1985). The significance of these observations seems to have been unclear to many, and understandably so given the technical limitations of primary astrocyte cultures and novelty of the findings. Based simply on the available data, it would be accurate to frame astrocytic function solely in terms of homeostatic regulation. On the other hand, it would not necessarily be unreasonable to infer beyond the data and see a more dynamic brain element entirely – astrocytes as intrinsic and essential components for information processing or storage.

Time would tell.

Astrocytic Ca^{2+} signaling: The Great Glial Revolution arrives

The landscape of glial biology was massively transformed in the early 1990s because of the development of reliable cell-permeable chemical Ca^{2+} indicator dyes, in particular fluo-3 (Minta et al., 1989). This new generation of indicators provided significant advantages over previous dyes, such as fura-2, being excited by visible light as opposed to UV and offering greater convenience for fluorescence microscopy. It was now more tractable to examine, in real time, some of the signaling events downstream of astrocytic metabotropic receptor activation.

Prior to the release of fluo-3, one group had already characterized glutamate-induced elevations of intracellular Ca^{2+} in cultured astrocytes using fura-2 (Enkvist et al., 1989). These data confirmed a lingering suspicion within the field – that the breakdown of membrane lipids and generation of IP_3 following receptor activation is likely accompanied by release of Ca^{2+} from ER stores. But the real hype ignited with a study released the following

year, wherein researchers observed oscillatory elevations in cytosolic Ca^{2+} in primary cultured astrocytes (using fluo-3) which could propagate as waves throughout the entire astrocyte network (Cornell-Bell et al., 1990). According to the authors, “These propagating waves of calcium suggest that networks of astrocytes may constitute a long-range signaling system within the brain” (Cornell-Bell et al., 1990, p. 470).

Building on this work, it was demonstrated that in organotypic brain slices, in which local tissue structure and network architecture is preserved, astrocytes display similar single-cell Ca^{2+} elevations and multi-cell Ca^{2+} waves (Dani et al., 1992). These results clearly indicated, to the authors, “that astrocytes may have a much more dynamic and active role in brain function than has been generally recognized” (Dani et al., 1992). Additional work by other groups suggested that these astrocytic Ca^{2+} elevations often coincide with electrical bursts from neurons in co-cultures, but also that neuronal activity does not always elicit the relatively slowly propagating astrocytic Ca^{2+} signals (Murphy et al., 1993).

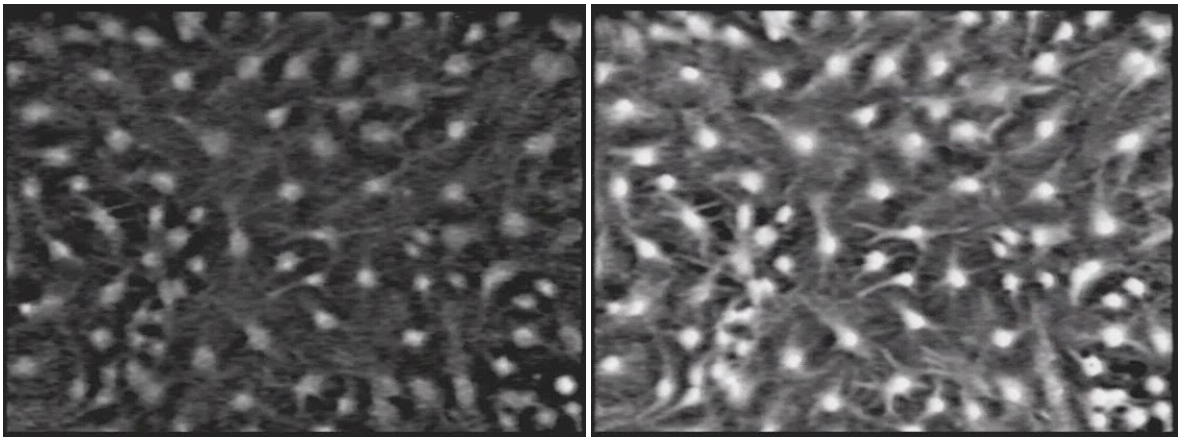

Figure 5 (Cornell-Bell et al., 1990). *Intercellular Ca^{2+} waves in cultured astrocytes*. Primary astrocyte cultures loaded with fluo-3 Ca^{2+} indicator dye before (left) and after (right) application of glutamate. Increased fluorescence intensity indicates an increase in free cytosolic Ca^{2+}.

After this point in time the focus of the greater glial biology community would shift almost exclusively to astrocytic Ca^{2+} signaling. At the deeper conceptual level astrocyte biology became synonymous with astrocytic Ca^{2+} signaling, much in the same way that neuronal function is understood through the action potential and synaptic release. This is ultimately the spirit of what I term the "Great Glial Revolution" – the paradigm shift from "astrocytes as nerve-glue" to "astrocytes as active partners in brain function". In most cases, astrocytic "activity" is understood in terms of Ca^{2+} dynamics. This framework will be explored more fully in Chapter 4.

Glial biology within the last two decades has seen a period of rapid conceptual and technical innovation. Cultures were replaced by acute slices, and gradually labs are developing in vivo experimental capability. Genetically-encoded Ca^{2+} indicator proteins like GCaMP have surpassed indicator dyes in sensitivity, signal-to-noise, and response amplitude, further facilitating sophisticated long-term in vivo studies. All of these developments helped to shape our current understanding of the diverse functions of astrocytes, specifically astrocytic Ca^{2+} signaling, in the brain.

Simultaneously though, the field has become known for the raw contention and starkly opposing beliefs among different investigators and groups on topics like gliotransmission (Fiacco et al., 2007; Agulhon et al., 2010) or the function of astrocytes in neurovascular coupling (Nizar et al., 2013; Takata et al., 2013), topics which will be discussed in more detail later in this chapter. This is a strange situation we find ourselves in. As the number of observations grows and as more "facts" accumulate, seemingly the less confident we are in what we "know" about astrocytes. It is as if there is an inverse relationship between volume of results obtained and knowledge gained – the opposite of what one would anticipate. This phenomenon will be addressed in Chapter 4.

THE MODERN ERA: PUTATIVE FUNCTIONS OF ASTROCYTES

Gliotransmission

Gliotransmission is the idea that astrocytes actively release substances in a Ca^{2+}-dependent manner for the purpose of modulating neuronal activity. Intrinsic to this notion is the concept of the “tripartite synapse” composed of the pre- and post-synaptic neuronal elements and the perisynaptic glial compartment.

In the first demonstration of signaling from astrocytes to neurons, researchers applied a focal electrical stimulation to single astrocytes, which prompted a spreading Ca^{2+} wave among multiple astrocytes and led to increases in intracellular Ca^{2+} in neurons resting atop the astrocyte layer (Nedergaard, 1994). A similar effect was noted by a separate group that same year (Parpura et al., 1994). Application of bradykinin to cultured astrocytes resulted in the release of glutamate into the culture medium, which was sufficient to induce elevations of neuronal Ca^{2+} through NMDA receptors (Parpura et al., 1994).

It was possible that these results were artifacts of astrocytes in culture conditions, perhaps due to functional dedifferentiation (Juurlink and Hertz, 1985). In an effort to address such concerns, several labs began testing for astrocytic Ca^{2+} elevations in response to neurotransmitter receptor stimulation in acute slice preparations in which local brain network architecture is preserved. Agonists of adrenergic receptors were reported to evoke Ca^{2+} elevations in hippocampal astrocytes in situ (Duffy and MacVicar, 1995). Electrical stimulation of the hippocampal Schaffer collateral neuronal fiber pathway similarly induced elevations of astrocytic Ca^{2+} within the stratum radiatum of the CA1 region that were dependent on metabotropic glutamate receptor activity (Porter and McCarthy, 1996). It was subsequently demonstrated that astrocytes in situ displayed Ca^{2+} elevations in response to a variety of neurotransmitters including GABA (Kang et al., 1998), acetylcholine (Shelton

and McCarthy, 2000; Araque et al., 2002), and histamine (Shelton and McCarthy, 2000). This proved true when washing on receptor agonists or stimulating fiber pathways to evoke synaptic release of neurotransmitters.

Astrocytic Ca^{2+}-induced release of glutamate could produce a diverse range of effects on neuronal activity. Most notably, astrocytic Ca^{2+} elevations led to NMDAR-dependent slow inward currents (SICs) in adjacent neurons and reduce the magnitude of excitatory and inhibitory post-synaptic currents (Araque et al., 1998b). Astrocytic Ca^{2+} elevation could also alter the frequency of miniature post-synaptic potentials (Araque et al., 1998a). These data opened the possibility for modulation of neuronal network dynamics via astrocytic Ca^{2+} signaling in response to neuronal activity. For instance, interaction of astrocytes and inhibitory neurons could lead to astrocyte-mediated potentiation of miniature inhibitory post-synaptic currents in pyramidal neurons (Kang et al., 1998). Results such as these gave rise to the notion of the “tripartite synapse” in which astrocytes constitute an equal partner with pre- and post-synaptic neuronal elements (Araque et al., 1999).

Initially it was unclear the mechanism by which astrocytic Ca^{2+} elevation led to changes in neuronal activity. One early study concluded that gap junction-mediated communication was important for astrocyte-to-neuron communication (Nedergaard, 1994). In contrast, other studies suggested a strictly Ca^{2+}-dependent release mechanism (Parpura et al., 1994), perhaps involving Ca^{2+}-dependent exocytosis events similar to those operating in neuronal synaptic release (Jeftinija et al., 1996).

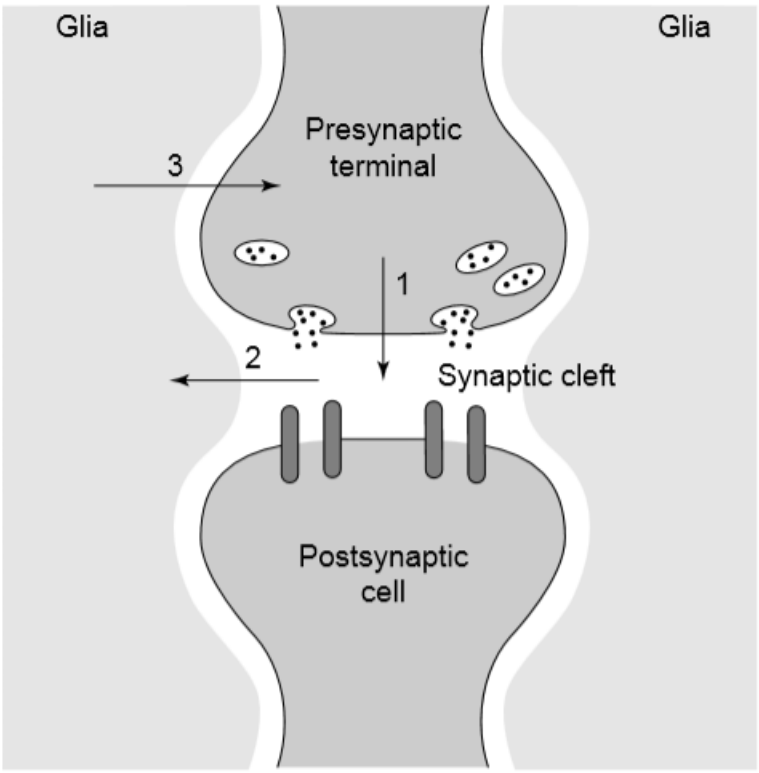

Figure 6 (Araque et al., 1999). *The tripartite synapse*. Neurons signal to one another via neurotransmitter release at the synaptic cleft (1). Neurotransmitter spillover during neuronal firing is detected by astrocytes surrounding in the synapse (2). Astrocytes signal back to neurons (3), thereby modulating neuronal activity as active partners in synaptic transmission.

Cultured astrocytes were previously shown to express certain proteins associated with neuronal vesicular release such as synaptobrevin II and syntaxin (Parpura et al., 1995). Results were later reported that injection of the light chain of the Botulinum B neurotoxin, which selectively cleaves the vesicle-associated SNARE protein synaptobrevin, into astrocytes inhibited the astrocyte-induced glutamate response in neighboring neurons in culture (Araque et al., 2000). Electron micrographs indicated that astrocytes express vesicular glutamate transporters (VGLUT1/2) and SNARE proteins on small vesicular compartments adjacent to neuronal structures (Bezzi et al., 2004).

While Ca^{2+}-dependent exocytosis is traditionally the primary mechanism believed to underlie gliotransmission-like effects, other non-vesicular release pathways have been proposed as well. These include reversal of glutamate transporters (Szatkowski et al., 1990), release from volume-sensitive membrane channels (Kimelberg et al., 1990; Takano et al., 2005), release through functional hemichannels composed of gap junction subunits (Ye et al., 2003), lysosomal exocytosis (Zhang et al., 2007), and release through other membrane channels such as the two-pore K^+ channel TREK-1 or the Ca^{2+}-activated anion channel BEST1 (Woo et al., 2012).

In many ways, studies examining gliotransmission have been the driving force behind progress in glial biology for decades now. In particular, this area of research pushed the further characterization of astrocytic Ca^{2+} dynamics. Questions about whether astrocytes are involved in modulating neuronal activity date back to the very beginnings of glial biology.

However, fewer ideas within the glial field are more hotly contested and controversial than gliotransmission. On the surface, this is due in large part because of discrepant results between different studies and investigative groups. For instance, uncaging IP_3 within astrocytes to evoke Ca^{2+} elevations is sufficient to increase the frequency of AMPA spontaneous excitatory post-synaptic currents (Fiacco and McCarthy, 2004; Fiacco et al., 2007). But evoking astrocytic Ca^{2+} by expressing and stimulating an exogenous Gq-GPCR does not alter neuronal activity (Fiacco et al., 2007; Wang et al., 2013a). Clamping astrocytic Ca^{2+} with BAPTA blocks long-term potentiation (LTP) in neighboring neurons (Henneberger et al., 2010). Yet a genetic mouse line lacking astrocytic IP_3R-dependent Ca^{2+} elevation and displays no observable Ca^{2+} signaling, the IP_3R2 KO, has normal basal neuronal activity (Petravicz et al., 2008) and plasticity (Agulhon et al., 2010). In contrast, a mouse line inducibly expressing a dominant negative synaptobrevin II in astrocytes (dn-SNARE) to prevent Ca^{2+}-dependent exocytosis produced defects in synaptic transmission and plasticity related to the inability of astrocytes to release adenosine

(Pascual et al., 2005). However, a separate genetic model designed to block astrocytic exocytosis using tetanus neurotoxin (TeNT) found no effect on basal synaptic activity (Lee et al., 2014). Furthermore, not all studies are in agreement on whether astrocytes express the necessary Ca^{2+}-dependent exocytosis machinery (Li et al., 2013).

These discrepancies are not minor. Rather many of these observations are flatly inconsistent with the idea that astrocytes actively regulate neuronal activity in a Ca^{2+}-dependent manner, especially if Ca^{2+} elevations are a function of Gq-GPCR and downstream IP_3R activity. The prominent belief among many investigators is that discrepant results are due to technical limitations (Agulhon et al., 2008; Volterra et al., 2014) or inevitable given the complexity of the brain and the processes under study (Araque et al., 2014; Volterra et al., 2014). In general the field holds that astrocytic Ca^{2+} signaling plays an incredibly important and complex role in modulating neuronal activity (see Figure 8), though this may occur by a diverse set of mechanisms.

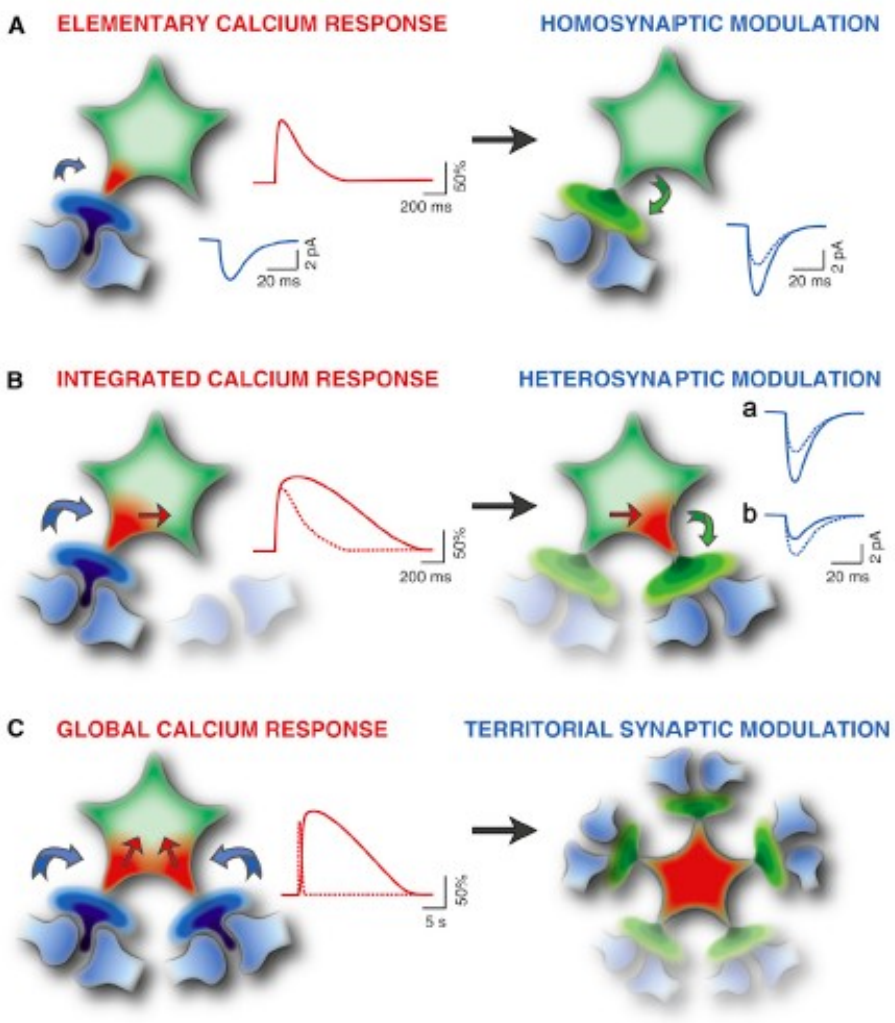

Figure 7 (Volterra et al., 2014). *Current thinking on astrocytic Ca^{2+} and synaptic modulation*. Different types of Ca^{2+} signals can be evoked in astrocytes depending on the nature and strength of local synaptic activity. In turn, these varying astrocytic Ca^{2+} elevations could function on diverse spatial or temporal scales to modulate activity of individual neurons or neuronal networks.

Astrocyte-to-Neuron Lactate Shuttle (ANLS)

Neurons in the brain do not directly contact the vascular surface (Mathiisen et al., 2010; McCaslin et al., 2011), or if they do this contact is extremely minimal. This creates a barrier for delivery of nutrients and metabolites from the blood stream to neurons. Astrocytes maintain close contact with essentially every cellular component of the brain including blood

vessels. Based on this fact, early researchers inferred that astrocytes might play the critical role of “feeding” neurons. Some of the initial studies examining this possibility in insect model systems were consistent with the idea (Wigglesworth, 1956, 1958, 1959, 1960).

The idea gained substantial traction with the finding that astrocytic glutamate uptake stimulates the utilization of glucose and production of lactate through aerobic glycolysis in cultured astrocytes (Pellerin and Magistretti, 1994). This was significant when considered alongside the previous finding that lactate can sustain neuronal activity in brain slices in the absence of glucose (Schurr et al., 1988). It was also becoming appreciated that glycogen, a stored form of glucose within cells, is primarily localized to astrocytes in brain (Ignacio et al., 1990). Subsequent histochemical studies characterized the expression profiles of lactate dehydrogenase enzymes (Bittar et al., 1996; Pellerin et al., 1998) and monocarboxylate transporters (Pellerin et al., 1998) in neurons and astrocytes. These data were consistent with the idea that astrocytes might generate lactate for neuronal consumption.

In vivo, the activity-dependent release of lactate in striatum was shown to be dependent on glutamate uptake when microdialysis was used to measure lactate levels and deliver glutamate uptake inhibitors in behaving rodents (Demestre et al., 1997). ^{13}C nuclear magnetic resonance spectroscopy revealed that neurons metabolize lactate in vivo (Hassel and Brathe, 2000). In acute brain slices, synaptic function was maintained preferentially by endogenous monocarboxylic glycolytic intermediates like lactate or pyruvate as opposed to endogenous glucose during bath glucose deprivation (Izumi et al., 1997). These various data supported the hypothesis that neuronal metabolism is fueled by lactate released by astrocytes following astrocytic breakdown of glucose via glycolysis (Magistretti and Pellerin, 1999).

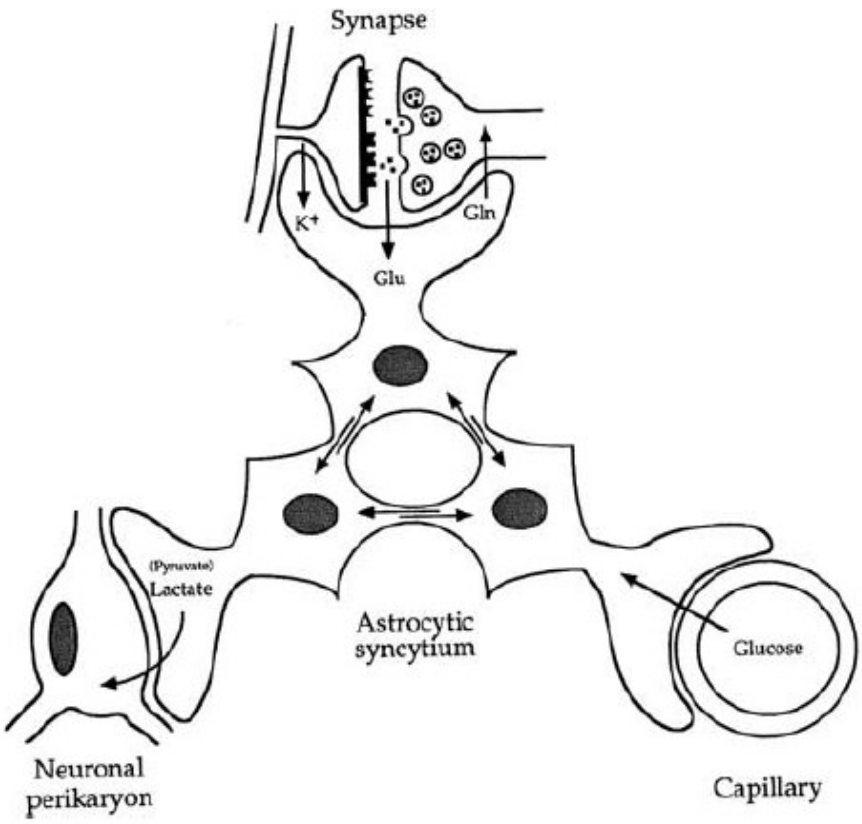

Figure 8 (Pellerin and Magistretti, 2012). *Basis of astrocyte-neuron lactate shuttle*. Glutamate uptake by astrocytes near synapses stimulates astrocytic glucose uptake and utilization by glycolysis. Lactate (or pyruvate) produced through glycolysis can be released to fuel neuronal metabolism.

However, the astrocyte-neuron lactate shuttle (ANLS) hypothesis was called into question by other groups. Interestingly, the initial observation of glutamate-induced utilization of glucose by astrocytes in culture was found to be heavily dependent on culture conditions, with many conditions yielding negative results (Hertz et al., 1998). Analyses of enzyme kinetics and substrate availability within the brain appeared inconsistent with ANLS (Chih et al., 2001).

Additional studies making use of novel techniques swayed the general opinion to favor ANLS. Multiphoton imaging of native NADH fluorescence in brain slices revealed a partitioning of glycolytic and oxidative metabolism between astrocytes and neurons, with

dendrites displaying immediate oxidative metabolism during periods of activity followed by delayed astrocytic glycolysis (Kasischke et al., 2004). Activity-stimulated glucose utilization observed in real time in vivo using blood-born fluorescent glucose analogues like 6-deoxy-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose (6-NBDG) indicated that astrocytic glucose uptake was markedly increased during brain activity whereas neuronal glucose uptake remained constant (Chuquet et al., 2010). The loss of neuronal activity caused by systemic insulin-induced hypoglycemia was prevented by infusion of lactate into the brain (Wyss et al., 2011). Brain activity led to an increase in lactate oxidation in vivo, and lactate was preferred as an energy substrate when both lactate and glucose were available (Wyss et al., 2011).

The importance of lactate shuttling to neurons was explored in two recent in vivo studies. Glycogenolysis and lactate release by astrocytes were found to be essential for long-term memory formation and LTP in the hippocampus (Suzuki et al., 2011). Impeding astrocyte-neuron lactate transport by disrupting monocarboxylate transporter expression produced amnesia, but could be reversed with application of exogenous lactate provided that neuronal lactate uptake was intact (Suzuki et al., 2011). Similar effects were noted when glycogenolysis was impaired pharmacologically (Newman et al., 2011), suggesting that astrocytic glycogen breakdown and lactate release are necessary for memory formation. Importantly, application of lactate could rescue memory deficits but application of glucose could not, suggesting that neurons preferentially use lactate as an energy source during physiological activity (Newman et al., 2011; Suzuki et al., 2011). Lactate might have other functions in the brain other than as a metabolic substrate or byproduct. In the locus coeruleus, astrocyte-derived lactate can excite neurons to release noradrenaline independent of lactate uptake into neurons and involving a cAMP-dependent step (Tang et al., 2014).

Not all observations agree with the astrocyte-neuron lactate shuttle hypothesis, however. Lactate release from astrocytes depends on glucose oxidation through glycolysis, but there is evidence that oxidative metabolism and glutamate oxidation are both used to fuel astrocyte metabolism (Hertz et al., 2007; Dienel, 2013). Many perisynaptic astrocytic processes contain mitochondria, permitting glutamate oxidation for ATP generation (Dienel, 2013). In a number of different experimental preparations neurons are capable of upregulating glucose transport and glycolytic processes to meet energy needs (Dienel, 2012). There is a net release of lactate from the brain during periods of activity, suggesting lactate is not locally oxidated (Dienel, 2012, 2013). Mathematical modeling indicates that astrocytic glycogen breakdown serves the purpose of preserving extracellular glucose levels for neuronal glycolysis and oxidative metabolism, rather than generation of lactate for shuttling to neurons (DiNuzzo et al., 2010, 2012). An alternative hypothesis to ANLS which considers these data states that lactate might be an “opportunistic”, glucose-sparing substrate under the proper conditions or in certain circumstances, but that glucose is probably the major fuel for neurons (Dienel, 2012).

It should be noted that the debate on the validity of ANLS is primarily over whether or not lactate is the preferred fuel source for neurons under normal conditions. Astrocytes are understood to play a critical role in maintaining neuronal energy demand, though whether this is through lactate shuttling, glucose shunting, or other mechanisms is unclear.

Gap junction coupled astrocyte networks

While it was known that direct, low-resistance connections exist between glial cells (Kuffler and Potter, 1964), the first observation of gap junctions in astrocyte membranes was made later using freeze-etching technique (Dermietzel, 1974), with functional dye-coupling

confirmed in brain slices (Gutnick et al., 1981). Astrocytes are extensively connected via gap junctions, which allow the passage of ions and electrical currents, and small molecules less than 1000 Da in size (Giaume and McCarthy, 1996). Astrocyte coupling was initially proposed as a syncitium-like organization, although focus within the field has shifted away from this idea towards functional and plastic networks that operate in concert with neuronal networks (Giaume and Liu, 2012).

One important aspect of astrocytic networks is that gap junctional communication between astrocytes is modified by neuronal activity (Rouach et al., 2004). This phenomenon was first reported in an astrocyte-neuron co-culture system, in which dye- and electrical-coupling between astrocytes was enhanced by the presence of cerebellar neurons (Fischer and Kettenmann, 1985). Dye-coupling was increased in astrocytes after stimulation of the frog optic nerve (Marrero and Orkland, 1996). Conditions that are known to induce plasticity or significant neuronal activity changes, such as an enriched environment, increased expression of genes related to astrocytic coupling, like Cx30, in addition to genes related to neuronal structure and activity (Rampon et al., 2000). A direct relationship between neuronal activity and astrocytic gap junctional communication was demonstrated in striatal astrocyte-neuron co-cultures. The presence of neurons in the culture augmented the degree of functional coupling between astrocytes, as assessed by intercellular Ca^{2+} wave propagation (Rouach et al., 2000). Modulation of gap junctional communication could also be modulated in the reverse direction. The removal of cultured neurons by application of neurotoxin or suppression of spontaneous neuronal activity with pharmacological agents both decreased glial coupling (Rouach et al., 2000).

The anatomic and functional compartmentalization of neuronal networks appears to be mirrored by those of astrocyte networks. The barrel field of the rodent somatosensory cortex contains well-defined compartmentalization, with characteristic Layer IV cortical “barrels” in which input from single whiskers is processed. Dye coupling and

immunochemical data indicate that interconnected astrocyte networks are also arranged into barrels. In particular, gap junctional communication is much stronger within barrels than between barrels, and genetic models that lack neuronal barrel organization also lack astrocytic barrel organization (Houades et al., 2008). Similar structural segregation was observed in the olfactory bulb (Roux et al., 2011), where single glomeruli process input derived from single odorants. Suppression of neuronal activity in vivo by application of tetrodotoxin (TTX) or sensory deprivation reduces intraglomerular gap junctional coupling by modulation of Cx30, but not Cx43, through a mechanism dependent on extracellular K^+ and K^+ influx into astrocytes (Roux et al., 2011).

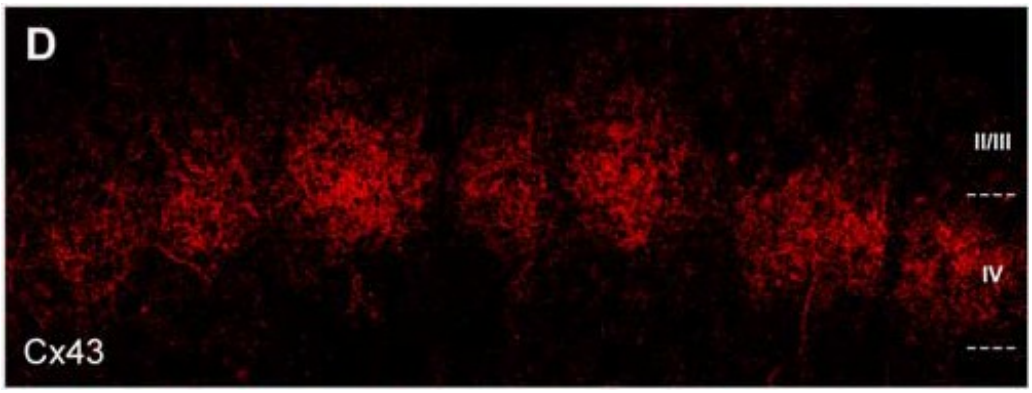

Figure 9 (Houades et al., 2008). *Gap junction-coupled astrocyte networks.* Astrocytes are extensively coupled by gap junctions composed of Cx43 and Cx30 that allow intercellular passage of ions and small molecules (<1000 Da). The structural organization of astrocytic coupling often mirrors local neuronal architecture, as in rodent barrel cortex above.

The importance of gap junctional coupling within astrocyte networks is still being explored, but initial studies highlight the role of intercellular astrocyte communication in metabolic support and regulation of basal synaptic transmission. Glucose or other metabolic substrates can be transported throughout astrocyte networks via gap junctions. In the

absence of extracellular glucose, coupled astrocyte networks are able to maintain neuronal activity by shuttling glucose or lactate across multiple cells to areas of energetic demand (Rouach et al., 2008). In contrast, neuronal activity cannot be maintained in a genetic mouse model lacking astrocytic Cx43 and Cx30 (Rouach et al., 2008). Intercellular glucose transport could possibly be a function of Na^{2+} currents and intercellular Na^{2+} waves generated by astrocytic glutamate uptake (Bernardinelli et al., 2004). The nature of evoked astrocytic Na^{2+} signals in acute slices depends on the strength of stimulation, with Na^{2+} transients propagating across multiple astrocytes with stronger stimulus intensities (Langer and Rose, 2009). Further, spread of Na^{2+} throughout astrocyte networks is impaired by interference with gap junctional communication and absent altogether in the Cx43, Cx30 double KO mouse line (Langer et al., 2012), indicating that astrocytic Na^{2+} signals travel intercellularly through gap junctions.

Interfering with astrocytic gap junctional coupling also leads to defects in synaptic transmission including increased basal transmission and release probability, impaired LTP, and enhanced LTD (Pannasch et al., 2011). These defects are attributed to increases in extracellular glutamate and K^+ levels, which are in turn due to slower clearance rates by Cx43, Cx30-deficient astrocytes (Pannasch et al., 2011), which is consistent with impairments in Na^{2+} uptake or intercellular dissipation noted in other studies (Langer et al., 2012). Synaptic transmission in mice with deficient astrocytic gap junctional coupling could also altered by changes in the ability of astrocyte networks to buffer extracellular K^+ levels. For example, while radial K^+ redistribution in hippocampal slices does not require intact astrocytic coupling, the presence of gap junctions facilitates K^+ clearance and increases the threshold for epileptiform events (Wallraff et al., 2006).

Astrocytes also form heterotypic Cx47/43 or Cx32/30 gap junctions with oligodendrocytes (Orthmann-Murphy et al., 2007), the myelin-forming glial cells of the central nervous system. A dysmyelinating phenotype and vacuolation are observed in the

Cx43, Cx30 double KO mouse brain (Lutz et al., 2009). These findings suggest that intercellular communication among coupled astrocyte networks or between astrocytes and oligodendrocytes is critical for myelin integrity and therefore for neuronal function beyond the synapse.

The majority of studies in the field of glial biology have considered astrocytes as single units, insofar as experimental design is concerned – for example, uncaging ions or molecules in single astrocytes. Functional coupling between astrocytes as part of larger functional networks is one of the most intriguing and unique characteristics of these cells. Though it is still a relatively unexplored area, available data already indicates that gap junctional communication is likely a central component of astrocytic function in the brain.

Neurovascular Coupling

As the brain processes information, blood flow to active regions is increased. This vascular phenomenon is termed functional hyperemia and also commonly referred to as neurovascular coupling. It is believed to occur to keep pace with increased neuronal metabolic demand, replenishing glucose and oxygen that is consumed at the onset of activity. While the first observation of functional hyperemia was made by Roy and Sherrington in the year 1890, the mechanisms of neurovascular coupling are still debated.

The first tractable hypothesis put forward posited that glial K^+ siphoning from synaptic compartments to the vascular surface linked neuronal activity to changes in vascular tone and blood flow (Paulson and Newman, 1987). Focus promptly shifted however, with work in the 1980s and 1990s establishing that astrocytes express a wide variety of G protein-coupled receptors (GPCRs) that signal through the major G protein

systems (Gq, Gs, Gi) that could be stimulated by neuronal activity in primary cultures or *in situ* (Murphy and Pearce, 1987; Porter and McCarthy, 1997).

Further studies revealed that large astrocytic Ca^{2+} signals can cause arteriole dilations or constrictions in acute slices or retinal preparations. Specifically, stimulating neuronal activity (Zonta et al., 2003; Metea and Newman, 2006; Straub et al., 2006) or activation of astrocytic Gq-GPCRs using agonists (Zonta et al., 2003; Mulligan and MacVicar, 2004) or directly uncaging Ca^{2+} or IP_3 in single astrocytes (Mulligan and MacVicar, 2004; Straub et al., 2006) leads to slow vascular changes correlated to evoked Ca^{2+} elevations in astrocytes. Interfering with astrocytic signals using mGluR antagonists (Zonta et al., 2003), inhibitors of ER Ca^{2+} release (Straub et al., 2006) or injecting individual astrocytes with BAPTA (Mulligan and MacVicar, 2004) decreases the magnitude of the evoked vascular changes. Similar results were obtained when uncaging Ca^{2+} in perivascular astrocyte endfoot structures in vivo (Takano et al., 2006). Astrocytic Ca^{2+} elevations activate arachidonic acid metabolism and release of various vasoactive products (Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Takano et al., 2006; Petzold et al., 2008).

There is evidence though that multiple pathways contribute to neurovascular coupling. A Ca^{2+}-dependent K^+ current can be stimulated in endfeet that can dilate or constrict arterioles depending on the magnitude of the Ca^{2+} signal (Filosa et al., 2006; Girouard et al., 2010). In vivo, manipulations that interfere with glial glutamate uptake also blunt vascular responses (Gurden et al., 2006; Schummers et al., 2008), although it is unclear how much of this effect might be indirect – for instance, due to modulation of astrocytic Ca^{2+} excitability.

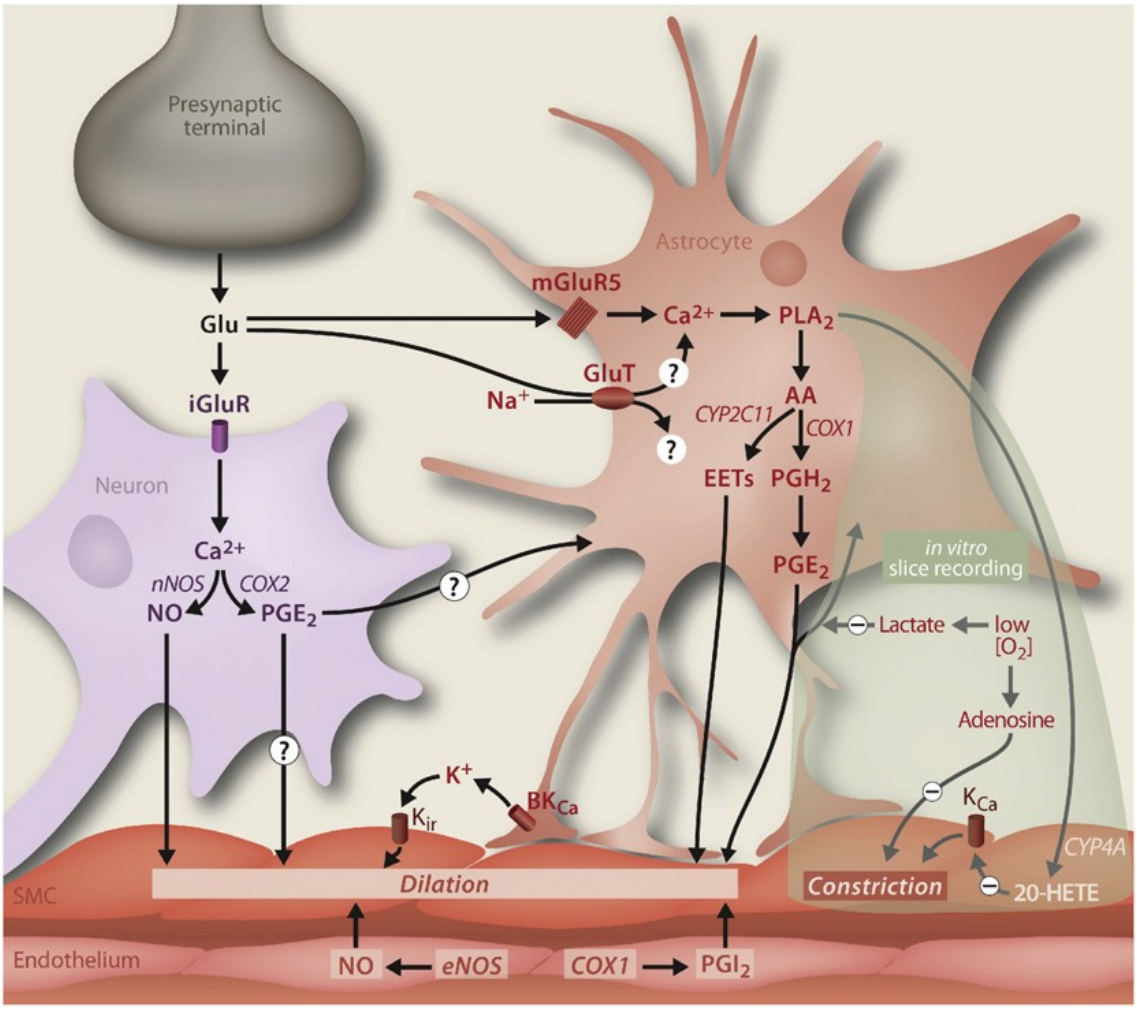

Figure 10 (Petzold and Murthy, 2011). *Current model of neurovascular coupling.* Neuronal activity stimulates astrocytic Gq-GPCRs leading to Ca^{2+} elevation and Ca^{2+}-dependent release of vasoactive arachidonic acid metabolites. This model is based largely on in situ studies.

However, not all experimental results are in agreement with the current neurovascular coupling model. In general, the onset of astrocytic Ca^{2+} response in vivo trails the stimulus onset by at least a full second and up to several seconds depending on the study (Wang et al., 2006; Schummers et al., 2008; Nimmerjahn et al., 2009). The blood flow increase, on the other hand, begins within the first second following the stimulus (Devor et al., 2007; Tian et al., 2010). Most studies have demonstrated that evoking a Ca^{2+} signal in

astrocytes can cause arteriole dilations (Zonta et al., 2003; Mulligan and MacVicar, 2004; Filosa et al., 2006; Metea and Newman, 2006; Takano et al., 2006; Girouard et al., 2010) or shown separately that physiological stimulus evokes blood flow increases (Kleinfeld et al., 1998; Devor et al., 2007; Tian et al., 2010) or astrocytic Ca^{2+} elevations (Wang et al., 2006; Petzold et al., 2008; Schummers et al., 2008; Nimmerjahn et al., 2009). A primary prediction of the model is that perivascular astrocytic Ca^{2+} elevation should precede the vascular response. Much of the available correlative in vivo data do not support this.

Recent studies have tested the causal relationship between astrocytic Ca^{2+} signals and vascular responses in vivo. Electrical forepaw stimulation in anesthetized mice led to reliable dilations of cortical arterioles, but adjacent astrocyte endfeet displayed inconsistent Ca^{2+} elevations (Nizar et al., 2013). When Ca^{2+} elevations were observed, they were delayed relative to the onset of blood flow increase (Nizar et al., 2013). Further, vascular responses to stimuli in vivo are preserved in the IP_3R2 KO mouse model, in which astrocytic IP_3R-dependent Ca^{2+} signaling is eliminated (Nizar et al., 2013; Takata et al., 2013). In contrast, a separate study described rapid astrocytic Ca^{2+} signals, the amplitude of which correlate well with the amplitude of evoked blood flow increases (Lind et al., 2013). Traditional microscopic frame imaging (1-2 Hz) is too slow to detect these rapid signals. Furthermore, in vivo experiments with anesthetized animals are confounded by the possibility that astrocytic signaling is directly impaired by anesthesia (Thrane et al., 2012).

The purpose of the present thesis research project was to, on a general level, address critical gaps within this field and specifically to directly test the hypothesis that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling mediates neurovascular coupling in awake mice. Our lab has developed key genetic tools that allow for selective control of astrocytic Gq-GPCR and Ca^{2+} signaling in awake animals; a previously unattainable degree of in vivo experimental control. The Gq-DREADD (Designer Receptor Exclusively Activated by Designer Drugs) system entails a mutated muscarinic receptor that lacks affinity for

endogenous ligands, does not display significant basal activity, and is activated by a blood-brain barrier-permeable compound, Clozapine-N-Oxide (CNO), that does not stimulate endogenous receptors (Armbruster et al., 2007). This system allows for activation of Gq-GPCR signaling selectively in astrocytes in vivo simply by intraperitoneal injection of CNO. The IP_3R2 KO mouse model lacks the IP_3 receptor subtype expressed by astrocytes, effectively eliminating Ca^{2+} release from the ER in astrocytes without affecting neuronal function (Petravicz et al., 2008; Agulhon et al., 2010). Our results (Bonder and McCarthy, 2014) corroborate and expand upon those of recent in vivo studies (Nizar et al., 2013; Takata et al., 2013), further suggesting that our current understanding of neurovascular coupling is not correct.

CHAPTER 2: THESIS RESEARCH

Experimental evidence demonstrating that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling does not mediate neurovascular coupling in mouse visual cortex in vivo

OVERVIEW

Local blood flow is modulated in response to changing patterns of neuronal activity (Roy and Sherrington, 1890), a process termed neurovascular coupling. It has been proposed that the central cellular pathway driving this process is astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling, though in vivo tests of this hypothesis are largely lacking. We examined the impact of astrocytic Gq-GPCR and IP_3R-dependent Ca^{2+} signaling on cortical blood flow in awake, lightly sedated, responsive mice using multiphoton laser-scanning microscopy and novel genetic tools that enable the selective manipulation of astrocytic signaling pathways in vivo. Selective stimulation of astrocytic Gq-GPCR cascades and downstream Ca^{2+} signaling with the hM3Dq DREADD designer receptor system was insufficient to modulate basal cortical blood flow. We found no evidence of observable astrocyte endfeet Ca^{2+} elevations following physiological visual stimulation despite robust dilations of adjacent arterioles using cyto-GCaMP3 and Lck-GCaMP6s, the most sensitive Ca^{2+} indicator available. Astrocytic Ca^{2+} elevations could be evoked when inducing the startle response with unexpected air puffs. However, startle-induced astrocytic Ca^{2+} signals did not precede corresponding startle-induced hemodynamic changes. Further,

neurovascular coupling was intact in lightly sedated, responsive mice genetically lacking astrocytic IP_3R-dependent Ca^{2+} signaling (IP_3R2 KO). These data establish that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling does not mediate neurovascular coupling in visual cortex of awake, lightly sedated, responsive mice.

INTRODUCTION

Understanding how cerebral vascular dynamics are coupled to neuronal activity is of intense scientific and clinical interest. Blood flow changes serve as the basis for functional MRI (Kim and Ogawa, 2012), which is the best method for noninvasively imaging human brain activity in real time. The underlying cellular and molecular mechanisms driving neurovascular coupling have remained elusive and controversial (Mishra et al., 2011; Nizar et al., 2013; Takata et al., 2013).

Substantial evidence utilizing pharmacological approaches to manipulate astrocytic signaling in situ supports the hypothesis that functional hemodynamics are mediated by Gq-GPCR-linked Ca^{2+}-dependent processes (Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Straub et al., 2006; Gordon et al., 2008; Girouard et al., 2010; He et al., 2012; Stobart et al., 2013). It remains unknown to what degree functional hemodynamics in the acute slice mirror processes of neurovascular coupling in vivo. It was recently reported that cortical arteriole dilations in anesthetized mice occur in the absence of observable Ca^{2+} elevations in adjacent astrocyte compartments (Nizar et al., 2013).

It is also unclear whether traditional experimental tools reveal physiology or artifacts of non-physiological manipulations (Fiacco et al., 2007; Agulhon et al., 2010; Wang et al., 2013a). Whereas chelating astrocytic Ca^{2+} blocks neurovascular coupling in situ (Mulligan and MacVicar, 2004; Gordon et al., 2008), functional hyperemia is intact in anesthetized IP_3R2 KO mice (Nizar et al., 2013; Takata et al., 2013), in which astrocytic IP_3R-dependent Ca^{2+} signaling is eliminated (Petravicz et al., 2008). However, astrocytic behavior could be markedly altered by anesthetics (Thrane et al., 2012). Anesthesia is known to increase basal blood flow in brain and alter functional hyperemia (Masamoto et al., 2009). Consequently, accurate dissection of the pathways mediating neurovascular coupling

requires the development and application of models that enable experimentation under near-physiological conditions.

In this study, we utilized cutting-edge genetic tools and physiological stimuli to test the hypothesis that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling mediates neurovascular coupling in awake, lightly sedated, responsive mice. We used the hM3Dq DREADD designer receptor system (Armbruster et al., 2007; Agulhon et al., 2013) to selectively stimulation astrocytic Gq-GPCR signaling cascades in vivo. To selectively eliminate IP_3R-dependent Ca^{2+} release downstream of Gq-GPCR activity, we employed the IP_3R2 KO mouse model (Petravicz et al., 2008). These genetic technologies overcome the major limitations of pharmacological tools such as non-selective drugs, caged molecules or chelators, and enable precise control over astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling in vivo. In addition to cell-permeable Ca^{2+} dyes, we utilized genetically-encoded Ca^{2+} sensors, cyto-GCaMP3 and Lck-GCaMP6s, for enhanced detection of astrocytic Ca^{2+} dynamics (Chen et al., 2013; Shigetomi et al., 2013). Overall, our experimental approach represents a significant advance over previous methods.

Our results establish that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling is not a central mediator of neurovascular coupling in visual cortex of lightly sedated, responsive mice. Additionally, our study introduces an experimental approach and genetic tools that can facilitate in vivo investigation on the cellular mechanisms that underlie functional hyperemia under near-physiological conditions.

MATERIALS AND METHODS

Mice

All mice were housed in the animal facilities at the University of North Carolina, Chapel Hill in accordance with Institutional Animal Care and Use Committee guidelines. IP_3R2 KO mice were generated as described previously (Li et al., 2005). IP_3R2 +/- mice were interbred to generate homozygous full mutant mice (IP3R2–/–) and littermate controls (IP_3R2 +/+). GFAP-cre mice were provided by Dr. Michael Sofroniew at the University of California Los Angeles. The Gfap-cre transgene was designed containing Cre recombinase and the entire mouse glial fibrillary acidic protein (/*Gfap*/) gene, driven by the Gfap promoter sequence, as described (Gregorian et al., 2009). All mice were maintained on a C57Bl/6 background and C57Bl/6 littermates were used as wild type controls. Both males and females were used for all studies.

Adeno-associated viral (AAV) injection for expression of GCaMP or hM3Dq

Mice P45-P90 were anesthetized with isoflurane (3-4% for induction, 1-2% for surgery, 100% oxygen). A vertical incision was made slightly lateral to midline, exposing primary visual cortex. The skull directly above the injection site was thinned to allow penetration by a glass pipette containing AAV diluted in Cortex Buffer artificial cerebral spinal fluid (Holtmaat et al., 2009). 0.5-1 uL of AAV solution was injected at a rate of 0.05 uL/min using a Harvard Apparatus 11 Plus pump and a syringe-to-pipette coupling system (Hamilton, #55751-01). AAVs were injected at the following titers: AAV8-GFAP-GCaMP3 and AAV8-GFAP-Lck-GCaMP6s, 1-1.2 x 10^{10} genome copies/uL; AAV8-GFAP-DIO-

GqDREADD-mCherry, 1 x 10^9 genome copies/uL. AAV was generated by UNC Vector Core Services. Following successful injection, the needle was left in the cortex for at least 5 minutes to allow for diffusion of the AAV away from the needle track. The incision was sealed with Vetbond and a surgical staple, covered with antibiotic ointment, and mice were given a single injection of antibiotic subcutaneously (ciprofloxacin, 5 mg/kg body weight dose). 4 weeks were given to allow for full transduction of virally-delivered genes and sufficient re-growth of thinned section of skull prior to installation of chronic optical windows.

Chronic optical imaging through Polished, Reinforced Thinned Skull (PoRTS) windows

Naïve adult mice or mice injected with AAV at least 4 weeks previously were anesthetized with isoflurane (3-4% for induction, 1-2% for surgery, 100% oxygen). Body temperature was maintained at 37.4° Celsius (Fine Science Tools TR-200). PoRTS optical windows were prepared as previously described (Drew et al., 2010), with a minor modification. Following skull thinning with a high-speed drill (Foredom), rather than polish by agitating grit slurry with a silicone whip we coated our silicone whips with grit powder. Free grit powder in the slurry tended to push through the weakened part of the bone where the glass pipette had previously punctured when delivering AAV. Coating the silicone whip with grit to create a gentle sanding device and prevented the above occurrence. 5 days were given for recovery from surgery, and cortical structures up to 200-250 um in depth could be imaged for up to several months. This technique avoids inflammation involved with chronic craniotomies (Xu et al., 2007; Drew et al., 2010), and offers greater convenience compared to traditional thinned skull preparations which require skull re-thinning and only allow for a limited number of imaging experiments (Yang et al., 2010).

Fluorescence immunochemistry

Mice were perfusion fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) three months after AAV injection and two months after PoRTS installation. Brains were removed and fixed in PFA for an additional 24 hours, rinsed in PBS, placed in 30% sucrose in PBS solution for 24 hours, frozen in OCT, and cut in 40 micron sections on a Reichert-Jung Cryo-cut 1800 cryostat. Block solution (20% normal goat serum, 0.1% triton X-100) was placed on the slides for 2 hours at room temperature prior to antibody treatments. For astrocytic GFAP staining, a Cy3-conjugated mouse anti-GFAP monoclonal antibody (Sigma) was used at a 1:500 dilution in PBS. For microglial staining, a rabbit anti-Iba1 monoclonal antibody (Wako) was used at a 1:500 dilution in PBS. For enhancement of GCaMP signal, mouse (Sigma) or rabbit (Invitrogen) anti-GFP antibodies were used at 1:500 dilution. Primary antibodies remained on the slides for 24 hours at 4°C, followed by appropriate secondary antibodies (Alexa Fluor) for two hours at room temperature at a 1:1000 dilution in PBS. Sections were rinsed in PBS and mounted with Vectashield fluorescence mounting medium with DAPI (Vector). Widefield images were acquired on a Zeiss Axioskop.

In vivo loading of Oregon Green BAPTA-1 and Sulforhodamine-101 dye

Adult mice (P60 to 6 months) were anesthetized with isoflurane (3-4% for induction, 1-2% for surgery, 100% oxygen). A 2–3 mm diameter craniotomy was opened over primary visual cortex. The dura mater was left intact. A 50-µg aliquot of Oregon Green–BAPTA (1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetatic acid)-1 (Invitrogen) was dissolved in a 20% Pluronic-127 (Invitrogen) solution in DMSO. After vortexing, this was diluted to 0.8 mM

in Cortex Buffer containing 30 μM sulforhodamine 101 to label astrocytes (Nimmerjahn et al., 2004). 40 nL of dye solution was injected at a depth of 250 um using similar methods as for AAV injection (see above). 1.2% agarose gel and a 3 mm cover slip were placed over the craniotomy, which was then sealed with dental cement (Parkell). For hydration and energy substrate a subcutaneous injection of Lactated Ringer's solution containing 5% dextrose was administered following surgery and prior to imaging.

Multiphoton imaging in lightly sedated, responsive mice

Initially, mice were anesthetized with isoflurane (3-4% for induction, 1-2% for maintenance, 100% oxygen). Chlorprothixene sedative was administered subcutaneously at doses between 0.4-3 mg/kg body weight. While anesthetized, mice were placed on a water-heated temperature controlled pad (Adroit Medical HTP-1500) and head-restrained underneath the microscope objective. In preparation for imaging, isoflurane was decreased and maintained well below anesthetizing levels (0-0.2%, 100% oxygen). The animal was allowed to stabilize under these conditions for at least 20 minutes prior to imaging. Under these conditions, mice are awake and responsive – they typically will not move during experiments, but if otherwise startled (by turning on the lights or touching the animal) they will struggle.

A custom two-photon microscope, converted from an Olympus Fluoview 300 system, with a 60x, 0.9 numerical aperture water immersion objective, and Hamamatsu photomultiplier tubes was used for imaging. Images were acquired using Fluoview 300 software at 1-2 Hz. For OGB-1/SR-101 experiments, imaging was performed at an imaging resolution of 0.84 microns/pixel (240 x 180 pixels). For GCaMP experiments, resolution was 0.42 microns/pixel (480 x 420 pixels). High-speed line-scanning (1.5 ms/line) was used to

directly measure blood flow within individual cortical capillaries (see below). For experiments requiring CNO injection, a 30-gauge catheter was inserted intraperitoneally to permit injection of vehicle or CNO solution during imaging without needing to touch or disturb the animal.

Visual Stimulation

VisionWorks software was used to generate drifting grating visual stimuli presented on a 7-inch VGA monitor (Lilliput 669GL) placed 10 cm from the mouse's eye at a 45 degree angle from normal. Stimuli consisted of 100% black/white contrast drifting square wave gratings (spatial frequency of 0.05 cycles/degree, drift speed of 2 cycles/second) presented at various angled orientations. A black, cardboard shroud was constructed and attached to the visual stimulus screen. This shroud fits just around the eye contralateral to optical window and prevents light from the stimulus screen from contaminating the imaging. Stimulus routines were designed such that orientations were presented in a unique, random order for each experimental set. Either short 5 second or longer 20 second stimuli were presented for all experiments. In the former case, orientations were 30 degrees apart, for a total of twelve unique stimuli. In the latter case, orientations were 45 degrees apart, for a total of 8 unqiue stimuli. Spike 2 software linked to a digitizer (Cambridge Electronic Design Limited, Micro 1401 DAQ) monitored outgoing triggers from the imaging acquisition system and the computer running VisionWorks.

Ca^{2+} imaging and analysis

ImageJ and Matlab software was used for image analysis. Image sequences were first motion-corrected using the Multistack Reg plugin for ImageJ. Ca^{2+} dynamics were obtained using custom Matlab scripts generously provided by Dr. Spencer Smith (University of North Carolina at Chapel Hill). These scripts allowed for semi-automated or freehand selection of regions of interest (ROIs). For OGB-1/SR-101 imaging data, ROIs were drawn around neuronal (OGB-1 positive, SR-101 negative) or astrocytic (OGB-1 positive, SR-101 positive) cell bodies. In the case of astrocytic ROIs, these were generated from the SR-101 channel to limit contamination from surrounding neuropil. The routine calculated the average pixel intensity $\Delta F/F_0$ with background noise subtracted for each ROI, and computed Ca^{2+} response averages based off precise trigger times tracked through Spike 2. Raw fluorescence data was mildly filtered using a 5-point period exponential moving average function (α=1/3) prior to averaging multiple responses.

For GCaMP imaging data, ROIs were drawn around astrocyte endfeet adjacent to cortical arterioles. Arterioles could be identified based on two criteria: 1) arterioles display spontaneous vasomotion whereas venules do not, and 2) blood in post-arteriole capillaries flows away from the vessel, whereas for venules blood flows towards the vessel. Endfoot ROIs were drawn slightly larger than the physical cellular structure because arteriole dilations often caused endfeet to move in the XY plane, producing false fluorescence changes. This necessitated drawing enlarged ROIs to ensure that the entire endfoot remained within the ROI during arteriole dilations. Contamination from surrounding neuropil regions was not a concern as GCaMP was selectively expressed in astrocytes.

For sampling spontaneous astrocytic Ca^{2+} dynamics in a non-biased manner (Figure 1B, 3C), we devised a grid analysis Matlab script. A grid of 20 x 20 pixel (approximately 8.35

x 8.35 microns) box ROIs was tiled across the imaging field, with fluorescence changes extracted as described above.

Blood flow imaging and analysis

For imaging blood flow, a high-molecular weight dextran-conjugated Rhodamine dye (Sigma #R9379) was dissolved into a 5% solution with saline and injected into the tail vein. Blood flow was measured in two ways. First, as in Figure 1C-E and 3E, F, relative volumetric blood flow changes can be estimated based on the degree of arteriole diameter change in response to stimuli. The estimation involves use of the Hagen-Poiseuille equation of fluid dynamics, which states that volumetric flux varies as a function of the fourth power of the vessel radius. To ascertain estimates of changes in vessel radius, images of vascular rhodamine were filtered using a 2-pixel median filter and binarized. Large ROIs were drawn around the vessel cross-section. By tracking fluorescence changes of the binarized images an area measurement in pixels was obtained. From this we derived changes in radius. Our estimated volumetric blood flow increases of 40% (Figure 3D, E, Figure 4B) to 50% (Figure 3F) correspond to arteriole dilations of approximately 8-12% in diameter, similar to what has been described in other studies (Takano et al., 2006). Tracking arteriole dilations permits simultaneous monitoring of cellular Ca^{2+} dynamics and blood flow changes in response to stimuli in vivo.

Volumetric blood flow changes were also directly measured by tracking erythrocyte velocities within cortical capillaries (Schaffer et al., 2006), as in Figure 3A and 4D, E. Capillary diameters generally remain constant. Consequently, changes in blood velocity are directly proportional to changes in volumetric flux through the capillary. Erythrocyte velocities were determined by line-scanning longitudinally through a length of capillary at high speed

(1.5 ms/line). Matlab scripts, generously provided by Dr. Chris Schaffer (Cornell University), were used to calculate erythrocyte velocities from the resulting XT images. Compared to estimating changes in arteriole radius, this method of measuring blood flow is direct and has much greater temporal resolution.

Blood flow response onset was estimated by determining the X-intercept of a line drawn through two points along the rising phase of the response, at 20% and 80% of maximum. Unfiltered capillary line-scanning blood flow data were used for this analysis to obtain high temporal resolution.

RESULTS

Selective stimulation of astrocytic hM3Dq does not alter basal visual cortical blood flow

Firmly elucidating the physiological role of astrocytic Gq-GPCR and Ca^{2+} signaling in neurovascular coupling has proven a difficult task due to reliance on pharmacological tools and acute slice preparations. To begin clarifying the role of astrocytic Gq-GPCR cascades and Ca^{2+} signaling in modulating cortical blood flow in vivo, we expressed the hM3Dq designer receptor and Lck-GCaMP6s in astrocytes using an AAV delivery system (see methods). The hM3Dq viral construct included a double-inverted open reading frame (DIO) element, which prohibits expression of hM3Dq in the absence of Cre-dependent recombination (Cardin et al., 2010). Astrocyte-selective expression of hM3Dq was therefore achieved by injecting the AAV into GFAP-Cre mice. Four weeks following AAV injection, mice were outfitted with a Polished, Reinforced Thinned Skull (PoRTS) cranial window (Drew et al., 2010), permitting chronic optical access to the visual cortex. Rhodamine dye was injected into the tail vein for monitoring changes in blood flow to stimuli based on direct measures within capillaries or arteriole diameter fluctuations (see Methods).

Expression of transgenes like GCaMP using AAV vectors does not appear to alter astrocytic electrical properties or induce reactivity as assessed by GFAP immunolabeling (Haustein et al., 2014). We tested for clear signs of astrocytic or microglial reactivity under our experimental conditions with fluorescence immunostaining of astrocytic GFAP and microglia following AAV injection and installation of PoRTS optical windows. GFAP immunolabeling is typically sparse in adult mouse cortex (Nolte et al., 2001) but can be induced by trauma to the brain, such as a craniotomy procedure (Xu et al., 2007; Holtmaat et al., 2009). Under our experimental conditions, GFAP immunolabeling in the cortex

ipsilateral to the AAV injection and PoRTS window were not detectably altered compared to the contralateral cortex (Figure 1A-C, n = 3 mice). GFAP labeling remained sparse in AAV-injected cortex (Figure 1A, B right panels), similar to the non-injected contralateral cortex (Figure 1A, B left panels). This was true even near the center of AAV injection close to where the injection needle penetrated (Figure 1C).

Altered microglial morphology is also indicative of cortical trauma (Xu et al., 2007). Iba1+ microglia from AAV-injected cortex (Figure 1D, E right panels) were indistinguishable from microglia in non-injected contralateral cortex (Figure 1D, E left panels) on the basis of gross morphology, indicating an absence of microglial reactivity near the injection and window site. These data suggest that expression of transgenes such as Lck-GCaMP6s in cortical astrocytes does not result in lasting glial reactivity.

Basal astrocytic Ca^{2+} activity in lightly sedated, responsive mice was readily detected by Lck-GCaMP6s (Figure 2A-C, see Methods). Diverse types of astrocytic Ca^{2+} elevations were detected throughout the imaging field such as “single peaks”, “multi-peaks” or “plateaus” (Figure 2C). These signals arose with an average frequency of 0.182 ± 0.012 SEM events/minute (n = 3 mice, 132 ROIs). “Multi-peak” or “plateau” signals varied greatly in duration and so we focused further quantification on basal “single peak” signals. The average basal “single peak” signal and distribution of “single peak” amplitudes are presented in Figure 2D and 2E respectively (n = 3 mice, 92 Ca^{2+} signals).

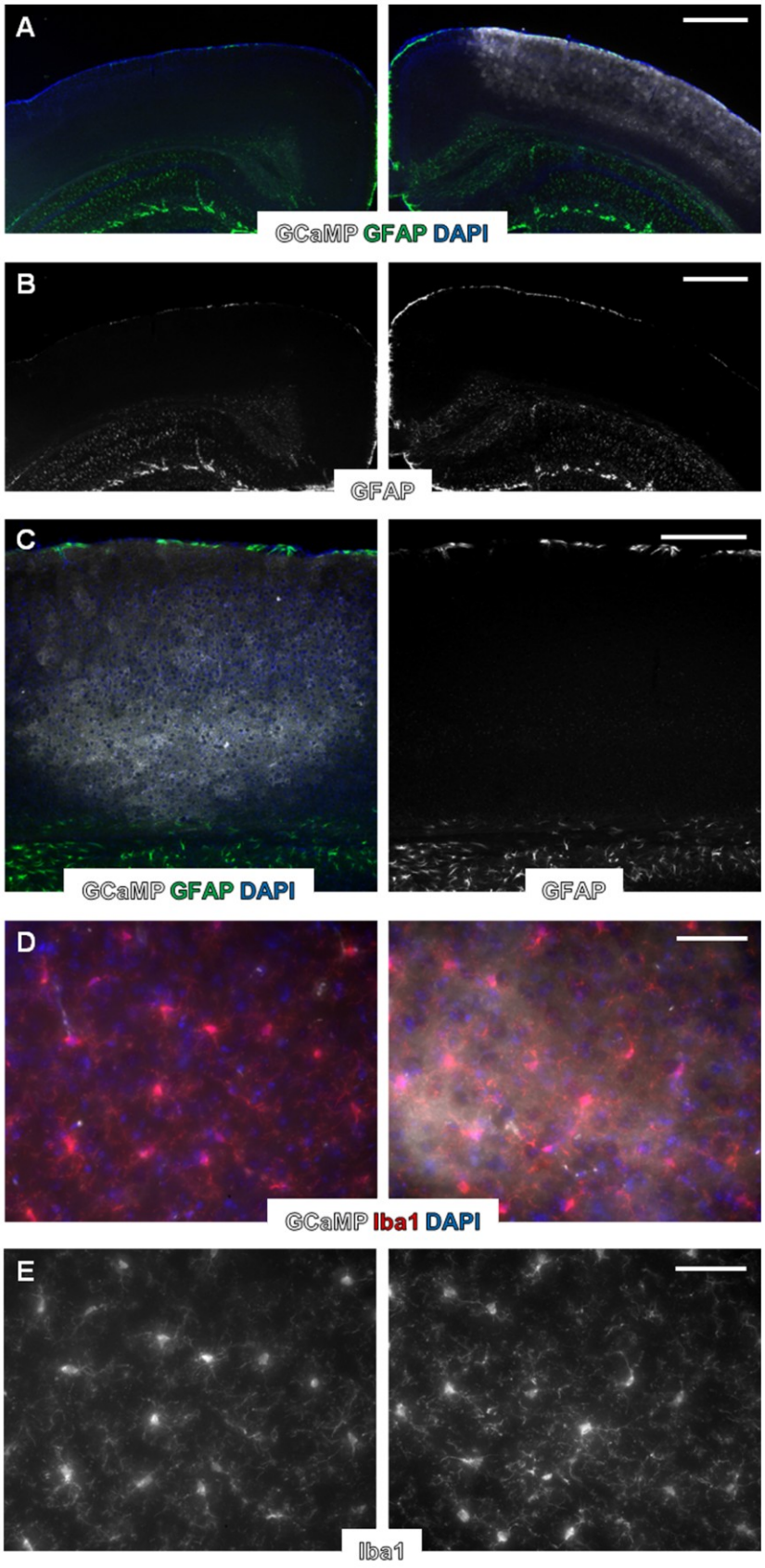
A
GCaMP GFAP DAPI
B
GFAP
C
GCaMP GFAP DAPI
GFAP
D
GCaMP Iba1 DAPI
E
Iba1

Figure 1. *Expression of transgenes using AAV vectors does not lead to lasting astrocytic reactivity or microglial activation*. (A) Fluorescence immunolabeling of nuclei (DAPI, blue), astrocytic GFAP (green), and Lck-GCaMP6s (gray) in visual cortex. Right panel shows AAV-injected cortex whereas left panel shows non-injected cortex from same animal. (B) Same images as in A but with GFAP only, more clearly displaying similarly sparse labeling in AAV- and non-injected cortices. (C) Fluorescence immunolabeling of nuclei (DAPI, blue), astrocytic GFAP (green), and Lck-GCaMP6s (gray) in visual cortex near the center of AAV injection and needle penetration. Right panel shows GFAP only. (D) Fluorescence immunolabeling of nuclei (DAPI, blue), microglia (Iba1, red), and Lck-GCaMP6s (gray) in visual cortex. Right panel shows AAV-injected cortex whereas left panel shows non-injected cortex from same animal. (E) Same images as in D but with Iba1 only, more clearly displaying similar gross microglial morphology in AAV- and non-injected cortices. Scale bars in A, B represent 500 microns, in C represents 250 microns, and in D, E represent 50 microns.

In the following experiments we focused on astrocyte endfeet (Figure 2A, black arrow) adjacent to penetrating cortical arterioles (Figure 2A, blue arrow). Intraperitoneal injection of CNO markedly increased astrocytic Ca^{2+} activity in mice expressing astrocytic hM3Dq (Figure 2F), but not in wild type mice (data not shown). The pattern of evoked Ca^{2+} elevation was dependent on CNO dose. Lower doses (typically 0.2 mg/kg) elicited "oscillatory-like" Ca^{2+} signals (Figure 2F upper panel, n = 6 mice) whereas higher doses (typically 1 mg/kg) resulted in continuous "plateau-like" signals lasting several minutes (Figure 2F lower panel, n = 7 mice). The latter of these signals has not been reported in vivo in response to physiological stimuli and likely reflects a supra-physiological stimulation of Gq-GPCR cascades. However, the kinetics of "oscillatory-like" CNO-evoked Ca^{2+} elevations closely mirrored those of basal "single peak" elevations (compare Figure 2D and 2G, black trace). In addition, the average amplitude of CNO-evoked "oscillatory-like" Ca^{2+} elevations was within the range of amplitudes of basal "single peak" signals (compare Figure 2E and 2G, black trace). These data suggest that the hM3Dq system is capable of evoking astrocytic Ca^{2+} elevations that fall within the physiological range of basal astrocytic Ca^{2+} dynamics in terms of kinetics and amplitude.

Despite the robust increase in astrocytic Ca^{2+} activity, basal blood flow remained unchanged following CNO injection (Figure 2F). This was the case regardless of "oscillatory-

like" (Figure 2F, upper traces, n = 6 mice) or "plateau-like" (Figure 2F, lower traces, n = 7 mice) responses. It was possible that blood flow was transiently modulated during individual endfoot Ca^{2+} elevations. To investigate this possibility, we determined blood flow changes during periods of elevated astrocytic Ca^{2+}. Cortical blood flow was unaffected by hM3Dq - induced Ca^{2+} increases in perivascular astrocyte endfeet, regardless of "oscillatory-like" (Figure 2G, n = 6 mice, 37 Ca^{2+} elevations) or "plateau-like" responses (Figure 2H, n = 7 mice, 7 Ca^{2+} elevations). Importantly, spontaneous arteriole vasomotion (fluctuation in arteriole diameter) was observed and functional hyperemia was readily evoked by physiological visual stimuli (Figure 2I, n = 7 mice, 379 stimulus trials). Therefore, the lack of blood flow modulation by CNO is not due to an inability to evoke blood flow changes in our recording conditions. These data suggest that selective stimulation of astrocytic Gq-GPCR cascades that evoke downstream Ca^{2+} signaling is not sufficient to modulate basal cortical blood flow in lightly sedated, responsive mice.

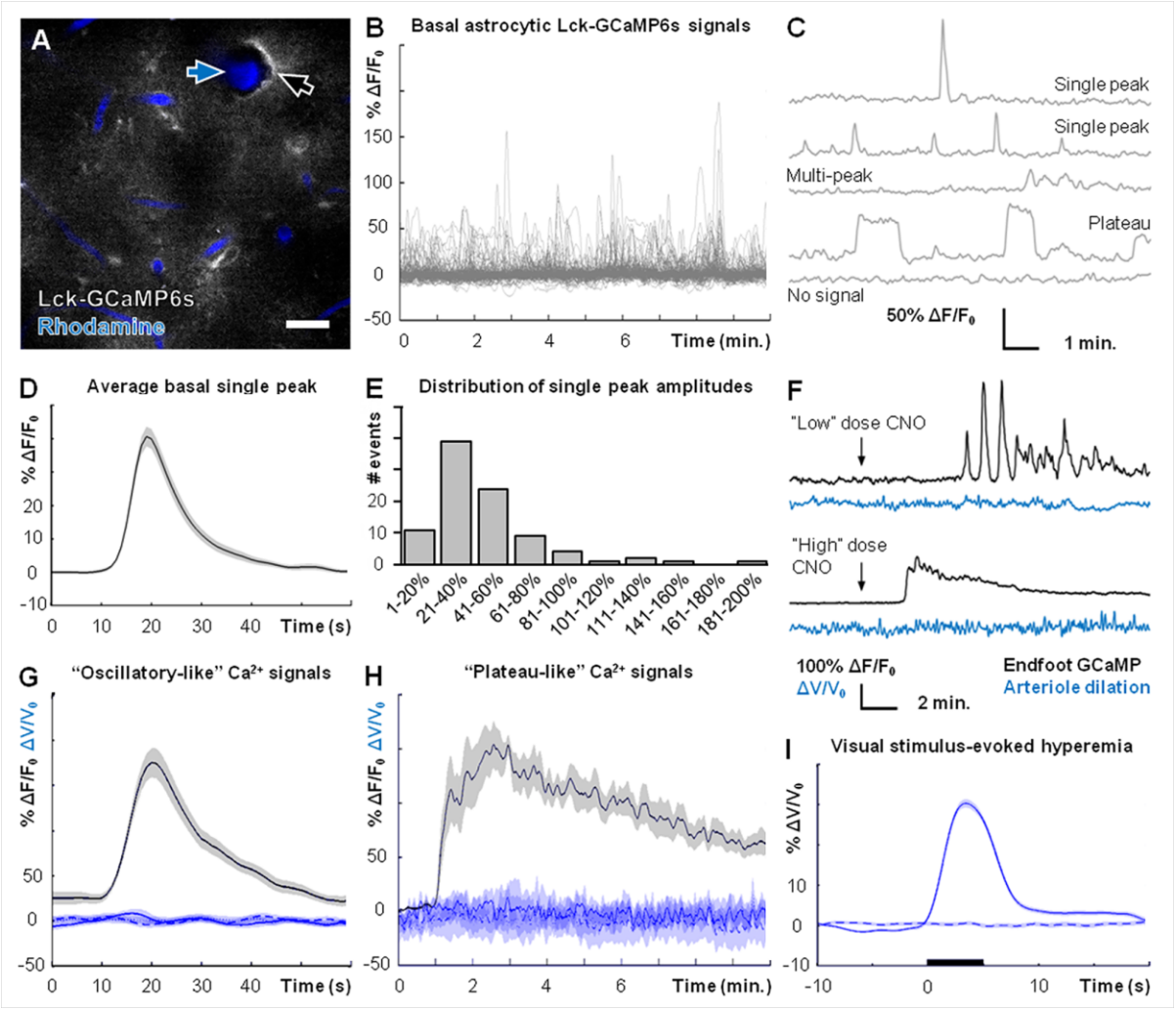

Figure 2. *Basal cortical blood flow is unaffected by stimulation of astrocytic hM3Dq*. (A) Field image of astrocytic Lck-GCaMP6s (gray) and intravascular rhodamine (blue), showing astrocyte endfeet (black arrow) and a cortical arteriole (blue arrow). (B) Non-biased sampling of Lck-GCaMP6s using a grid analysis (see Methods) reveals robust basal astrocytic Ca^{2+} dynamics. (C) Representative examples of diverse basal astrocytic Ca^{2+} signals detected by Lck-GCaMP6s (n = 3 mice, 132 ROIs). (D) Average basal astrocytic "single peak" Ca^{2+} elevation detected by Lck-GCaMP6s (n = 3 mice, 92 Ca^{2+} signals). (E) Histogram of basal astrocytic "single peak" Ca^{2+} elevations detected by Lck-GCaMP6s (n = 3 mice, 92 Ca^{2+} signals). (F) Representative traces of different types of astrocytic Ca^{2+} responses (black traces) that can be evoked by adjusting CNO dose, and basal blood flow during same CNO trials (blue traces). (G) Average blood flow (solid blue line, n = 6 mice, 37 trials) during CNO-induced astrocytic "oscillatory-like" Ca^{2+} signals (black line, n = 6 mice, 37 Ca^{2+} elevations). Dashed and dotted blue lines are averages from vehicle-injection and baseline (no injection) trials respectively. (H) Same information as D but during CNO-induced astrocytic "plateau-like" Ca^{2+} signals (n = 7 mice, 7 trials). Dashed and dotted blue lines are averages from vehicle-injection and baseline (no injection) trials respectively. (I) Average cortical blood flow increase (n = 7 mice, 379 stimulus trials) following visual stimulation (black bar). Dashed trace is during no-stimulus trials (n = 7 mice, 384 trials). Scale bar in A represents 20 microns. Shaded regions in D, G-I represent standard error of mean.

Visual cortical astrocytes do not display observable somatic Ca^{2+} elevations following visual stimulation

In the prior section, we provided a detailed analysis on the correlation between basal cortical blood flow in vivo during experimental elevations of astrocytic Gq-GPCR-linked Ca^{2+} signaling. Next, we characterized astrocytic Ca^{2+} dynamics following the presentation of physiological drifting grating visual stimuli to lightly sedated, responsive mice (Figure 3A). Following an acute craniotomy surgical procedure we loaded Layer II/III of the visual cortex with Oregon Green BAPTA-1 AM (OGB-1) and Suflorhodamine-101 (SR-101) dye to monitor somatic neuronal and astrocytic Ca^{2+} dynamics during stimulus presentations (Figure 3A).

Neuronal cell bodies exhibited elevations in intracellular Ca^{2+} that correlated with visual stimulus presentation (Figure 3B upper panel, black traces, Figure 3C, n = 4 mice, 87 cells, 3132 stimulus trials). A population of Layer II/III cortical neurons did not respond during visual stimuli but exhibited spontaneous Ca^{2+} spiking activity (Figure 3B lower panel, gray traces, n = 4 mice, 82 cells). We termed this population “non-responsive neurons”. In a separate set of experiments, visual stimuli evoked robust increases in cortical blood flow within capillaries (Figure 2I, n = 7 mice, 379 stimulus trials). These results serve as strong positive controls both for visual system responsiveness to the stimuli and the ability to evoke robust blood flow changes in lightly sedated, responsive mice.

Despite strong neuronal and blood flow responses to visual stimuli, a similar analysis did not reveal observable stimulus-correlated Ca^{2+} activity within astrocyte cell bodies (Figure 3B middle panel, blue traces, n = 4 mice, 35 cells). However, averaging across all data sets identified a small, relatively rapid astrocytic Ca^{2+} response temporally correlated to the stimulus (Figure 3D, n = 4 mice, 35 cells). Several points suggest that this response is contamination from neuropil signal in the Z-plane. First, averaging across data sets for the non-responsive neuronal population reveals the same small, rapid Ca^{2+} elevation (Figure

3E, gray trace, n = 4 mice, 82 cells 3132 stimulus trials), suggesting a shared source of contamination. Second, the astrocytic signal was not observed when imaging GCaMP with expression restricted to astrocytes (Figure 4F-I). In the absence of neuronal and neuropil indicator signal, no contamination effect was observed. Recent studies describe a similar contamination in their analyses of astrocytic Ca^{2+} dynamics in vivo (Lind et al., 2013; Nizar et al., 2013). These data demonstrate that astrocytes in lightly sedated, responsive mice do not display somatic Ca^{2+} elevations following visual stimulation.

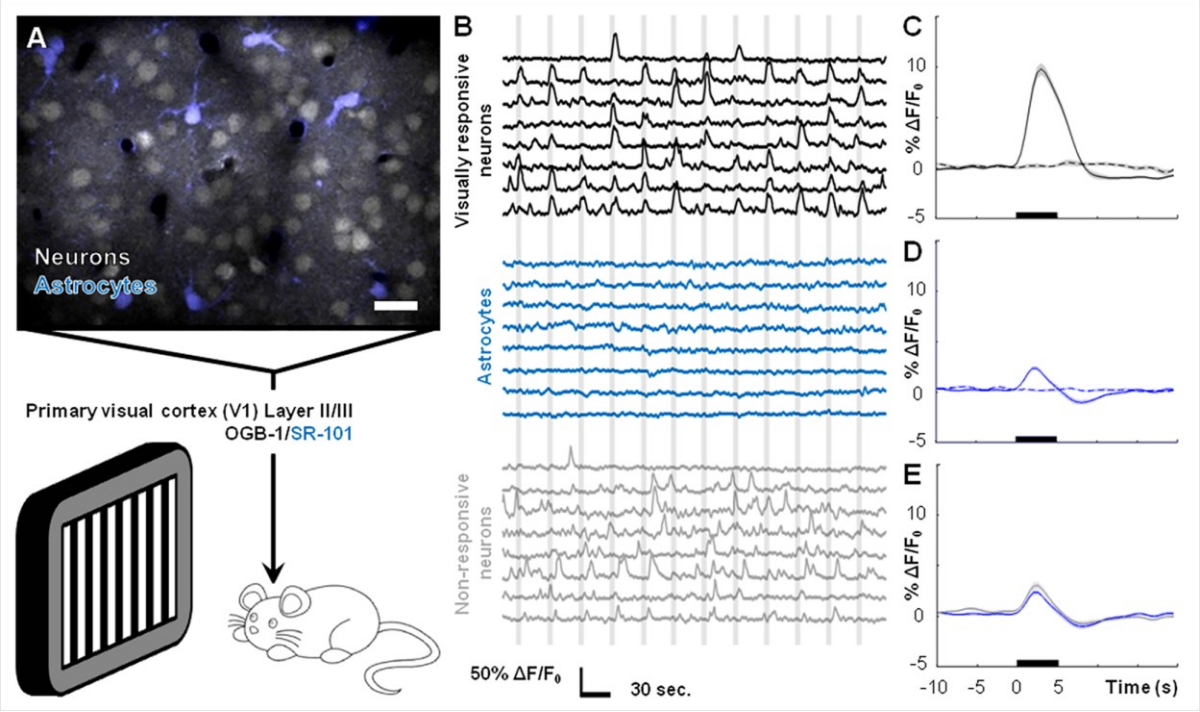

Figure 3. *Mouse visual cortical astrocytes do not display observable somatic Ca^{2+} elevations following visual stimulation*. (A) Field image showing Layer II/III neurons and astrocytes loaded with OGB-1 (gray)/SR-101 (blue) dye. Drifting grating visual stimuli were presented to lightly sedated, responsive mice. (B) Representative traces of fluorescence signals from ROIs drawn around the cell bodies of visually-responsive neuron (black traces), astrocytes (blue traces) and visually non-responsive neurons (gray traces) during stimulus presentations (vertical gray bars). (C) Average neuronal Ca^{2+} increase to visual stimuli (n = 4 mice, 87 cells, 3132 stimulus trials). (D) Average astrocytic Ca^{2+} dynamics during visual stimuli (n = 4 mice, 35 cells, 1152 stimulus trials). (E) Average astrocytic (blue) and non-responsive neuronal (gray, n = 4 mice, 82 cells, 3132 stimulus trials) Ca^{2+} dynamics during visual stimuli, indicating contamination from neuropil signal. Scale bar in A

represents 20 microns. For C-E, stimuli were presented during time frame indicated by black bars, dashed lines indicate no-stimulus trials, and shaded regions represent standard error of mean.

Perivascular astrocyte endfeet do not display observable Ca^{2+} elevations following visual stimuli

It is increasingly appreciated that the larger share of astrocytic Ca^{2+} activity occurs in finer cellular processes ("microdomains") and not the soma (Di Castro et al., 2011; Panatier et al., 2011; Shigetomi et al., 2013; Sun et al., 2014; Volterra et al., 2014). It is believed that Ca^{2+} elevation within astrocyte endfeet is likely more crucial for mediating neurovascular coupling than Ca^{2+} signaling elsewhere in the cell (Attwell et al., 2010; Petzold and Murthy, 2011; Newman, 2013; Howarth, 2014). To monitor Ca^{2+} dynamics in astrocyte endfeet with enhanced sensitivity, we expressed cyto-GCaMP3 (Figure 4A) or Lck-GCaMP6s (Figure 2A) in astrocytes and simultaneously measured cellular Ca^{2+} signaling and blood flow changes following presentation of visual stimuli to lightly sedated, responsive mice.

Similar to Lck-GCaMP6s (Figure 2B), cyto-GCaMP3 readily detected basal astrocytic Ca^{2+} dynamics (Figure 4B) including "single peaks", "multi-peaks" and "plateaus" (not shown). These signals arose with an average frequency of 0.111 ± 0.01 SEM events/minute (n = 3 mice, 181 ROIs). The decreased frequency of events revealed by cyto-GCaMP3 compared to Lck-GCaMP6s can most likely be attributed to the increased sensitivity of GCaMP6s (Chen et al., 2013), with additional sensitivity conferred by the membrane-tethering of Lck-GCaMP6s (Shigetomi et al., 2013). The average basal "single peak" Ca^{2+} signal detected using cyto-GCaMP3 (Figure 4C, n = 3 mice, 94 Ca^{2+} signals) was similar kinetically to the average "single peak" detected using Lck-GCaMP6s (Figure 2D, Figure 4D). The slightly more rapid decay of the average "single peak" detected by cyto-GCaMP3 can most likely be attributed to the faster kinetic profile of GCaMP3 compared to GCaMP6s

(Chen et al., 2013). The distribution of basal "single peak" Ca^{2+} elevations detected by cyto-GCaMP3 is displayed in Figure 4E (n = 3 mice, 94 Ca^{2+} signals).

Surprisingly, astrocyte endfoot Ca^{2+} signals did not deviate from baseline values during visual stimuli even though cortical arterioles exhibited robust and reliable dilations (Figure 4F, n = 9 mice). Ca^{2+} signals measured using cyto-GCaMP3 (Figure 4F, top 3 black traces) were similar to those measured using Lck-GCaMP6s (Figure 4F, bottom black trace). Clear arteriole dilations were observed regardless of GCaMP variant expressed (Figure 4F, upper panel, blue traces). Averaging across data sets confirmed that robust and reliable arteriole dilations occur in the absence of observable astrocyte endfoot Ca^{2+} elevations (Figure 4G, n = 9 mice, 464 stimulus trials).

Recognizing that astrocytic Ca^{2+} elevations, when they do take place, are delayed relative to the onset of blood flow responses (Wang et al., 2006; Schummers et al., 2008; Tian et al., 2010), it has been suggested that rather than playing a role in initiating functional hyperemia astrocytic Ca^{2+} signaling might instead be important for maintaining or modulating blood flow during prolonged stimulus presentations (Schulz et al., 2012; Lind et al., 2013). However, we found no evidence of endfoot Ca^{2+} elevations when presenting 20 second-long stimuli (Figure 4H, n = 5 mice, 187 stimulus trials), compared to the earlier 5 second-long stimuli (Figure 4F, G).

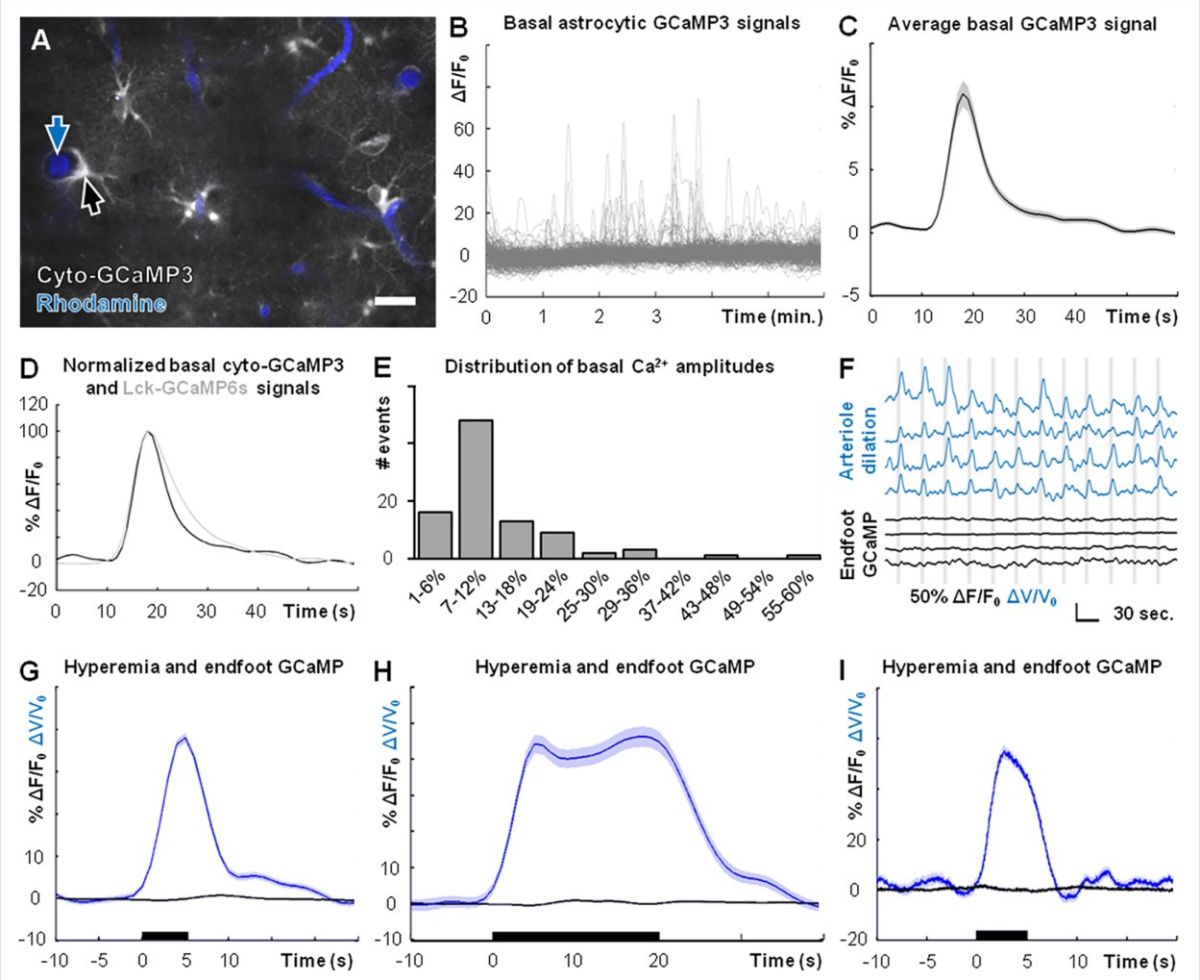

Figure 4. *Cortical arterioles dilate in the absence of observable Ca^{2+} elevations in perivascular astrocyte endfeet*. (A) Field image of cyto-GCaMP3 (gray) and intravascular rhodamine (blue), showing astrocyte endfeet (black arrow) and cortical arterioles (blue arrow). (B) Non-biased sampling of cyto-GCaMP3 using a grid analysis (see Methods) reveals robust basal astrocytic Ca^{2+} dynamics. (C) Average basal astrocytic "single peak" Ca^{2+} elevation detected by cyto-GCaMP3 (n = 3 mice, 94 Ca^{2+} signals). (D) Average basal astrocytic "single peak" Ca^{2+} elevations detected by cyto-GCaMP3 (black trace) and Lck-GCaMP6s (gray trace, see also Figure 2D), normalized to respective maxima. (E) Histogram of basal "single peak" amplitudes detected by cyto-GCaMP3 (n = 3 mice, 94 Ca^{2+} signals). (F) Representative simultaneous measures of blood flow changes based on arteriole dilations (blue traces) and astrocyte endfoot Ca^{2+} dynamics (black traces) during visual stimulation (vertical gray bars). The top three traces for each are from experiments using cyto-GCaMP3. The bottom trace is from an experiment using Lck-GCaMP6s. (G) Average blood flow (blue trace, n = 9 mice, 464 stimulus trials) and astrocyte endfoot Ca^{2+} (black trace, n = 9 mice, 464 stimulus trials, pooled from cyto-GCaMP3 and Lck-GCaMP6s experiments) dynamics following 5 second-long visual stimuli (black bar). (H) Average blood flow (blue trace, n = 5 mice, 187 stimulus trials) and astrocyte endfoot Ca^{2+} (black trace, n = 5 mice, 187 stimulus trials, pooled from cyto-GCaMP3 and Lck-GCaMP6s experiments) dynamics following 20 second-long visual stimuli (black bar). (I) Average blood flow (blue trace) and astrocyte endfoot Ca^{2+} (black trace, Lck-GCaMP6s) dynamics assessed by high-speed multi-photon line-scanning (n = 3 mice, 183 stimulus trials) following 5 second-long visual stimuli (black bar). Scale bar in A is 20 microns. Shaded regions in C, G-I represent standard error of mean.

A recent study described a type of rapid, neuronal activity-dependent astrocytic Ca^{2+} signal (Lind et al., 2013). To test for the presence of this signal in visual cortical astrocytes following visual stimulation, we simultaneously imaged astrocyte endfoot Lck- GCaMP6s dynamics and arteriole dilations using high-speed line-scanning (see Methods), achieving a temporal resolution of 3.5 ms per time point. We found no evidence of observable rapid Ca^{2+} elevations within astrocyte endfeet despite robust dilations of adjacent cortical arterioles (Figure 4I, n = 3 mice, 183 stimulus trials). Together these data suggest that arteriole diameter and cortical blood flow can be modulated in lightly sedated, responsive mice in the absence of observable Ca^{2+} elevations in astrocyte cell bodies or endfeet.

Interestingly, positive Ca^{2+} elevations that correlated very well with stimulus-evoked arteriole dilations in terms of amplitude and kinetics were observed by linescanning in neuropil regions of wild type mice expressing GFAP-Lck-GCaMP6s (Figure 5A, n = 4 mice, 315 stimulus trials). In addition, similar Ca^{2+} elevations were observed following visual stimulation in IP3R2 KO mice (Figure 5B, n = 2 mice, 111 stimulus trials), in which astrocytic IP_3R-dependent Ca^{2+} signaling is genetically ablated. These data were initially quite exciting as IP_3R-independent, activity-related astrocytic Ca^{2+} has not been previously described.

However, we have observed neuronal expression of transgenes driven by the GFAP promoter when using AAV delivery. For instance, injection of AAV8-GFAP-Cre into Ai9 mice in which tdTomato is expressed in a Cre-dependent fashion leads to extensive neuronal tdTomato labeling (data not shown). Fluorescence immunolabeling experiments carried out on AAV8-GFAP-Lck-GCaMP6s-injected mice revealed sparse labeling of cortical neurons, mainly in Layer V pyramidal neurons (Figure 5C, n = 3 mice). These data suggest that the positive Ca^{2+} elevations witnessed following visual stimulation could be due to dendritic expression of Lck-GCaMP6s.

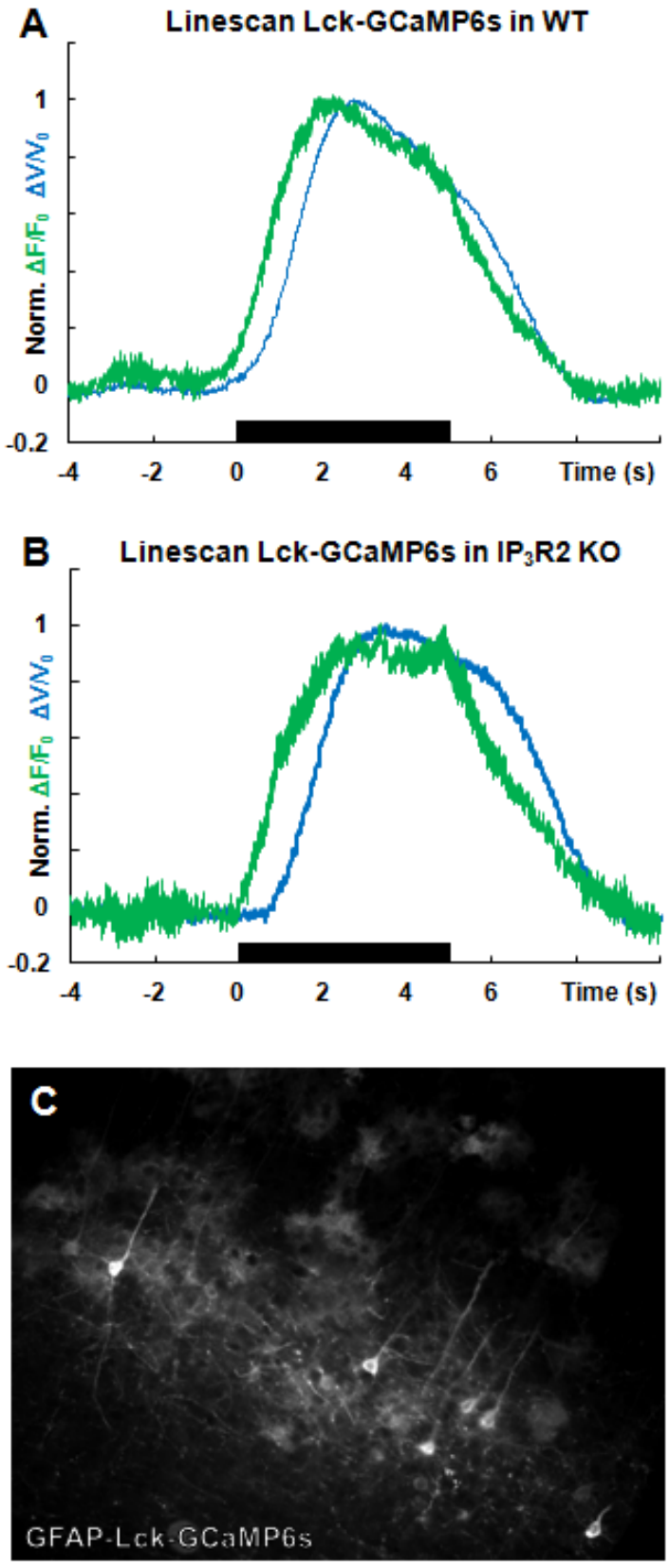
A
Linescan Lck-GCaMP6s in WT
Norm. ΔF/F0 ΔV/V0
1
0
-0.2
-4
-2
0
2
4
6
Time (s)
B
Linescan Lck-GCaMP6s in IP3R2 KO
Norm. ΔF/F0 ΔV/V0
1
0
-0.2
-4
-2
0
2
4
6
Time (s)
C
GFAP-Lck-GCaMP6s

Figure 5. *Neuropil regions display Ca^{2+} elevations that correlate very well with stimulus-evoked arteriole dilations in terms of amplitude and kinetics.* (A) Average blood flow (blue trace) and neuropil Ca^{2+} (green trace, GFAP-Lck-GCaMP6s) dynamics, normalized to respective maxima, in wild type mice assessed by high-speed multi-photon line-scanning (n = 3 mice, 315 stimulus trials) following 5 second-long visual stimuli (black bar). (B) Average blood flow (blue trace) and neuropil Ca^{2+} (green trace, GFAP-Lck-GCaMP6s) dynamics, normalized to respective maxima, in IP_3R2 KO (n = 2 mice, 111 stimulus trials) following 5 second-long visual stimuli (black bar). (C) Fluorescence immunolabeling in AAV8-GFAP-Lck-GCaMP6s-injected visual cortex reveals neuronal expression of Lck-GCaMP6s.

Startle-inducing air puff stimuli elicit astrocytic Ca^{2+} elevations and cortical blood flow changes

Despite both GCaMP variants readily detecting basal astrocytic Ca^{2+} signals (Figure 2B and 4B) we were unable to observe positive Ca^{2+} elevations following visual stimulation (Figure 3D and 4F-I). We next sought an alternative stimulation method that could reliably evoke astrocytic Ca^{2+} elevations under our experimental conditions and serve as a positive control. The cortex contains widespread noradrenergic projections from the locus coeruleus, and electrical stimulation of this nucleus readily elicits Ca^{2+} elevations in cortical astrocytes (Bekar et al., 2008). Noradrenergic projections to the cortex appear to play a prominent role in mediating concerted cortical astrocytic Ca^{2+} dynamics in fully awake mice, particularly when engaged through the startle response (Ding et al., 2013; Paukert et al., 2014). We therefore monitored visual cortical astrocytic Ca^{2+} dynamics with Lck-GCaMP6s while inducing the startle response with unexpected 3-4 second-long air puffs (Ding et al., 2013).

Startling lightly sedated, responsive mice with air puffs resulted in field-wide elevations of astrocytic Ca^{2+} (Figure 6A, n = 5 mice), thus demonstrating the ability for astrocytes to display Ca^{2+} elevations following a physiological stimulus under our experimental conditions. The distribution of response amplitudes is displayed in Figure 6B, with failures occurring in 7 of 46 total air puffs (n = 5 mice, 46 air puffs). Air puffs also elicited a distinct pattern of blood flow changes on average, with an initial increase followed by a dip

below baseline, and a final recovery to baseline or slight overshoot (Figure 6C, blue trace, n = 5 mice, 46 air puffs). Interestingly, on average the astrocytic Ca^{2+} elevations do not appear to precede the initial blood flow changes (Figure 6C, n = 5 mice, 46 air puffs). These data indicate that startling lightly sedated, responsive mice with unexpected air puffs is sufficient to evoke widespread astrocytic Ca^{2+} elevations and alter cortical blood flow. However, astrocytic Ca^{2+} elevations do not precede the initial blood flow increase, suggesting that astrocytic Ca^{2+} signaling does not mediate the onset of vascular dynamics during the startle response.

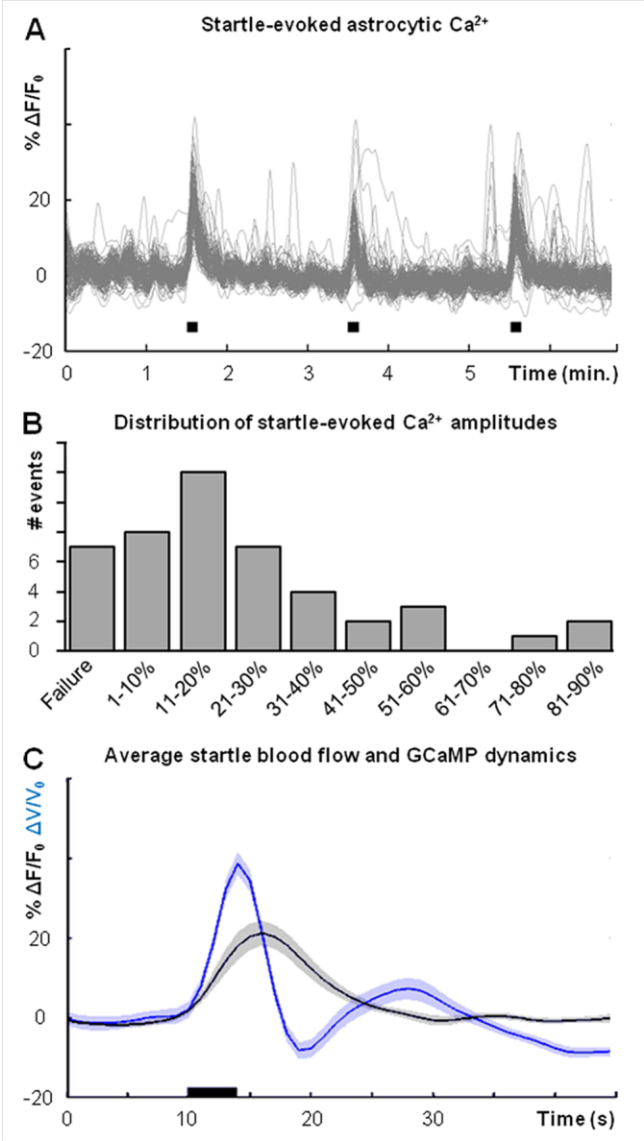

Figure 6. *Air puff startle elicits widespread astrocytic Ca^{2+} elevation and cortical blood flow changes.* (A) Non-biased sampling of air puff (black boxes) startle-evoked astrocytic Ca^{2+} (Lck-GCaMP6s) signals using a field-wide grid analysis (see Methods). (B) Histogram of astrocytic Ca^{2+} elevation amplitudes following air puff startle (n = 5 mice, 46 stimulus trials). (C) Average blood flow changes (blue trace) and astrocyte endfoot Ca^{2+} signals (black trace, Lck-GCaMP6s) following air puff startle, indicated by black bar (n = 5 mice, 46 stimulus trials). Shaded regions in C represent standard error of mean.

Genetic deletion of astrocytic IP_3R-dependent Ca^{2+} signaling does not alter neurovascular coupling in lightly sedated, responsive mice

Two recent studies demonstrated that functional hyperemia is intact in anesthetized IP_3R2 KO mice in vivo (Nizar et al., 2013; Takata et al., 2013), in which astrocytic IP_3R-dependent Ca^{2+} activity is eliminated (Petravicz et al., 2008). Accurate interpretation of these results is difficult because of the confounding effects of anesthesia or anesthetized state on astrocytic signaling (Thrane et al., 2012) and blood flow dynamics (Masamoto et al., 2009). To determine if functional hyperemia is altered in lightly sedated, responsive IP_3R2 KO mice, we loaded the visual cortex with OGB-1/SR-101 dye and monitored neuronal and astrocytic Ca^{2+} dynamics following visual stimulation in IP_3R2 KO mice and wild type littermate controls.

Previous studies demonstrated that basal neuronal activity and plasticity are unaffected by the loss of astrocytic IP_3R-dependent Ca^{2+} signaling (Petravicz et al., 2008; Agulhon et al., 2010). Similarly, in vivo the percentage of total neurons sampled that were responsive to visual stimuli was not statistically different between IP_3R2 KO visual cortex and controls (Figure 7A, n = 4 mice each genotype, WT n = 169 cells, KO n = 177 cells, student's t-test, p = 0.7328). Average neuronal Ca^{2+} spiking response to visual stimulation did not differ between genotypes (Figure 7B, n = 4 mice each genotype, WT n = 87 cells, 3132 stimulus trials, KO n = 88 cells, 3168 stimulus trials). As anticipated, we did not observe Ca^{2+} elevations in astrocyte cell bodies from IP_3R2 KO mice (Figure 7C and inset, n = 4 mice each genotype, WT n = 35 cells, 1152 stimulus trials, KO n = 30 cells, 1080 stimulus trials). These data suggest that the visual cortical circuitry of IP_3R2 KO mice and wild type littermate controls is similarly responsive to visual stimuli.

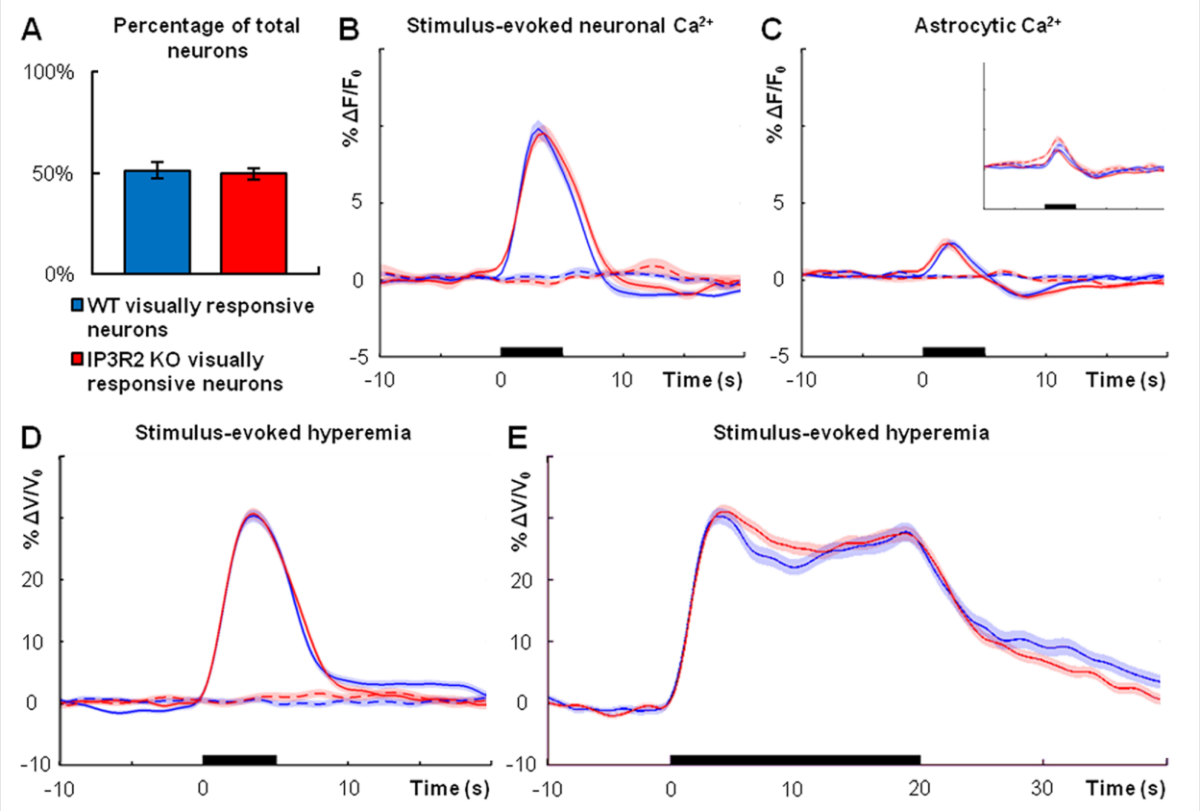

Figure 7. *Neurovascular coupling is intact in visual cortex of lightly sedated, responsive IP_3R2 KO mice*. (A) Percentage of neurons (OGB-1 positive/SR-101 negative) that are responsive to visual stimuli (n = 4 mice each genotype, WT n = 169 total cells, KO n = 177 total cells, student's t-test p = 0.7328). (B) Average neuronal Ca^{2+} increases in wild type (blue, n = 4 mice, 87 cells, 3132 stimulus trials) and IP_3R2 KO (red, n = 4 mice, 88 cells, 3168 stimulus trials) following 5 second-long visual stimulation (black bar). Dashed traces are averages from trials in which no stimuli were presented. (C) Average astrocytic Ca^{2+} dynamics following 5 second-long visual stimulation (black bar) in wild type (blue, n = 4 mice, 35 cells, 1152 stimulus trials) and IP_3R2 KO (red, n = 4 mice, 30 cells, 1080 stimulus trials). Dashed traces are averages from trials in which no stimuli were presented. Inset additionally displays wild type (dashed blue trace) and IP_3R2 KO (dashed red trace) non-responsive neuron Ca^{2+} signals during visual stimuli, indicating contamination from neuropil signal. (D) Average blood flow increase following 5 second-long visual stimulus (black bar) in wild type (blue, n = 7 mice, 379 stimulus trials) and IP_3R2 KO (red, n = 8 mice, 414 stimulus trials). Dashed traces are averages from trials in which no stimuli were presented. (E) Average blood flow increase to 20 second-long visual stimulus (black bar) in wild type (blue, n = 7 mice, 189 stimulus trials) and IP_3R2 KO (red, n = 6 mice, 227 stimulus trials). Dashed traces are averages from trials in which no stimuli were presented. Error bars in A and shaded regions in B-E represent standard error of mean.

Functional hyperemia was completely intact in lightly sedated, responsive IP_3R2 KO mice following visual stimulus presentation (Figure 7D, WT n = 7 mice, 379 stimulus trials, KO n = 8 mice, 414 stimulus trials). Similar results were observed with 20 second-long visual stimulation (Figure 7E, WT n = 7 mice, 189 stimulus trials, KO n = 6 mice, 227 stimulus trials). Across a range of studies, observable astrocytic Ca^{2+} elevations in response to Gq-GPCR agonists (Petravicz et al., 2008; Chen et al., 2012; He et al., 2012; Navarrete et al., 2012; Nizar et al., 2013) or electrical field stimulation (He et al., 2012; Wang et al., 2012; Haustein et al., 2014) or afferent stimulation (Takata et al., 2011; Navarrete et al., 2012; Nizar et al., 2013) are absent in IP_3R2 KO animals in situ and in vivo. Thus, these data suggest that astrocytic IP_3R-dependent Ca^{2+} signaling is not centrally involved in initiating functional hyperemia or modulating the sustained phase of neurovascular coupling in lightly sedated, responsive mice. Importantly, these data corroborate the critical results of recent in vivo studies (Nizar et al., 2013; Takata et al., 2013).

DISCUSSION

Astrocytes are reported to alter vessel diameter by means of intracellular Ca^{2+} elevations (Attwell et al., 2010; Petzold and Murthy, 2011; Newman, 2013; Howarth, 2014). Astrocytes contact many synapses and astrocyte endfeet surround over 99% of cerebral vascular surface (Mathiisen et al., 2010; McCaslin et al., 2011). It is hypothesized that spillover of neurotransmitter from synapses stimulates astrocytic Gq-GPCRs, resulting in IP_3R-dependent Ca^{2+} release from ER stores (Duffy and MacVicar, 1995; Porter and McCarthy, 1996; Araque et al., 2002; Newman, 2005), Ca^{2+}-dependent release of vasoactive molecules onto arteriole smooth muscle (Zonta et al., 2003; Mulligan and MacVicar, 2004; Takano et al., 2006), thereby modulating blood flow. However, accurate dissection of the cellular pathways underlying neurovascular coupling has proven difficult. Mechanistic studies have relied on acute slice preparations and pharmacological tools with direct in vivo tests largely lacking.

A recent study questions our current mechanistic understanding of neurovascular coupling. Using dyes to monitor neuronal and astrocytic Ca^{2+} dynamics in anesthetized mice, Nizar et al. (2013) demonstrated that arteriole dilation could occur in the absence of observable endfoot Ca^{2+} elevation, astrocytic Ca^{2+} elevations did not precede dilation of adjacent arterioles following footshock, and arteriole dilations in IP_3R2 KO mice were intact. However, this study was limited by its reliance on computational processing to isolate astrocytic Ca^{2+} signal from contaminating neuropil signal and the use of general anesthesia, which interferes with astrocytic signaling (Thrane et al., 2012) and alters brain blood flow (Masamoto et al., 2009).

Our study is the first utilizing the DREADD designer receptor system, GCaMP, and chronic PoRTS optical windows for assessing the role of Gq-GPCR and Ca^{2+} signaling cascades in neurovascular coupling in vivo in the absence of general anesthesia, thereby

representing a significant advance experimentally on numerous fronts. We analyzed the effects of selective manipulations of astrocytic Gq-GPCR and IP_3R-dependent signaling on neurovascular coupling in visual cortex of awake, lightly sedated, responsive mice, and simultaneously monitored vascular and astrocytic Ca^{2+} dynamics following visual stimulation. Our data strongly argue that astrocytic Gq-GPCR cascades and IP_3R-dependent Ca^{2+} signaling are neither sufficient nor necessary for functional hyperemia in vivo. These pathways do not play as central a role in neurovascular coupling as previously reported.

First, selective stimulation of astrocytic Gq-GPCR signaling cascades was not sufficient to modulate basal cortical blood flow in vivo (Figure 2). Previous studies used bath application of Gq-GPCR agonists (Zonta et al., 2003; He et al., 2012), Ca^{2+} uncaging (Metea and Newman, 2006; Girouard et al., 2010; Stobart et al., 2013), or electrical afferent or field stimulation (Filosa et al., 2006; Girouard et al., 2010; He et al., 2012) to demonstrate that elevations in astrocytic Ca^{2+} are sufficient to alter vascular diameter in situ. Uncaging Ca^{2+} in endfeet is sufficient to modulate arteriole diameter in vivo (Takano et al., 2006). Whether pharmacological signaling manipulations in situ are representative of physiology is unclear. Receptor agonists are not cell-selective and could modulate vascular diameter through cell types other than astrocytes. Laser uncaging does not engage the full fabric of Gq-GPCR cascades. Acute slice preparations lack blood flow and intraluminal vascular pressure, and astrocytes might rapidly enter a state of reactive gliosis (Takano et al., 2014).

To overcome such concerns, we utilized the hM3Dq DREADD system (Armbruster et al., 2007). The hM3Dq enables stimulation of the entire Gq-GPCR cascade selectively in astrocytes in vivo, an advance over previous methods. The hM3Dq system has been successfully used by many investigators to dissect the contribution of cell-specific signaling cascades in physiological processes in vivo (Wess et al., 2013). We observed robust CNO-induced Ca^{2+} elevations within perivascular astrocyte endfeet in mice expressing hM3Dq in

astrocytes, yet the diameters of adjacent arterioles remained at baseline values. Contrasting with previous studies, our observations indicate that stimulation of astrocytic Gq-GPCR cascades is not sufficient to modulate basal cortical blood flow in vivo. It is possible that astrocytic Gq-GPCR signaling could modulate physiologically-evoked hemodynamics, an interesting subject for future studies.

Second, arteriole dilations following visual stimulation occurred independently of observable Ca^{2+} elevations in astrocyte cell bodies (Figure 3) and arteriole-adjacent endfeet (Figure 4). We observed robust spontaneous Ca^{2+} dynamics when cyto-GCaMP3 or membrane-bound Lck-GCaMP6s was expressed in astrocytes. GCaMP3 allows for greater detection of "microdomain" Ca^{2+} events in astrocytes compared to bulk-loaded dyes (Shigetomi et al., 2013) and GCaMP6s is the most sensitive Ca^{2+} indicator currently available (Chen et al., 2013). In addition to Gq-GPCR-linked IP_3R-dependent events, basal Ca^{2+} currents through plasma membrane TRP-family channels have been described (Shigetomi et al., 2012; Dunn et al., 2013). Endfoot TRPV4 channels might play a role in amplifying neurovascular responses by facilitating Ca^{2+}-induced Ca^{2+} release (Dunn et al., 2013). However, we found no evidence of observable Ca^{2+} elevations in astrocyte endfeet of lightly sedated, responsive mice following visual stimulation, indicating that observable arteriole-adjacent endfoot Ca^{2+} is not required for functional hyperemia in vivo regardless of the pathway engaged.

We detected positive astrocytic Ca^{2+} signals and blood flow changes when startling lightly sedated, responsive mice with unexpected air puffs (Figure 6). Interestingly, the startle-evoked astrocytic Ca^{2+} signals did not precede the onset of the blood flow increases. However, it is possible that the blood flow decrease following the initial increase (Figure 6C) could be mediated by astrocytic Ca^{2+}-dependent processes. It is known that norepinephrine levels in the cortex differentially regulate functional hemodynamics in vivo (Bekar et al., 2012). These effects could be due to direct action of astrocytes on blood vessels, or

perhaps by astrocyte-driven modulation of local neuronal circuitry. It has been suggested that astrocytic Ca^{2+} signaling might play a more prominent role in mediating startle or stress networks, with less critical involvement in local sensory processing (Ding et al., 2013; Sun et al., 2013; Paukert et al., 2014), an idea consistent with our findings. It will be interesting to explore these possibilities in future studies.

Infusing BAPTA into perivascular astrocytes in situ blocks Ca^{2+} elevations to bath-applied Gq-GPCR agonists or laser uncaging and prevents arteriole dilation (Mulligan and MacVicar, 2004). Interfering with astrocytic Ca^{2+} dynamics with receptor antagonists reduces vascular response in situ (Filosa et al., 2006) and in vivo (Zonta et al., 2003; Takano et al., 2006). However, functional hyperemia in response to footshock (Nizar et al., 2013) or whisker stimulation (Takata et al., 2013) is intact in anesthetized IP_3R2 KO mice, in which astrocytic IP_3R-dependent Ca^{2+} signaling is eliminated (Petravicz et al., 2008). The IP_3R2 KO model has been used by numerous groups to study astrocytic IP_3R-dependent cellular processes in situ and in vivo (Petravicz et al., 2008; Di Castro et al., 2011; Takata et al., 2011; Chen et al., 2012; He et al., 2012; Navarrete et al., 2012; Wang et al., 2012; Nizar et al., 2013; Haustein et al., 2014).

We observed that neurovascular coupling was intact in lightly sedated, responsive IP_3R2 KO mice (Figure 7), strongly indicating that astrocytic IP_3R-dependent Ca^{2+} signaling is not necessary for functional hyperemia in vivo. It is insightful that in IP_3R2 KO acute slices, vascular responses to agonists or electrical field stimulation are eliminated (He et al., 2012), a result opposite of our findings and those of two other in vivo studies (Nizar et al., 2013; Takata et al., 2013). It is plausible that mechanisms of neurovascular coupling or vascular responsiveness differ in acute brain slice compared to in vivo.

A recent study characterized rapid astrocytic Ca^{2+} signals following stimuli in vivo, the amplitude of which correlates with the amplitude of functional hyperemia (Lind et al., 2013). A previous study described rapid Ca^{2+} elevations (time to peak ~0.5 seconds) in 5% of

cortical astrocytes in anesthetized mice (Winship et al., 2007). Computational methods were used to isolate astrocytic Ca^{2+} signals from contaminating neuropil signals in both studies. In contrast, our use of GCaMP overcomes the problem of signal contamination by selectively labeling astrocytes. With Lck-GCaMP6s we did not detect rapid Ca^{2+} signals in astrocytes following visual stimulation (Figure 4). The presence or absence of rapid astrocytic Ca^{2+} signals may be a function of the strength of synaptic input to different cortical regions or the nature and strength of stimulus used.

Our results strongly suggest that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling does not play a central role in mediating neurovascular coupling in the intact brain. Neuron-born mediators, perhaps involving the activity of specific interneuron subtypes (Cauli et al., 2004; Kocharyan et al., 2008; Cauli and Hamel, 2010), might be primary drivers of neurovascular coupling in vivo. It is also possible that astrocytes contribute to neurovascular coupling but through signaling processes other than Gq-GPCR-linked IP_3R-dependent Ca^{2+} elevation. Ionic processes have been proposed to mediate neurovascular coupling (Paulson and Newman, 1987; Witthoft et al., 2013), however this possibility is relatively unexplored. Ion fluxes occur in concert with neurotransmitter uptake (Brew and Attwell, 1987; Langer and Rose, 2009) and ion buffering (Kofuji and Newman, 2004; Zhou et al., 2009) during local neuronal firing. In the future it will be interesting to further elucidate these relatively unexamined cellular processes and assess potential roles they might have in mediating neurovascular coupling in vivo.

CHAPTER 3: FUTURE DIRECTIONS IN NEUROVASCULAR COUPLING

THE CHALLENGE: AN ALTERNATIVE NEEDED

Astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} elevation is the major signaling pathway implicated in mediating neurovascular coupling (Attwell et al., 2010; Petzold and Murthy, 2011; Newman, 2013; Howarth, 2014). Due to near-complete coverage of cerebral vasculature by astrocyte endfeet (Mathiisen et al., 2010; McCaslin et al., 2011), neuron-born signals must necessarily be transmitted to the vascular surface by way of astrocytic processes. Certain signaling molecules, like gaseous nitric oxide (NO), are able to diffuse directly to the vascular surface. However, genetic elimination of neuronal type I NO synthase (NOS) does not result in any defect in functional hyperemia to physiological whisker stimulation (Ma et al., 1996), suggesting that neuronal NO does not control neurovascular coupling in vivo.

As more sophisticated in vivo studies are performed (Nizar et al., 2013; Takata et al., 2013), the less likely it appears that astrocytic Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling underlies functional hyperemia. Serious consideration must be given to alterative hypotheses. Unfortunately, there are no alternative proposed mechanisms with enough direct experimental support to be considered viable at this point in time.

Glial K^+ siphoning hypothesis of neurovascular coupling

Originally it was posited that redistribution of extracellular K^+ by astrocytes from synaptic compartments to the vascular surface during neuronal activity mediates neurovascular coupling (Paulson and Newman, 1987). The rationale behind this hypothesis was briefly discussed in Chapter 1. Recent mathematical modeling supports the viability of the idea (Witthoft et al., 2013), but beyond this there is not much direct data on glial K^+ siphoning and neurovascular coupling. The hypothesis is not yet widely considered.

Astrocytes display a passive electrical conductance (Kuffler and Potter, 1964; Meeks and Mennerick, 2007) and are electrically coupled via gap junctions (Adermark and Lovinger, 2008; Meme et al., 2009). Astrocytes behave like K^+ electrodes because of their passive K^+ conductance. Interestingly, astrocytic whole-cell current tracks nearly linearly with evoked excitatory postsynaptic potentials and evoked action potentials in acute slice (Sibille et al., 2014). A significant portion of passive astrocytic K^+ conductance is mediated by inward rectifying channels, specifically Kir4.1 (Djukic et al., 2007), which are expressed in perisynaptic and perivascular astrocyte compartments (Higashi et al., 2001). Astrocytes also express the two-pore K^+ channels TWIK-1 and TREK-1 that could contribute to K^+ redistribution as well (Zhou et al., 2009).

In addition to passive conductance properties, astrocytes actively take up excess extracellular K^+ released from neurons during bouts of activity. Kir4.1 channels partly mediate astrocytic K^+ uptake (Chever et al., 2010), but active uptake appears to depend more on Na^+/K^+ ATPase function (D'Ambrosio et al., 2002). K^+ taken up at perisynaptic astrocyte compartments is “siphoned” from regions of high K^+ concentration to areas of low K^+ concentration via glial networks (Holthoff and Witte, 2000; Amzica et al., 2002; Kofuji and Newman, 2004). Gap junction coupling facilitates but is not necessary for K^+ clearance in the hippocampus (Wallraff et al., 2006). In retina, Muller glial cell K^+ conductance acts to

shunt excess K^+ from the retina into the vitreous fluid (Kofuji and Newman, 2004). An analogous notion is that in brain excess K^+ is siphoned from the parenchymal side of the astrocytic network to the perivascular side. It is known that mild elevations of extracellular K^+ have a strong vasodilatory effect on cortical arterioles (Knot et al., 1996; Horiuchi et al., 2002). Thus K^+ siphoning by astrocytes could in theory provide a method for coupling neuronal activity directly to vascular dynamics.

Conducted vasodilation via vascular endothelium

One cornerstone of the current mechanistic model of neurovascular coupling is that astrocytes release a vasoactive signal directly onto cerebral vascular smooth muscle. This is believed to be the case because smooth muscle surrounding cerebral arteries and arterioles permits dilation or constriction of the vessels, thereby allowing active modulation of blood flow (Nishimura et al., 2007). For this reason, studies have focused specifically on peri-arteriolar astrocyte endfeet in examining the ability of astrocytic Ca^{2+} to modulate vessel diameter (Mulligan and MacVicar, 2004; Takano et al., 2006; Bonder and McCarthy, 2014).

It has been assumed that signaling directly to smooth muscle is required for smooth muscle hyperpolarization/relaxation or depolarization/constriction but this is not necessarily true. It is well recognized that in the peripheral vascular system localized dilations of penetrating arterioles are conducted upstream to increase blood flow throughout an entire vascular tree (Bagher and Segal, 2011). This phenomenon is known as “conducted vasodilation”. While a number of potential mechanisms might contribute to conducted vascular responses, ultimately Ca^{2+} and electrical signaling pathways along a tightly coupled endothelium and at myo-endothelial junctions between endothelial and smooth muscle cells are paramount (Bagher and Segal, 2011). Isolated cerebral arteries and arterioles display

the ability to conduct local vasodilatory signals along entire lengths of the vessel (Dietrich et al., 1996; Horiuchi et al., 2002).

There is evidence that conducted vasodilation operates in the brain in vivo under normal conditions and similarly depend on signaling along the vascular endothelium. Local electrical stimulation of cerebellar parallel fibers in anesthetized rats evoked blood flow increases in and outside the stimulated area (Iadecola et al., 1997). Importantly, vessel dilations were observed in areas where no field potential response could be detected, suggesting that the dilation was conducted along the vessel itself rather than evoked by local neuronal activity (Iadecola et al., 1997). Selective disruption of the vascular endothelium using light-dye treatment, which involves generation of reactive oxygen species that selectively disrupt integrity of the endothelium but leaves the vessel and smooth muscle intact, results impairments in cerebral vascular dilations in vivo (Rosenbaum, 1986; Chen et al., 2014). Electrical hindpaw stimulation leads to increases in total hemoglobin in the somatosensory cortex of anesthetized rats, indicative of increases in blood flow (Chen et al., 2014). Wide-field disruption of endothelial integrity broadly impairs cortical blood flow increases following hindpaw stimulation in vivo whereas local light-dye treatment in a single vessel impairs dilation only upstream of the treatment site (Chen et al., 2014), an incredibly interesting finding. These data indicate that the vascular endothelium is critical for proper neurovascular coupling in vivo and that conducted vasodilation might be a prominent aspect of endothelium-mediated cerebral vascular responses.

This is a critical conceptual shift for the field of neurovascular coupling. Neurovascular signals need not contact arteriole smooth muscle directly at the site of dilation. Instead, neurovascular signals can originate from many hundreds of microns or even millimeters downstream of the dilation site being observed. Furthermore, neurovascular signals need not be released onto vascular smooth muscle. The endothelium is capable of transmitting rapid, long-range electrical or Ca^{2+} signals and communicating

these signals into smooth muscle cells (Socha et al., 2011). It is possible therefore that vascular dilation could ultimately originate within capillary endothelium and propagate upstream to smooth muscle within feeding arterioles. These possibilities are now beginning to be discussed and investigated by certain research groups (Chen et al., 2014), a positive development for the field.

Intersection with astrocytic Ca^{2+} signaling

Astrocytic Ca^{2+}, at least not Gq-GPCR-linked IP_3R-dependent Ca^{2+} signaling, does not appear to directly mediate neurovascular coupling in vivo (Nizar et al., 2013; Takata et al., 2013; Bonder and McCarthy, 2014). On the other hand, many in situ studies (Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Straub et al., 2006) and some in vivo work (Takano et al., 2006) specifically identified this signaling cascade as controlling functional blood flow increases in the brain. A “strong” conclusion drawn from the available data is that mechanisms of vascular diameter change in an acute slice are different from the actual mechanisms operating in vivo. A corollary to this is that acute slice results are experimental artifacts either due to use of pharmacological tools like Ca^{2+} uncaging (Wang et al., 2013a) or the conditions in acute slices and their impact on glial function or reactivity (Takano et al., 2014). Therefore, it is possible that astrocytic Ca^{2+} signaling is completely uninvolved in mediating neurovascular coupling in vivo.

A more conservative conclusion is that astrocytic Ca^{2+} signaling is involved in neurovascular coupling under certain circumstances or is an indirect modulator of the process. Conditions in acute slice might shift the prominence of astrocytic Ca^{2+} signaling in altering vascular diameter, or pharmacological tools might stimulate this signaling cascade to such a degree that it becomes prominent in mediating vascular responses. In either case,

Ca^{2+} can be made to interact with a signaling pathway that ultimately controls functional hyperemia under normal conditions. Based on this, we can use our knowledge of pathway intersections to identify putative alternative mechanisms. It is possible that astrocytic Ca^{2+} elevation exerted its effect on vascular diameter in previous studies by acting through another unidentified pathway. I will examine intersections of Ca^{2+} signaling and K^+ conductance in astrocytes for the purpose of providing evidence in support of glial K^+ siphoning as the mechanism coupling neuronal activity to functional hemodynamics.

A link between astrocytic Ca^{2+} elevation and K^+ flux is well established in the literature. Expression of Ca^{2+}-activated K^+ channels, particularly BK channels, is high in astrocyte endfeet (Price et al., 2002) and it has been shown in situ (Filosa et al., 2006) and in vivo (Girouard et al., 2010) that elevations of astrocytic Ca^{2+} can stimulate K^+ fluxes through these channels that alter vascular diameter. Ca^{2+}-dependent uptake of extracellular K^+ by astrocytes, attributed to stimulation of Na^+/Ca^{2+} exchanger and Na^+/K^+ ATPase activity, has been described in acute slice (Wang et al., 2012). Interestingly, this effect was only observed deeper in the slice and not superficially (Wang et al., 2012). Potentially, this is due to the greater influence of bath solution flow in regulating extracellular ionic environment superficially in the slice, whereas intrinsic homeostatic mechanisms are more prominent deep within the slice. A similar hypothesis could explain why intracellular Ca^{2+}-dependent pathways appear to be critical for neurovascular changes in acute slice but not in vivo.

In addition, two-pore K^+ channels that contribute to passive astrocytic K^+ conductance like TREK-1 (Zhou et al., 2009) are activated by molecules implicated in neurovascular coupling or augmenting blood flow. TREK-1 is stimulated by arachidonic acid (Goldstein et al., 2001), one of the primary astrocyte-derived neurovascular mediators, and isoflurane (Patel et al., 1999), which increases brain blood flow in vivo (Masamoto et al., 2009). It is possible that known vasoactive substances, such as anesthetics, influence brain blood flow through their impacts on astrocytic K^+ currents.

The K^+ siphoning hypothesis is therefore able to be reconciled with previous in situ findings, alleviating discrepancy across studies. I believe this is a strong point of K^+ siphoning as an alternative mechanism of neurovascular coupling. However, it must be noted that the "strong" conclusion – that in situ studies largely characterized experimental artifact – is logically valid and not without support. We were able to evoke large and long-lasting astrocytic Ca^{2+} elevations using the hM3Dq DREADD system without observing any change in basal blood flow in vivo (Chapter 2, Figure 2). The DREADD system has advantages over other signaling manipulation tools. For example, unlike Ca^{2+} uncaging the DREADD allows for activation of the entire fabric of Gq-GPCR signaling rather than a lone Ca^{2+} elevation devoid of signaling context. But the DREADD also has its limitations. We do not know if it is trafficked or its activity regulated (i.e. phosphorylation or receptor turnover) in the same way as endogenous receptors. Also, under normal conditions there would never be stimulation of just astrocytic receptors in the absence of transporter activity or changes in extracellular ion concentrations, as is the case with CNO-activation of DREADD. In summary, intersections of astrocytic Ca^{2+} signaling and K^+ flux pathways might be insightful and relevant in considering alternative mechanisms, but this point can be debated.

Drawing inferences from available data

Recent in vivo work has identified a rapid form of Ca^{2+} response within astrocytes that correlates well with vascular responses in magnitude and kinetics when the contralateral ramus infraorbitalis of the trigeminal nerve was stimulated electrically (Lind et al., 2013). Interestingly, the evoked Ca^{2+} signals differ somewhat between astrocytic cell bodies and endfeet, with the most correlation to vascular responses occurring in the perivascular endfeet (Lind et al., 2013). The onset of this signal is ~100 ms following

stimulus onset, with rapid rising and slower decay phase, in total only several hundred milliseconds in duration (Lind et al., 2013).

The authors of this study did not extensively investigate the source of the fast astrocytic Ca^{2+} elevation, only briefly noting that it was impaired by glutamate receptor antagonists (Lind et al., 2013). We estimated that the onset of functional hyperemia in visual cortex of awake, responsive mice is approximately 300 ms following onset of visual stimulation (Bonder and McCarthy, 2014). It must be noted that most GPCR cascades do not function on this rapid a time scale. For a variety of GPCRs, receptor-G-protein interaction occurs within 50 ms and G protein activation occurring within 0.3-2 seconds (Lohse et al., 2008; Vilardaga, 2010). Accumulation of cAMP downstream of Gs-GPCR activation takes place on the order of tens of seconds (Lohse et al., 2008). There are rare exceptions – for instance, closure of cGMP-gated cation channels involving intermediate enzymatic steps takes place within about 200 ms following light-activation of rhodopsin in the retina (Makino et al., 2003).

We cannot discount the possibility that GPCR-mediated intermediate steps involved in neurovascular coupling operate on the most rapid timescales reported for this class of signaling systems. But even granting this much, the entire neurovascular cascade must be transmitted through multiple cellular compartments and undergo at least two full steps of release and diffusion across the extracellular space (see Figure 1). The number of steps increases if pericytes are included as additional elements, as some groups contend (Peppiatt et al., 2006) – vascular signals initiated at pericytes must still be transmitted upstream to arteriole smooth muscle. Cellular architecture is a limiting factor in neurovascular responses given the sub-second timescale they operate on. It is unlikely that multistep Gq-GPCR-mediated enzymatic cascades and multiple release events and diffusion across the extracellular space could be completed within 300 ms to account for the onset of blood flow increase that we observed in our study (Bonder and McCarthy, 2014).

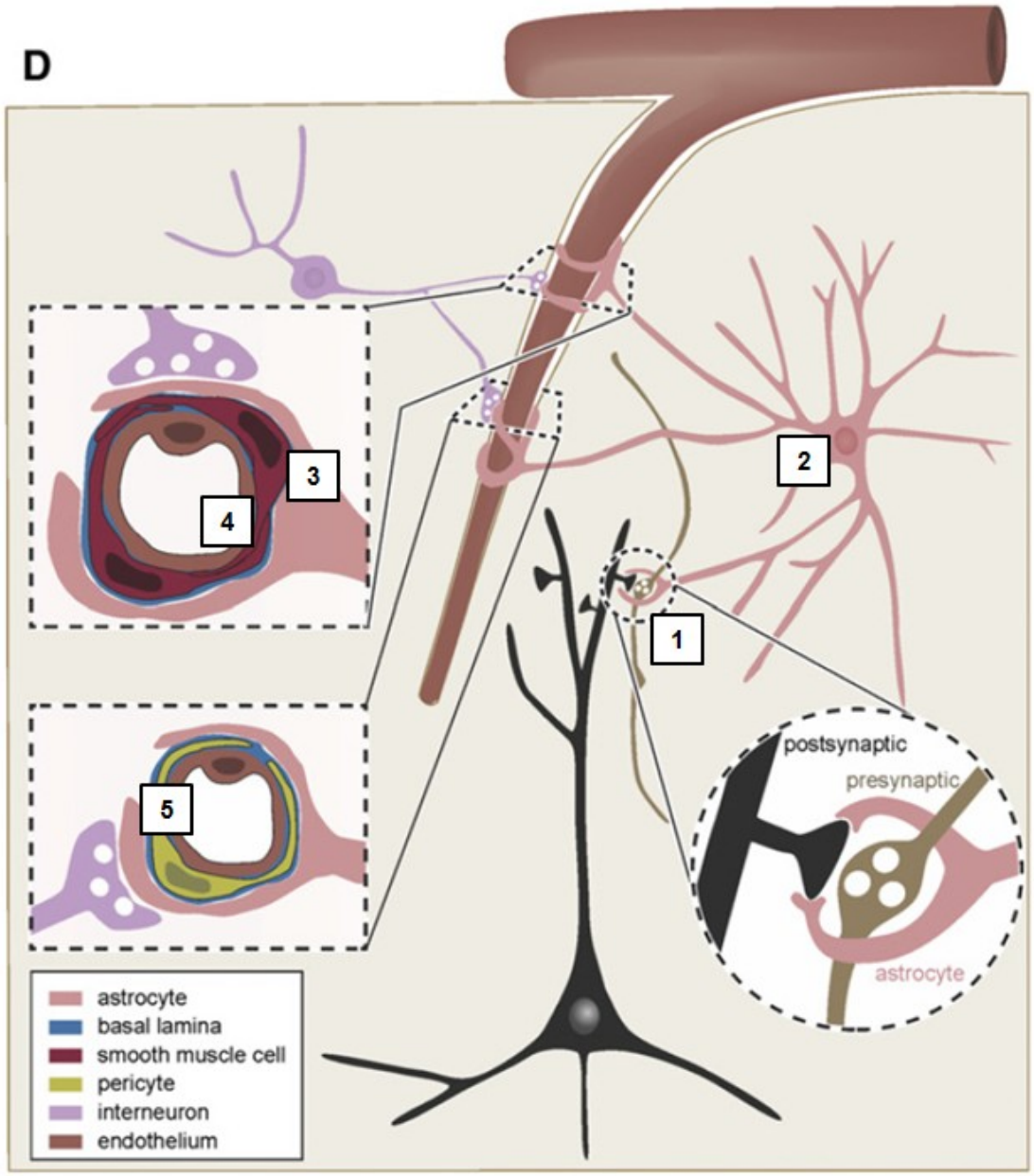

Figure 1 (Petzold and Murthy, 2011). *Schematic of the neurovascular unit.* To reach the vascular surface neurovascular signals must: (1) diffuse from the synapse, (2) pass through astrocyte compartments, (3) diffuse across extracellular space to the vascular surface, and (4) be converted to a vasodilatory signal within vascular endothelium or smooth muscle. Pericytes (5) lie underneath astrocyte endfeet directly on top of the capillary endothelium. If pericytes are considered additional elements of the neurovascular unit, this adds another layer of signal transduction or diffusion.

Further, Lind et al. (2013) noted that neuronal somatic fast calcium responses occurred with an onset of ~100 ms as well. It is unrealistic that astrocytic Gq-GPCR cascades are able to progress at the same rate as neuronal Ca^{2+} elevations driven primarily by voltage-gated calcium channel currents and ion fluxes. Based on this, I assert that the fast astrocytic Ca^{2+} signal reported by Lind et al. (2013) is not mediated by Gq-GPCRs or IP_3R-dependent Ca^{2+} release.

Instead, we suggest that this astrocytic Ca^{2+} signal might be indicative of rapid ion flux taking place within coupled astrocyte networks during bouts of neuronal activity rather than being a receptor-driven event. In addition to extracellular K^+ clearance, electrochemically-driven glutamate uptake elicits an inward Na^+ current in astrocytes (Bergles and Jahr, 1997). Astrocytes also express a host of ion transporters, such as the Na^+/Ca^{2+} exchanger (Rojas et al., 2007) and the $Na^+/K^+/2Cl^-$ cotransporter (Larsen et al., 2014), as well as non-ion-selective connexin-based hemichannels (Chever et al., 2014) and ion-selective TRP channel family members (Shigetomi et al., 2012; Dunn et al., 2013). These many ion fluxes are likely interdependent and can operate on the rapid timescales necessary to account for the experimentally observed onset of functional hyperemia. The Ca^{2+} signal reported in Lind et al. (2013) might therefore also be an expression of many simultaneous, rapid ionic processes.

This is an exciting proposition considering the excellent correlation of amplitude and kinetics between fast astrocyte endfoot Ca^{2+} signals and evoked blood flow responses (Lind et al., 2013). Rapid Ca^{2+} elevation could, in itself, mediate functional hyperemia. This seems unlikely based on our fast linescan data reported in Chapter 2 (Figure 4I), in which no Ca^{2+} elevation was observed following visual stimulation in awake, responsive mice but vascular dilations were robust. An alternative hypothesis is that simultaneous, co-dependent rapid ion flux pathways, such as K^+ siphoning, would be similarly correlated to vascular responses

and control functional hyperemia. In other words, fast Ca^{2+} signals are in this sense a "marker" for ionic fluxes. This was not considered in Lind et al. (2013)

If true, it is curious that we did not observe fast astrocyte endfoot Ca^{2+} elevations in our experimental conditions, since similar ionic astrocytic processes would be assumed to occur. It is possible that the presence or absence of fast Ca^{2+} elevations depends on the nature and dynamics of neuronal activity in the brain region in question. Mouse visual cortex, where we performed our experiments, is not segregated into functional neuronal groups as is the mouse somatosensory cortex, which contains discrete hindlimb-, forelimb- or whisker-processing regions. Instead orientation selectivity is dispersed in a "salt-and-pepper" fashion. A consequence of this is that, for any single stimulus, an astrocyte in mouse visual cortex "senses" activity from only one or several neurons simultaneously whereas an astrocyte in somatosensory cortex "senses" activity from many neurons. Weaker or less prominent ionic fluxes would be expected to occur in any single astrocyte in the functionally dispersed mouse visual cortex compared to the functionally segregated somatosensory regions, perhaps explaining why we did not observe fast Ca^{2+} signals (again, assuming these signals are coordinated with ionic fluxes). We offer a similar explanation as to why we did not observe large somatic Ca^{2+} elevations in astrocytes (Chapter 2, Figure 2) whereas other groups have in different cortical regions (Wang et al., 2006; Petzold et al., 2008; Schummers et al., 2008; Nimmerjahn et al., 2009).

Arguments against glial K^+ siphoning mediating neurovascular coupling

To my knowledge, there is only one study which attempted to directly test the hypothesis that glial K^+ siphoning mediates neurovascular coupling. In acute retinal preparations, directly depolarizing Muller glia to stimulate K^+ extrusion onto retinal

vasculature did not result in changes in vessel diameter (Metea et al., 2007). It should be noted that it was not demonstrated that depolarizing glia resulted in elevations in extracellular K^+ as intended. In addition, in a genetic mouse model lacking Kir4.1 channels (Kofuki et al., 2000) light-evoked vessel dilations were intact in acute retinal preparations (Metea et al., 2007). The authors therefore concluded that neurovascular coupling is not mediated by glial K^+ siphoning.

Conceptually, there are valid reasons for interpreting the findings of this study conservatively. First, as previously discussed, astrocytes in the brain express numerous K^+ conductance routes. Much of retinal Muller glial K^+ conductance appears to be mediated by Kir4.1 channels (Kofuki et al., 2000), though this was determined mainly by examining alterations in the light-evoked electroretinogram which is perhaps a less sensitive assay than direct measures of K^+ conductance. I believe it is pertinent to reiterate the point that bath solution flow in acute preparations could have blunted intrinsic processes of ionic homestasis or ionic signaling, or that Muller glia could have entered a state of reactivity (Takano et al., 2014), impacting the results of the study. The possibility remains that Kir4.1 KO Muller glia are capable of carrying K^+ currents sufficient to achieve a workable K^+ siphoning effect. This could explain the presence of intact light-evoked vascular diameter changes in the Kir4.1 KO.

More importantly, vasculature in the retina is not completely surrounded by glial processes (Ochs et al., 2000). In the outermost retinal layer I, coverage of vessels by non-Muller cells approaches 50% on average (Ochs et al., 2000). Horizontal cells also make frequency contacts with retinal vasculature (Knabe and Ochs, 1999). Given the significantly differing cellular architecture of the retinal neurovascular unit compared to that in brain, it is possible that mechanisms of neurovascular coupling in retina differ from those in brain. The precedent exists, as peripheral functional blood flow increases are mediated by direct neuronal afferents onto arteries and arterioles (Bagher and Segal, 2011).

PROPOSED EXPERIMENTAL DESIGN TO TEST THE K^+ SIPHONING HYPOTHESIS

Overview

Early notions of astrocytic K^+ conductance asserted that Kir4.1 channels were solely or primarily responsible for carrying K^+ current (Metea et al., 2007). In contrast, I argue that the phenomenon of glial K^+ siphoning is a complex process involving the concerted actions of multiple K^+ conductance routes. There is also the possibility that other ionic processes, such as Ca^{2+} elevation, could interact with and influence glial K^+ fluxes. The experimental consequence of this complexity is that inhibition or genetic deletion of single proteins or channels will not obliterate the phenomenon of glial K^+ siphoning in its entirety. This makes the hypothesis difficult to effectively test directly, that is, to design an experimental targeting strategy that will block K^+ outright. Instead, I propose that this hypothesis be tested through a series of correlation studies utilizing different genetic models or pharmacological agents. Specifically, one would examine the correlation between functional hyperemia in vivo and properties of astrocytic K^+ conductance in vivo or in situ assessed by patching astrocyte endfeet.

For instance, we performed preliminary experiments measuring functional hyperemia in response to visual stimuli in the Kir4.1 cKO mouse line, in which Kir4.1 was genetically eliminated selectively in the visual cortex by injection of AAV8-GFAP-Cre. These initial results suggest that blood flow increases in the Kir4.1 cKO might be delayed and lower in amplitude compared to wild type control mice (Figure 2). If the glial K^+ siphoning hypothesis is true, then outward K^+ currents in astrocyte endfeet should be similarly blunted in amplitude or kinetics. Available data, though limited, is so far consistent with this idea. The Kir4.1 cKO displays slower K^+ clearance and lower average local field potential power spectrum (Chever et al., 2010). Correlation of results across a range of models and

molecular targets, with proper controls, builds support for the hypothesis that astrocytic K^+ siphoning mediates neurovascular coupling in vivo. Potential difficulties with interpreting experimental results will be discussed.

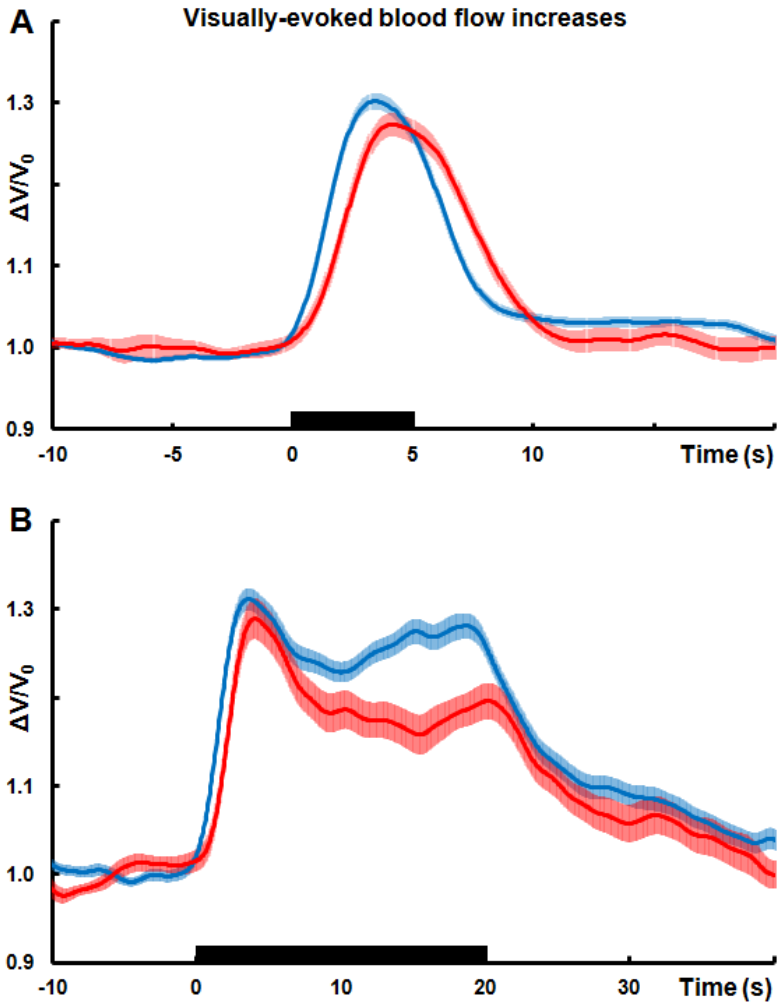

Figure 2. *Functional hyperemia might be impaired in the Kir4.1 cKO*. (A) Average cortical blood flow increases in wild type (blue trace, n = 7 mice, 379 stimulus trials) and Kir4.1 cKO (red trace, n = 2 mice, 47 stimulus trials) following presentation of 5 second-long drifting grating visual (black bar) stimuli to awake, responsive mice. (B) Average cortical blood flow increases in wild type (blue trace, n = 7 mice, 189 stimulus trials) and Kir4.1 cKO (red trace, n = 2 mice, 40 stimulus trials) following 20 second-long stimuli (black bar). Shaded regions in A and B represent standard error of mean.

Suggested Genetic Models and Pharmacology

Kir4.1 cKO (Djukic et al., 2007; Chever et al., 2010) – Kir4.1 is a weakly inwardly rectifying K^+ channel expressed predominantly in astrocytes in brain and a major carrier of astrocytic passive K^+ conductance.

TREK-1 KO (Zhou et al., 2009; Namiranian et al., 2010) – TREK-1 is a two-pore domain K^+ channel contributing to astrocytic passive K^+ conductance.

GLT1 KO (Tanaka et al., 1997) – GLT1 is one of the two primary glutamate transporters in brain, expressed predominantly by astrocytes.

GLAST KO (Watase et al., 1998) – GLAST, the glutmate-aspartate transporter, is another major glutamate transporter in brain, expressed predominantly by astrocytes.

Cx43 cKO / Cx30 KO (Wallraff et al., 2006) – Connexin 43 and connexin 30 are the two connexin proteins expressed in astrocytes that comprise astrocytic gap junctions.

TWIK-1 KO (Wang et al., 2013b) – TWIK-1 is a two-pore domain K^+ channel contributing to astrocytic passive K^+ conductance.

Aqp4 KO (Strohschein et al., 2011) – Aquaporin 4 is a water-permeable channel expressed in astrocytes that helps maintain cellular osmolarity, and tissue water and ionic homeostasis.

Na^+/Ca^{2+} exchanger (NCX) KO (Wakimoto et al., 2000; Wang et al., 2012) – NCX extrudes Ca^{2+} from cells, though its activity can be reversed allowing for Ca^{2+} uptake if intracellular Na^+ levels are sufficient.

Inhibition of Na^+/K^+ ATPase with ouabain (Larsen et al., 2014) – Na^+/K^+ ATPase activity produces an efflux of Na^+ ions and influx of K^+ ions into cells. In astrocytes, Na^+/K^+ ATPase activity is responsible for rapid clearance of extracellular K^+ during neuronal activity.

Inhibition of $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) with bumetanide (Larsen et al., 2014)

Inhibition of Na^+/Ca^{2+} exchanger (NCX) with SN-6 or SEA0400 (Wang et al., 2012)

Required Controls and Potential Difficulties

In addition to the precise mechanisms coupling neuronal activity to vascular dynamics, neurovascular coupling is a function of neuronal and vascular responsiveness. In other words, any genetic or pharmacological manipulation that alters neuronal activity or the ability of cerebral vessels to dilate will in turn affect neurovascular coupling, independent of any changes to the intermediate coupling mechanisms. Thus, there are two primary concerns that must be addressed in this experimental paradigm – basal neuronal activity and basal vascular responsiveness must be compared between the variable and control groups. In the optimal situation, neither of these parameters would differ between variable and control groups, therefore any difference in functional hyperemia can be attributed to the intermediate mechanisms coupling neuronal activity to vascular dynamics.

Basal neuronal activity can be assessed in situ or in vivo. In acute slice preparations spontaneous and evoked postsynaptic currents or field potentials should be measured in the same cortical region where functional hyperemia is being measured in vivo. The visual cortex is particularly attractive for these studies because the circuitry is well understood (Bear and Rittenhouse, 1999). Similar electrophysiological measures are feasible in vivo, though Ca^{2+} dyes like OGB-1 are more effective for evaluating circuit dynamics or simultaneously monitoring activity within many neurons at a single-cell level. Basal and evoked Ca^{2+} spiking frequency, amplitude, and signal kinetics are obtainable with Ca^{2+} dyes. For example, in our study (Bonder and McCarthy, 2014) we compared the amplitude and kinetics of visual stimulus-evoked neuronal somatic Ca^{2+} elevations and the fraction of

visually-responsive neurons between wild type controls and IP_3R2 KO mice (Chapter 2, Figure 3). These measures provided the basic picture that single cell responses to stimuli and network dynamics in general were not substantially altered in the IP_3R2 KO visual cortex. It was also well-established that in this particular genetic model, basal neuronal activity is not different from wild type controls (Petravicz et al., 2008; Agulhon et al., 2010).

Ultimately, it will be most informative to investigate synaptic and neuronal activity both in situ and in vivo. In certain instances, synaptic defects could be detected in acute slice preparations but the impact on neuronal function in vivo would not be intuitively inferred or known. For example, the Kir4.1 cKO displays normal pyramidal neuron membrane properties, whole-cell currents and excitability with normal input-output relationships and paired-pulse facilitation, but decreased spontaneous EPSC frequency and amplitude (Djukic et al., 2007). Does this mean that visually-evoked neuronal responses in vivo would be normal or decreased? Might the number of visually-responsive neurons differ? The only way to know is to perform the in vivo experiment. In addition, genetic or pharmacological manipulations can selectively or primarily impair synaptic plasticity as opposed to basal synaptic transmission. The Kir4.1 cKO has enhanced short-term plasticity (Djukic et al., 2007; Sibille et al., 2014). Ostensibly the mechanisms of short-term plasticity are not relevant during basic visual stimulus processing, but this can only be determined by monitoring neuronal activity in vivo.

One additional measure that will be helpful in cases where neuronal activity is significantly impacted by the genetic or pharmacological manipulation under study is to monitor elevation of extracellular K^+ during evoked bouts of neuronal activity using K^+ electrodes. The theory behind the K^+ siphoning hypothesis is that K^+ flows down its concentration gradient from synaptic compartments where K^+ concentration is elevated to perivascular compartments where K^+ concentration is lower. Astrocytes, as passive conductors of K^+ current, act as the pipeline for K^+ current to flow as dictated by this

gradient. Therefore the particulars of single neuron activity or even network dynamics are not as important as the resultant elevation of extracellular K^+. In other words, the degree of extracellular K^+ concentration elevation is the relevant measurable endpoint. This principle is valid in theory but needs to be demonstrated experimentally.

Intrinsic vascular responsiveness is another factor that can be impacted by genetic or pharmacological manipulations. An effective way to test for this is to apply known vasoactive stimuli to isolated vessels in situ. Such stimuli include elevated extracellular K^+ (Knot et al., 1996; Horiuchi et al., 2002) or ATP (Namiranian et al., 2010). Whole-cell currents from smooth muscle cells should be examined by patch-clamp technique. By these methods Namiranian et al. (2010) determined that basal cerebral vessel reactivity does not differ between wild type controls and TREK-1 KO mice. In vivo, intrinsic vascular responsiveness can be examined by intravenous injection of compounds that act as vasodilators or vasoconstrictors by acting directly at the vessel. These include nitroglycerine which is converted to NO within blood vessels (Tassorelli et al., 1999) or adrenoceptor agonists and antagonists (Tsai et al., 1989). Such treatments will also induce systemic cardiovascular changes, which could introduce confounding influence in interpreting experimental results, more so in germ-line KO models which could have cardiovascular differences from wild type animals. It is therefore recommended that selective, conditional genetic manipulations and local pharmacological treatments be used when available.

The greatest challenge in the experimental design I have outlined will occur in instances where neuronal activity or vascular responsiveness are substantially altered in the variable experimental group. In these cases, it will be difficult or impossible to attribute changes in functional hyperemia specifically to impairments in astrocytic K^+ siphoning versus impairments in neuronal or vascular reactivity. Interestingly, this is not something the field has generally paid enough attention to in the past. Many studies have used antagonist applications in vivo or bath washes in situ to target astrocytic signaling cascades. With

blunted neurovascular coupling observed compared to the non-drug condition, these various studies concluded that the signaling cascade in question is directly involved in mediating neurovascular coupling (Zonta et al., 2003, Mulligan et al., 2004, Takano et al., 2006). However, these investigators failed to take into account alterations in basal neuronal or vascular responsiveness. For example, a 50% decrease in functional hyperemia to a given stimulus following drug application - is this effect due to a 50% decrease in the fidelity of intermediate neurovascular signaling (as authors typically assume) or due to a 50% decrease in the intrinsic ability of vessels to dilate?

I believe that future studies must take adopt more holistic viewpoint in studying the phenomenon of neurovascular coupling. Besides proposing a relatively novel idea in K^+ siphoning that considers a broader perspective, I also attempted to create an experimental design and strategy more carefully controls for potential confounding variables.

CHAPTER 4: CONCLUSIONS

IS ASTROCYTIC Ca^{2+} SIGNALING CRUCIAL FOR BRAIN FUNCTION?

Prominent literature reviews continue to argue that astrocytic Ca^{2+} signaling is a crucial mediator of brain function and an active component of information processing (Araque et al., 2014; Volterra et al., 2014). Some evidence is consistent with these claims but other data is flatly incompatible.

The IP_3R2 KO mouse line: a case study in cognitive dissonance

The IP_3R2 KO mouse line genetically lacks the IP_3R subtype expressed by astrocytes (Holtzclaw et al., 2002), thereby eliminating Ca^{2+} release from the ER. Astrocytes express various potential routes for regulating basal intracellular Ca^{2+} levels, such as TRP family channels (Shigetomi et al., 2012, Dunn et al., 2013). No published studies have identified modes of neuronal activity-dependent elevation of astrocytic Ca^{2+} other than Gq-GPCR-linked Ca^{2+} release from the ER via IP_3Rs. Accordingly, the IP_3R2 KO mouse line lacks all observable neuronal activity-dependent Ca^{2+} elevation (Petravicz et al., 2008). Surprisingly, IP_3R2 KO mice are overtly normal and indistinguishable from wild type controls (Petravicz et al., 2008). This directly contradicts the conclusions of many prior and subsequent studies. If astrocytic Ca^{2+} signaling were important for normal brain function, the IP_3R2 KO line should possess multiple severe cognitive defects.

Two arguments are frequently provided to explain the apparent contradiction of the IP_3R2 KO. One argument suggests that there are developmental mechanisms that compensate for the loss of IP_3R-dependent Ca^{2+} signaling. This is a typical, reasonable criticism of germ-line knockout genetic lines. However, in this case it is most likely not a concern. Observable neuronal activity-dependent astrocytic Ca^{2+} increases are clearly absent in IP_3R2 KO mice. Therefore, Ca^{2+} signaling itself is not compensated for by non-IP_3R-dependent pathways. The most common examples of total compensation occur in cases of similar proteins or protein isoforms being able to cover the loss of another protein's function. But ions like Ca^{2+} do not have alternate "isoforms" so it is unclear what could so precisely and completely compensate for the loss of ion signaling in the IP_3R2 KO.

In addition, a cKO of IP_3R2 using Cre-lox recombination under the control of the glial GFAP promoter produces a mouse that similarly lacks astrocytic Ca^{2+} elevations but also displays no overt behavioral phenotypes (Petravicz et al., 2014). Conditional models typically suffer less from developmental compensation than germ-line knockouts because the onset of genetic deletion is a function of the onset of promoter activity. The fact that the IP_3R2 cKO also appears normal suggests that the lack of significant defects in the germ-line IP_3R2 KO is not due to developmental compensation.

The second major argument proposed to explain the IP_3R2 KO discrepancy contends that IP_3R-independent Ca^{2+} signaling persists in the fine processes of astrocytes, but traditional imaging techniques (i.e. Ca^{2+} dyes) are not able to detect this activity. This point is valid but also irrelevant to the present argument. It is true that bulk-loaded Ca^{2+} dyes do not provide sufficient signal-to-noise in the neuropil to detect microdomain signals. Even if they did, the dyes do not necessarily load selectively in astrocytes, making it difficult if not impossible to discern astrocytic Ca^{2+} activity from neuronal in these regions. But on the other hand, all of the documented roles of astrocytic Ca^{2+} signaling have been determined from studies monitoring large amplitude Ca^{2+} signals readily observed using dyes. In most

cases these signals are measured from the cell body, not the processes. If unobservable levels of astrocytic Ca^{2+} signaling are sufficient to preserve largely normal brain function in the IP_3R2 KO, then even the smallest observable elevations of astrocytic Ca^{2+} should produce incredibly robust effects on neuronal activity or vascular diameter. Yet, large elevations of astrocytic Ca^{2+} can be induced using exogenous receptor systems without producing any effect on neuronal activity (Fiacco et al., 2007; Agulhon et al., 2010) or basal blood flow (Bonder and McCarthy, 2014).

GCaMPs are beginning to reveal the nature and dynamics of microdomain Ca^{2+} signaling in fine astrocyte processes. A recent study identified residual basal but not neuronal activity-dependent Ca^{2+} dynamics in IP_3R2 KO astrocytes in the hippocampal mossy fiber pathway (Haustein et al., 2014). It is possible that residual neuronal activity-dependent Ca^{2+} signaling could persist in astrocytic microdomains in other brain regions in vivo, though there is no data yet to suggest this is true. Regardless, these are not the types of Ca^{2+} dynamics implicated in the roles of astrocytes in greater brain function.

Most likely the IP_3R2 KO mouse model does possess cognitive defects, but they may be too subtle to detect by typical behavior batteries. It is possible that these defects might become exaggerated and detectable under certain conditions. For example, given the putative role of astrocytes in maintaining brain metabolic homeostasis, it would be interesting to examine the effect of limiting food intake long-term on IP_3R2 KO mice versus wild type controls. Unable to rely on sufficient levels of ambient glucose, neurons might require the breakdown of astrocytic glycogen and shuttling of metabolic substrates from astrocytes to neurons to function properly. Mechanisms such as these might be impaired in IP_3R2 KO mice but with access to limitless quantities of food the defects are negligible. There might also be social abnormalities in these mice as well, but these sorts of tests are always difficult to interpret.

“Funny-looking neurons”

Whether or not astrocytic Ca^{2+} signaling is crucial for brain function is actually less interesting to me than the fact that most of the field views answering this question in the negative as an absurd proposition. Why is this? Consider the same question in relation to any other signaling pathway – Gs, Gi, receptor tyrosine kinases, ERK, transcription factors, etc. We might anticipate deficits in genetic models that have eliminated these targets in astrocytes, simply by virtue of the fact that astrocytic function overall has been impacted. At the same time, we might not be surprised to find relatively little wrong. The data would be the data. But with Ca^{2+} signaling the field in general seems very intent on rationalizing the available data in such a way that astrocytic Ca^{2+} remains critical for brain function.

I believe that the reason for this is that astrocytic Ca^{2+} signaling, since the initial publication of intercellular Ca^{2+} waves by Cornell Bell et al. (1990), has been conceptualized in a manner analogous to neuronal action potentials. That is to say, Ca^{2+} signaling is considered the mode of astrocyte excitability, as action potentials are for neurons. As a colleague at UNC, Richard Weinberg, once accurately phrased it, astrocytes are viewed as “funny-looking neurons”. This concept requires further clarification.

All living cells have the ability to respond to the environment in some way. In this sense, all cells are "responsive". Responsiveness to the environment must be contrasted with “excitability”, which is a special property of certain cell types whose overall function is ultimately directed towards a discrete, singular output. The canonical example of such a cell in neurobiology is the neuron, its “mode of excitability” being the action potential. The function of neurons is to communicate and integrate information across distances. The action potential is the cellular switch that turns a neuron “on” or “off” in relation to performing this overall function. In other words, a mode of excitability defines binary states of cellular function. The cell is either currently performing its overall function, or it is not. Therefore, we

can discuss “cellular activity/activation” with the understanding that the “activity/activation” is related to the mode of excitability – i.e. “neuronal activity” is the action potential.

Neurons clearly do much more than simply fire action potentials – diverse molecular pathways are involved in guiding axon or dendrite growth, synapse formation and maintenance, governing a cell’s ability to fire, and so on. These are all examples of a neuron’s ability to respond to environmental factors. Genetic models that target these various pathways might impair neuronal function, but only because the ability to fire action potentials is impaired. Therefore, these diverse pathways of environmental responsiveness do not directly factor into “neuronal activity”, since this term specifically refers to and includes only action potentials.

In other words, the action potential is the readout of overall neuronal function. A brain that has been completely bathed in TTX and is devoid of action potentials is still a “dead” brain regardless of the massive and diverse plethora of environmental responsiveness activities taking place. That is ultimately what illustrates the difference between “responsiveness” and “excitability”.

Simply possessing the ability to respond to environmental factors (i.e. expressing receptor systems) does not automatically grant a cell the property of “excitability”. If a cell is not excitable, then we cannot refer to that cell’s “activity” in a general sense – there is no overarching “activity” indicative of a discrete, singular output. This is the crux of the discussion on astrocytes and Ca^{2+} signaling – astrocytic Ca^{2+} signaling has been confused into being a mode of excitability rather than being recognized as a mode of environmental responsiveness, the same as any other molecular pathway. In fact, the question has never even been asked, “Are astrocytes excitable cells?” Instead this has unjustifiably been assumed to be true.

This is clearly reflected in the language used in many glial biology publications. I will choose one particularly egregious recent publication to illustrate my point, titled:

"Norepinephrine controls astroglial responsiveness to local circuit activity" (Paukert et al., 2014). Already in the title, the authors reference astroglial "responsiveness", implying some overarching mode of cellular excitability distinct from other forms of environmental responsiveness. This mistaken conceptual phrasing is repeated continually throughout the article. For instance, in the abstract (bold added by me for emphasis):

> "However, little is known about **how astrocytes are engaged** during different behaviors in vivo. Here we demonstrate that norepinephrine **primes astrocytes to detect changes** in cortical network activity. We show in mice that locomotion **triggers simultaneous activation of astrocyte networks** in multiple brain regions. This **global stimulation of astrocytes** was inhibited by alpha-adrenoceptor antagonists and abolished by depletion of norepinephrine from the brain. Although astrocytes in visual cortex of awake mice were **rarely engaged** when neurons were activated by light stimulation alone, pairing norepinephrine release with light stimulation markedly enhanced astrocyte Ca^{2+} signaling. Our findings indicate that norepinephrine **shifts the gain of astrocyte networks** according to behavioral state, **enabling astrocytes to respond** to local changes in neuronal activity" (Paukert et al., 2014).

Phrases such as "astrocytes are/are not engaged" or "stimulation/response of astrocytes" imply logically that astrocytes are switching from an "off" to an "on" state or remaining "off" during different behaviors or with certain experimental manipulations. Similar language is found many studies, for example those that utilize channelrhodopsin (or similar systems) to "optogenetically activate astrocytes" or "photostimulate astrocytes" (Gourine et al., 2010; Li et al., 2012; Sasaki et al., 2012; Perea et al., 2013). Instead, researchers should phrase these techniques as "optogenetic activation/photostimulation of astrocytic Ca^{2+} signaling". The difference in wording is subtle but the conceptual implications are enormous.

Additionally, properties of astrocytic Ca^{2+} signaling have been described using the same terms and concepts as neuronal signaling. For instance (bold added by me for emphasis) – "Bidirectional **scaling** of astrocytic metabotropic glutamate receptor signaling following long-term changes in neuronal firing rates" (Xie et al., 2012), "Intracellular calcium oscillations in astrocytes: a **highly plastic**, bidirectional form of communication between neurons and astrocytes in situ" (Pasti et al., 1997), "Long-lasting changes of calcium oscillations in astrocytes: a new form of glutamate-mediated **plasticity**" (Pasti et al., 1995).

In common language, the term “plasticity” means flexibility or the ability to change. In this sense, it is not inaccurate to refer to the “plasticity” or “scaling” of astrocytic receptors or signaling. But in the discipline of neuroscience, the terms “plasticity” and “scaling” refer to particular molecular and cellular processes of neurons. In the publication titles cited above, by referring to astrocytic “plasticity” or “scaling” the authors were drawing unsupported parallels to neuronal processes even if this was not intended.

These types of phrases reveal the assumption made by many glial biologists that astrocytes are excitable cells, Ca^{2+} elevation being the mode of excitability. At the very least it demonstrates that many researchers’ thinking is biased towards viewing astrocytic function through a pre-conceived lens of neuronal function. This explains, completely in my mind, why there is such controversy surrounding the IP_3R2 KO mouse line. It has been conceptualized as a “silent astrocyte” model. The lack of overt phenotypes must therefore be interpreted to mean that astrocytes themselves are unnecessary for brain function. This truly is an unreasonable conclusion, but one based on a false premise. The IP_3R2 KO is not a “silent astrocyte” model – it simply lacks astrocytic IP_3R-dependent Ca^{2+} elevation, one of many astrocytic signaling pathways. In fairness, to my knowledge the specific term “silent astrocyte model” has never been used in formal literature. But given my prior discussion on language in publications, the dominant conceptualization of the IP_3R2 KO as a “silent astrocyte” model is logically (I would argue necessarily) implied.

An absence of Ca^{2+} elevation in astrocytes following some stimulation does not mean that astrocytic neurotransmitter transporters are not taking up excess glutamate at the synapse, that astrocytic glycogen is not being broken down or blood-born glucose is not being taken up by astrocytes, or that elevated extracellular K^+ is not being buffered away from active synaptic compartments by astrocytes. In what manner then are astrocytes “not engaged” in the absence of Ca^{2+} elevation? Each individual signaling pathway operating within the astrocyte must be considered in turn as possessing its own unique drivers and

outputs and contributing to a discrete range of functions. We should not point to one cellular readout (like Ca^{2+} elevation) and label it “astrocyte activity”. No such overarching astrocytic activity exists. Framing astrocytic function in these terms is misleading and inaccurate.

CONCLUSION

The early 1990s was a period of rapid conceptual shift in glial biology, frequently cited as a revolution. In reviewing literature dating back to the inception of the field, it becomes clear that some aspects of the "Great Glial Revolution" are products of a bit of revisionist history. For instance, it was never the dominant belief that astrocytes are solely passive, structural support elements. In Chapter 1 I made note of some of the hypotheses put forward by researchers in the late 1800s and early 1900s – that glia insulate nerve fibers (Ramon y Cajal, 1920), feed neurons (Golgi, 1885-1886), or take up waste or signaling substances released by neurons as they fire (Berlucchi, 2002). Similarly, we find researchers in the 1950s and 1960s asking questions like, "Do astrocytes modulate neuronal activity through electrical signaling?" "Do astrocytes feed metabolites to neurons?" Rather than a paradigm shift, experiments in the early 1990s really just provided feasible cellular and molecular mechanisms to justify the long-held belief that astrocytes are more than "nerve-glue".

Regardless, the last couple decades of glial research have been exciting and productive on a number of fronts, spurring development of new imaging and experimental tools like Ca^{2+} dyes, GCaMPs, DREADDs, optogenetics and multiphoton in vivo microscopy. Yet at the same time, endemic throughout the field is the seemingly contradictory notion that "we *really don't know* what these cells do". To quote a recent review and perspective article (Araque et al., 2014):

> "It is quite clear that astrocytes play a very large array of roles in multiple brain regions, utilizing a multitude of functional membrane receptors and signaling molecules. Yet, given the complexity and diversity at play, it is no surprise that the literature also reports some discrepant results regarding the roles played by astrocytes in the regulation of synaptic functions. These data highlight our limited understanding of the true nature of astrocytes and their interactions with neurons and point to future directions for research on neuron-glia interactions" (p. 735).

I find this quote both intriguing and telling, and it is by no means an outlier. The review by Murphy and Pearce (1987), which forms the backbone of my Chapter 1 discussion on astrocytic receptors, contains the following phrase in relation to the consequences of receptor activation: “In fact, very little is known [of the functions of these cells in the CNS], which makes difficult the selection of assays for measuring [functional] ‘endpoints’” (p. 386). Another review on astrocytic receptor systems coming a decade later (Porter and McCarthy, 1997) posits that: “…it is likely that future research will provide evidence for even more exciting and dynamic roles for astrocytes in CNS physiology and pathology” (p. 451).

We learn more facts about astrocytes with each new study, yet it feels as though we come no closer to a coherent understanding of their purpose in the brain, their “true nature”. Some astrocytic functions are generally accepted, such as metabolic support, even if the details are debated. But topics like the role of astrocytes in neurovascular coupling, which was explored in depth in this thesis and our publication (Bonder and McCarthy, 2014), or gliotransmission are fiercely contested. Neurovascular coupling research is essentially “back to square one” at this point. Still other areas of glial research, like defining the properties and functions of gap junction-coupled astrocytes networks, are in their infancy. I do not think it unreasonable to conclude that certain sub-fields have been under-represented throughout all the debate and frenzy.

I outlined what I believe is a fundamental roadblock to progress in glial research – we must consciously recognize and abandon the concept of astrocytic Ca^{2+} signaling as a mode of cellular “excitability”. By adopting this updated paradigm many present debates become immediately much less contentious. For example, that neuronal function is normal in the IP_3R2 KO (Petravicz et al., 2008; Agulhon et al., 2010) or unaltered by stimulating exogenous Gq-GPCRs (MrgA1) expressed in astrocytes (Fiacco et al., 2007; Agulhon et al., 2010) does not automatically negate the idea of gliotransmission. Rather than these findings

implying that “astrocytes do not release gliotransmitters”, they signify that “astrocytes do not release gliotransmitters in an IP_3R-dependent manner”.

To be fair, the scientists that published the initial results with the IP_3R2 KO and MrgA1 Gq-GPCR system (Fiacco et al, 2007, Petravicz et al., 2008, Agulhon et al., 2010) concluded only that their findings suggest that, “at least in the hippocampus, the mechanisms of gliotransmission need to be reconsidered” (Agulhon et al., 2010, p. 1250). However, other researchers interpreted these studies somewhat differently: “The field seems to be divided into three conflicting views, [one of which is that] Ca^{2+}-dependent astrocytic glutamate does not do anything important” (Woo et al., 2012, p. 25). This is an instructive illustration of how across the field, glial biologists do not approach ideas from the same fundamental conceptual viewpoint.

In my opinion we spend an inordinate amount of time debating details, rather than identifying and reconciling the foundational conceptual differences that give rise to our error and disagreement. With differing foundations for thought, it is unsurprising that we arrive at differing and often-times incompatible conclusions. The day this is no longer the case, a true “Great Glial Revolution” can take place.

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