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Permalink

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Journal

Journal of Neuroscience, 38(1)

ISSN

0270-6474

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Publication Date

2018-01-03

DOI

10.1523/jneurosci.0016-17.2017

Peer reviewed

Dual Perspectives

Dual Perspectives Companion Paper: Gliotransmission: Beyond Black-and-White, by Iaroslav Savtchouk and Andrea Volterra

Multiple Lines of Evidence Indicate That Gliotransmission Does Not Occur under Physiological Conditions

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A major controversy persists within the field of glial biology concerning whether or not, under physiological conditions, neuronal activity leads to Ca^{2+}-dependent release of neurotransmitters from astrocytes, a phenomenon known as gliotransmission. Our perspective is that, while we and others can apply techniques to cause gliotransmission, there is considerable evidence gathered using astrocyte-specific and more physiological approaches which suggests that gliotransmission is a pharmacological phenomenon rather than a physiological process. Approaches providing evidence against gliotransmission include stimulation of Gq-GPCRs expressed only in astrocytes, as well as removal of the primary proposed source of astrocyte Ca^{2+} responsible for gliotransmission. These approaches contrast with those supportive of gliotransmission, which include mechanical stimulation, strong astrocytic depolarization using whole-cell patch-clamp or optogenetics, uncaging Ca^{2+} or IP3, chelating Ca^{2+} using BAPTA, and nonspecific bath application of agonists to receptors expressed by a multitude of cell types. These techniques are not subtle and therefore are not supportive of recent suggestions that gliotransmission requires very specific and delicate temporal and spatial requirements. Other evidence, including lack of propagating Ca^{2+} waves between astrocytes in healthy tissue, lack of expression of vesicular release machinery, and the demise of the D-serine gliotransmission hypothesis, provides additional evidence against gliotransmission. Overall, the data suggest that Ca^{2+}-dependent release of neurotransmitters is the province of neurons, not astrocytes, in the intact brain under physiological conditions.

Key words: astrocyte; calcium; d-serine; glutamate; GPCR; IP3R

Introduction

The goal of this portion of the Dual Perspectives feature is to share information and evidence that have led us to reject the hypothesis that astrocytes participate in gliotransmission under physiological conditions. As such, this evidence has been built on "negative" data, which by definition is more difficult to defend, requires a higher level of scrutiny, and more often than not goes unpublished. However, we would like to emphasize at the outset the difference between "absence of evidence" versus "evidence of absence." "Absence of evidence" suggests that something has not been observed but has never been objectively and carefully tested. In the case of gliotransmission, there is strong and ample evidence from carefully designed and controlled studies leading to the conclusion that gliotransmission does not occur under phys-

iological conditions; that is, there is substantial evidence of absence. The reader is encouraged to read through both viewpoints and, with the information provided, form their own perspectives and conclusions regarding the existence of gliotransmission. We invite the reader to consider the following questions as they read through both perspectives: In weighing the evidence for and against gliotransmission, what methods or conditions were used in each case? Did one set of methods or conditions approach astrocyte physiology more closely than another? Are sufficient controls in place to eliminate potential involvement of astrocytic pumps, ion channels, and transporters that could also be modulated by ion fluxes, changes in membrane potential, G protein-coupled receptors (GPCRs), and/or Ca^{2+}? Finally, is gliotransmission necessary or important for brain function and behavior? Here we concisely highlight findings from a wide spectrum of laboratories that argue against gliotransmission being a physiological process.

Discovery of gliotransmission and methods used to stimulate astrocytes

Cultured astroglia *in vitro* directly signal to neurons and other astroglia through the Ca^{2+}-dependent exocytosis of neurotransmitters (Parpura et al., 1994, 1995; Araque et al., 1998a, b, 1999a,

2000, 2001; Parpura and Haydon, 2000); the term "gliotransmission" was created to describe this phenomenon (Bezzi and Volterra, 2001). The discovery of gliotransmission *in vitro* was very exciting as it challenged the classical view of nervous system function and quickly led to study of this process in acute brain sections. Pharmacological approaches were the easiest to use and were therefore the first used to stimulate astroglial Ca^{2+} elevations. Many of the early techniques used *in vitro*, including mechanical stimulation of astrocytes with a pipette tip, stimulating endogenous GPCRs with bath-applied agonists, uncaging Ca^{2+} within the cell, and chelating astrocyte Ca^{2+} by whole-cell dialysis of BAPTA, were carried over to work in intact tissue (for review, see Araque et al., 1999b; Bezzi and Volterra, 2001). While these techniques resulted in changes in neuronal synaptic activity interpreted as gliotransmission, they were limited by a lack of specificity or physiological relevance. For example, strong evidence from work *in vitro* indicated that stimulation of astrocytic Gq-GPCRs was sufficient to drive gliotransmission through release of Ca^{2+} from IP3 receptor-dependent internal stores (Parpura et al., 1994; Jeftinija et al., 1996; Pasti et al., 2001; Montana et al., 2006). However, in intact brain tissue, the application of agonists with the intent to stimulate astrocytic receptors directly stimulates these same receptors on neurons and other glia. Continual dialysis of astrocytes with BAPTA dilutes basal Ca^{2+} levels as well as other signaling molecules within the cell, making it nonspecific while also potentially buffering extracellular Ca^{2+} required for synaptic transmission. Overall, the methods used to study gliotransmission have either been nonselective (e.g., activation of endogenous Gq-GPCRs) or nonphysiological and wrought with potential artifacts (e.g., uncaging or chelating Ca^{2+}). Development and use of genetic approaches to study gliotransmission were transformative by ensuring selectivity of astrocyte stimulation in intact tissue while more closely replicating astrocyte Ca^{2+} release mechanisms.

Transgenic expression of a Gq-GPCR only in astrocytes and the concept of astrocytic "calcium codes" necessary for gliotransmission

Out of concern for limitations imposed by the available techniques used to stimulate astrocytes, a transgenic line of mice was developed to stimulate Ca^{2+} elevations only in astrocytes (to provide selectivity) and in a GPCR-dependent manner (to replicate endogenous astrocyte Ca^{2+} release mechanisms). This was achieved by driving expression of an endogenous Gq-GPCR to astrocytes that is not normally expressed in forebrain, and whose ligand cannot activate other forebrain GPCRs (Fiacco et al., 2007). The Gq-GPCR, Mas-related gene receptor A1 (MrgA1R), is endogenously expressed by nociceptive sensory terminals in the spinal cord but is not expressed in brain. Mice expressing the MrgA1R in astrocytes are phenotypically normal, breed well, and have normal lifespans, presumably because the receptor is expressed but never activated by endogenously released neurotransmitters. Approximately 90% of astrocytes express MrgA1R (Fiacco et al., 2007). The spatial and temporal pattern of Ca^{2+} activity evoked by MrgA1R stimulation was found to closely resemble the pattern of Ca^{2+} evoked by mGluR agonists in the same astrocytes (Fiacco et al., 2007), suggesting that the MrgA1R uses the same intracellular machinery as native astrocytic Gq-GPCRs, and does not interfere with Ca^{2+} elevations mediated by native astrocytic Gq-GPCRs (Fiacco et al., 2007). Importantly, MrgA1R Ca^{2+} elevations universally initiated in astrocyte processes (as opposed to the soma) and propagated into the entire visible extent of the astrocyte, including the fine processes (Fig. 1; Movie 1) (Fiacco et

al., 2007). Among the hippocampal astrocyte population, MrgA1R Ca^{2+} elevations were highly synchronous, making it easy to correlate astrocyte Ca^{2+} to any possible changes in neuronal activity. In contrast to findings observed in earlier studies of gliotransmission, which recorded Ca^{2+} elevations in the astrocyte soma only (Parri et al., 2001; Bowser and Khakh, 2004; Fellin et al., 2004; Perea and Araque, 2005; Serrano et al., 2006; D'Ascenzo et al., 2007), MrgA1R-evoked astrocyte Ca^{2+} elevations did not alter in any way neuronal excitatory synaptic activity or short- or long-term synaptic plasticity (Fiacco et al., 2007; Agulhon et al., 2010).

The MrgA1R agonist concentration used by Fiacco et al. (2007) (10 μM) produced very strong and sustained astrocytic Ca^{2+} elevations. It has since been suggested that these Ca^{2+} elevations are the wrong "code" for gliotransmission (Araque et al., 2014). This assertion seems unlikely for a number of reasons. First, as has already been pointed out, the Ca^{2+} elevations evoked by MrgA1R stimulation propagated into all visible astrocytic compartments where the putative diffuse, more scattered astrocytic vesicles have been suggested to reside (Bezzi et al., 2004; Crippa et al., 2006; Santello et al., 2011). Second, vesicular exocytosis of neurotransmitter increases as a function of Ca^{2+} concentration. In fundamental work, Llinás (1977) demonstrated that the amplitude of the postsynaptic potential increases stepwise with the amount of presynaptic Ca^{2+} influx. The strong Ca^{2+} elevations evoked by MrgA1R stimulation seem well suited to propagate into the small astrocytic compartments to induce exocytosis. Santello et al. (2011) discussed the importance of stronger and spatially larger synchronous astrocyte Ca^{2+} elevations for exocytosis of astrocytic glutamate in sufficient quantity to be detected by adjacent neurons. Third, approaches used to elevate astrocyte Ca^{2+} in reports of gliotransmission, including bath application of agonists to native astrocytic GPCRs, uncaging Ca^{2+} or IP$_3$, optogenetic stimulation, mechanical stimulation, and strong astrocytic depolarization via whole-cell voltage-clamp (Araque et al., 1999b; Bezzi and Volterra, 2001; Fiacco and McCarthy, 2004; Perea and Araque, 2007; Perea et al., 2014) do not produce universally local or subtle Ca^{2+} responses, suggesting that gliotransmission is not a finicky process dependent on delicate temporal or spatial requirements as is now being suggested (Araque et al., 2014; Sherwood et al., 2017). Fourth, Araque et al. (2014) referred to previous work in cultured astroglia where long-lasting astrocyte Ca^{2+} increases (similar to those generated by MrgA1R stimulation) were observed to trigger a solitary episode of gliotransmitter release at the onset of the Ca^{2+} increase (Pasti et al., 2001). However, this finding in itself refutes the idea that subtle and discrete changes in Ca^{2+} are required for gliotransmission: The long-lasting Ca^{2+} increases evoked by Pasti et al. (2001) did indeed result in gliotransmission (*in vitro*). Indeed, the sustained astroglial Ca^{2+} elevations evoked in those experiments released enough glutamate from astroglia to produce massive, 30 s duration Ca^{2+} elevations in cocultured sniffer cells. Such responses would not have been missed by Fiacco et al. (2007) who, in addition to performing continuous recordings of EPSCs in CA1 pyramidal neurons, also recorded neuronal Ca^{2+} activity. At no time during those recordings, including the onset and rising phase of the fast, widely synchronous astrocyte Ca^{2+} elevations, was there any change in neuronal activity or any effect on neuronal Ca^{2+} (Fiacco et al., 2007). It is important to note that, in the same study where MrgA1 stimulation failed to elicit gliotransmission, the nonphysiological approach of uncaging IP3 in astrocytes increased EPSC frequency in line with a previous study (Fiacco and McCarthy, 2004); similar findings have also been reported by Nedergaard and colleagues (Wang et al., 2013).
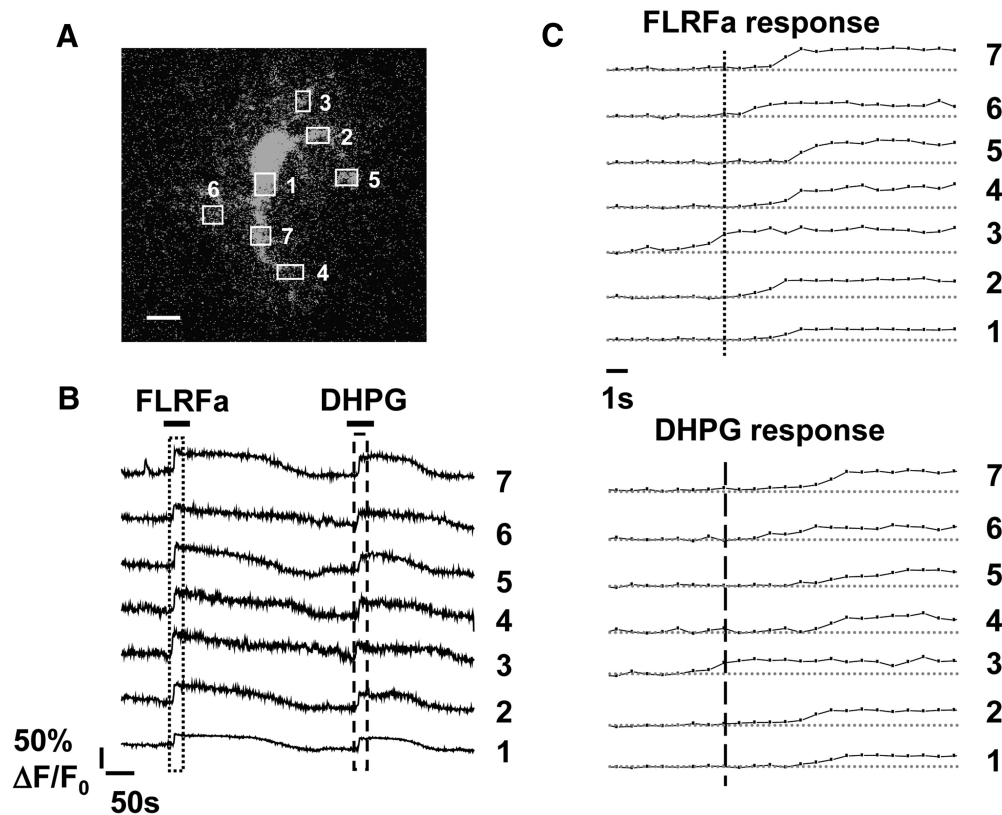
Figure 1. Stimulation of astrocytic MrgA1 receptors produces a widespread Ca^{2+} elevation that propagates throughout the cell, including the fine processes and with a pattern similar to a native Gq-GPCR. **A**, Numbered regions of interest over astrocytic compartments correspond to the fluorescence over time measurements recorded in the same regions (**B, C**). Increases in fluorescence indicate astrocyte Ca^{2+} elevations. **B**, Application of the MrgA1 receptor agonist FLRFa produced a Ca^{2+} response very similar to the one produced by application of the endogenous Group I mGluR agonist DHPG. Hatched boxes represent regions of expanded timescale shown in **C**. Stimulation of astrocytic MrgA1Rs evoked a Ca^{2+} elevation that initiated in a process and then propagated throughout the visible extent of the astrocyte, including the fine processes (see Movie 1 for this cell, where it is clearly evident that the Ca^{2+} elevation enters the fine astrocyte compartments, even though fluorescence intensity was recorded only in a small number of regions of interest). **C**, The Ca^{2+} elevation evoked by MrgA1R stimulation (top) produced a pattern nearly identical to that produced by DHPG application (bottom). Scale bars, 10 μM. Reprinted with permission from Elsevier Limited, copyright 2007.
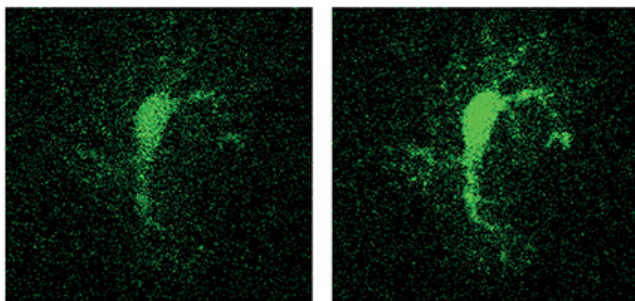
Movie 1. MrgA1-evoked astrocyte Ca^{2+} elevation propagates throughout all visible astrocytic compartments, including the fine processes with a similar pattern as DHPG. This movie shows the same astrocyte as in Figure 1. The first Ca^{2+} elevation occurs in response to application of the MrgA1 agonist FLRFa to activate astrocytic MrgA1 receptors, followed by application of 20 μM DHPG to stimulate Group I mGluRs. The Ca^{2+} elevation in response to stimulation of MrgA1Rs propagates throughout the entire visible astrocyte, including the fine processes. The pattern of the response, including initiation in an astrocyte process and propagation into the small compartments, is very similar to the pattern evoked by stimulation of Group I mGluRs. The movie shows 30 frames per second, which is sped up ~40×. Actual acquisition speed is one frame per 1.3 s.

Overall, the findings using the MrgA1 transgenic mice demonstrate that when astrocytic Gq-GPCR signaling cascades are selectively stimulated, the resultant fast and synchronous astrocytic Ca^{2+} elevations that propagate throughout the fine processes of

astrocytes do not produce gliotransmission. Importantly, stimulation of native astrocytic Gq-GPCRs that are enriched in astrocytes (the endothelin receptors) also increases astrocyte Ca^{2+} but produces no effect on neuronal synaptic transmission or short- or long-term plasticity (Fiacco et al., 2007; Agulhon et al., 2010). The data argue against a subtle Ca^{2+} code required to evoke gliotransmission, but rather just the opposite: A variety of very nonphysiological methods that evoke increases in astrocyte Ca^{2+} led to gliotransmission.

Significant attenuation of both local and large scale spontaneous and evoked astrocyte calcium elevations in IP3R2-deficient mice produces no effect on synaptic transmission, plasticity, or behavior

Stimulation of astrocytic Gq-GPCRs activates a phospholipase C signaling pathway leading to cytosolic Ca^{2+} elevations resulting from Ca^{2+} release from IP3 receptor-sensitive intracellular stores (Falkenburger et al., 2010; Gresset et al., 2012). Pharmacological inhibition of IP3 receptors or emptying Ca^{2+} stores was found to block exocytotic release of glutamate from cultured astroglia and prevent gliotransmission (Araque et al., 1998a, b; Montana et al., 2006). Based on strong immunohistochemical evidence suggesting that astrocytes exclusively express the IP3 receptor Type 2 (IP3R2) isoform (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007), IP3R2 knock-out (IP3R2$^{-/-}$ KO) mice were tested to determine whether removal of physiological sources of astrocyte Ca^{2+} impaired gliotransmission (Petravicz et al., 2008).

Petravicz et al. (2008) observed the following: (1) removal of IP3R2 prevented spontaneous and evoked Gq-GPCR-mediated astrocyte Ca^{2+} elevations; and (2) there was no effect of abolishing IP3R-sensitive sources of astrocyte Ca^{2+} on neuronal synaptic activity. A caveat of the work is that Ca^{2+} activity was not examined in the fine processes of IP3R2$^{-/-}$ astrocytes, leaving open the possibility that not all Ca^{2+} activity was completely abolished.

A comprehensive evaluation of evoked and spontaneous astrocyte Ca^{2+} activity in IP3R2$^{-/-}$ mice has since been provided by Srinivasan et al. (2015) and Agarwal et al. (2017) using genetically encoded Ca^{2+} indicators expressed in astrocytes in brain slices and in vivo. These studies have revealed that, although not completely abolished, a highly significant amount of spontaneous and evoked astrocyte Ca^{2+} activity is absent in IP3R2$^{-/-}$ mice, including the fast, local microdomain Ca^{2+} activity suggested to be essential for gliotransmission (Araque et al., 2014). Srinivasan et al. (2015) observed that all spontaneous Ca^{2+} activity, and Ca^{2+} elevations evoked by agonist application or startle are absent in the astrocyte soma in IP3R2$^{-/-}$ mice, along with ~60% of the local, spontaneous Ca^{2+} transients occurring in the fine processes. Fast, startle-evoked astrocyte Ca^{2+} elevations were also abolished in the fine processes of IP3R2$^{-/-}$ mice, leaving behind a very slow, nondecaying shift in baseline Ca^{2+}. Agarwal et al. (2017) obtained similar results as Srinivasan et al. (2015) with regard to Ca^{2+} activity abolished in the IP3R2$^{-/-}$ mice. The number of spontaneously active microdomains was reduced by 65% (in slices) and 64% (in vivo), and the number of events remaining in each domain was significantly attenuated in both preparations. Use of a membrane-tethered GCaMP3 in astrocytes biased reporting of astrocyte Ca^{2+} activity to the finest compartments, indicating that IP3R2 KO significantly abolished Ca^{2+} activity exhibiting the spatiotemporal dynamics recently suggested to be important for gliotransmission (Araque et al., 2014). As to evoked responses, application of the purinergic receptor agonist ATP, considered a key neurotransmitter/gliotransmitter regulating astrocyte Ca^{2+} activity and gliotransmission, did not evoke any astrocyte Ca^{2+} increases, consistent with loss of IP3 receptor-dependent Ca^{2+} release mechanisms. Norepinephrine was still able to elicit a small increase in astrocyte microdomain activity. Locomotion-induced norepinephrine release in vivo evoked on average 125 microdomain Ca^{2+} events from a baseline of 25 events in mice expressing IP3R2, but only 9 microdomain Ca^{2+} events from a baseline of 4 in IP3R2$^{-/-}$ mice (Agarwal et al., 2017). The conclusion that can be made from these data is that, although Ca^{2+} activity persists in IP3R2$^{-/-}$ mice, it is markedly and significantly attenuated.

If Ca^{2+}-dependent gliotransmission was an essential physiological process, the dramatic loss of astrocyte Ca^{2+} in IP3R2$^{-/-}$ mice would be expected to produce an equally dramatic impairment of synaptic function and animal behavior compared with control animals producing >60% more local spontaneous Ca^{2+} transients and much faster, more synchronized Ca^{2+} elevations in response to locomotor activity, sensory input, or startle. However, IP3R2$^{-/-}$ mice are healthy, viable, breed well, and live normal lifespans with no overt behavioral abnormalities (Petravicz et al., 2008, 2014). The significant loss of IP3R2-dependent Ca^{2+} signaling in astrocytes throughout the brain failed to affect tests of learning and memory, motor or sensory control, or measurements of anxiety and depression (Petravicz et al., 2014). Moreover, no differences were observed in evoked excitatory synaptic activity, post-tetanic potentiation (PTP)/LTP, or neurovascular

coupling compared with recordings in littermate control animals (Petravicz et al., 2008; Agulhon et al., 2010; Bonder and McCarthy, 2014). The fact that these mice do not exhibit a behavioral or electrophysiological phenotype is stunning given the large number of reports on astrocytic GPCR-dependent Ca^{2+} modulation of synaptic transmission and plasticity, as well as functional hyperemia (discussed further below). Overall, the data show that removal of IP3R2, the predominant source of physiological Ca^{2+} elevations in astrocytes, has no effect on neuronal activity and animal behavior. These findings provide strong evidence that astrocytes do not release gliotransmitters in a Ca^{2+}-dependent manner to actively control neuronal activity, synaptic transmission, and animal behavior. The most logical conclusion that can be made from these data is that gliotransmission does not occur in intact brain tissue under physiological conditions.

Neuronal receptors or astrocytic receptors?
One advantage provided by the MrgA1 transgenic approach is that stimulation of the receptors by bath-applied agonists is astrocyte selective. Because stimulation of astrocytic MrgA1 Gq-GPCRs did not result in gliotransmission (Fiacco et al., 2007; Agulhon et al., 2010), questions were raised as to differences between this approach versus those in which endogenous astrocytic receptors are stimulated. There are two competing hypotheses: The first is that the MrgA1 Ca^{2+} elevations are incapable of inducing gliotransmission. This is very improbable as discussed in the previous section: just about every report on gliotransmission uses approaches that produce universally nonphysiological increases in astrocyte Ca^{2+}, whereas MrgA1 stimulation induces physiologically relevant astrocyte Ca^{2+} increases. The competing hypothesis is that agonists applied with the intent to stimulate native astrocytic receptors directly stimulate receptors on excitatory neurons, inhibitory neurons, or other glia. The most commonly used agonists to stimulate astrocytic Ca^{2+} elevations, the mGluR agonists ($\pm$)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD) and 3,5-dihydroxyphenylglycine (DHPG), directly depolarize neurons, increase their firing rates, potentiate NMDA receptor currents, and depotentiate synaptic responses following LTP (Mannaioni et al., 2001; Heidinger et al., 2002; Zho et al., 2002; Rae and Irving, 2004). It would be difficult, if not impossible, to disentangle these direct effects from putative astrocyte-mediated ones (Fiacco et al., 2007; Agulhon et al., 2010).

In support of the hypothesis that direct stimulation of neurons has been misinterpreted as gliotransmission has come from observations made from stimulation of endogenous endothelin receptors. Evidence to date suggests that endothelin receptors are enriched in astrocytes and minimally expressed by neurons (Andersson et al., 2007; Zhang et al., 2014). Recent work has further indicated that stimulation of astrocytic endothelin receptors produces local Ca^{2+} elevations in the fine astrocyte processes in IP3R2$^{-/-}$ mice, even in the absence of soma responses (Srinivasan et al., 2015), thereby displaying the spatiotemporal dynamics recently suggested to be important for gliotransmission (Araque et al., 2014; Sherwood et al., 2017). However, stimulation of endogenous astrocytic endothelin receptors produces no effect on neuronal excitatory EPSCs, evoked EPSCs, protein tyrosine phosphatase, or LTP in the same recordings in which the Group I mGluR agonist DHPG affects these measurements (Fiacco et al., 2007; Agulhon et al., 2010). These observations lend further support to the idea that when GPCRs are selectively stimulated in astrocytes, whether the receptors are endogenously or transgeni-

cally expressed, the resultant Ca^{2+} elevations do not produce gliotransmission.

Intercellularly propagating astrocyte Ca^{2+} waves that would provide compelling support for Ca^{2+}-dependent gliotransmission are absent in healthy forebrain

One of the most convincing demonstrations of Ca^{2+}-dependent gliotransmitter release was provided by the Ca^{2+} waves that propagate between cultured astroglia (Cornell-Bell et al., 1990; Cornell-Bell and Finkbeiner, 1991). Poking a single astroglial cell in culture initiates a Ca^{2+} wave that propagates among hundreds of astroglia. The mechanism behind propagating Ca^{2+} waves is well defined. It requires IP3 receptors (Boitano et al., 1992; Charles et al., 1993) and involves Ca^{2+}-dependent release of ATP and stimulation of purinergic P2Y receptors on adjacent astroglia (Hassinger et al., 1996; Guthrie et al., 1999; Fam et al., 2000). Belonging to the family of Gq-GPCRs, P2YR stimulation results in activation of the PLC signaling cascade, production of IP3, and release of Ca^{2+} from internal stores. The store-liberated Ca^{2+} triggers release of the gliotransmitter ATP to stimulate P2YRs on adjacent astroglia to continue propagating the Ca^{2+} wave. Widespread astroglial Ca^{2+} waves were cited as strong evidence for gliotransmission (Araque et al., 1999b; Bezzi and Volterra, 2001; Gallagher and Salter, 2003).

While easily observed and well defined in culture, there is very little evidence for propagating Ca^{2+} waves between forebrain astrocytes in intact brain slices or *in vivo*. In response to afferent stimulation, sensory stimulation, or startle *in vivo*, are synchronous Ca^{2+} elevations involving many astrocytes simultaneously, not Ca^{2+} initiated in a single astrocyte that then propagates into adjacent astrocytes. This is an important distinction not always made clear in many studies, which often refer to these responses as "Ca^{2+} waves" due to their wave-like appearance. When stimuli are confined to a single hippocampal astrocyte (e.g., as opposed to puff application of a high concentration of agonist extracellularly), Ca^{2+} elevations do not propagate into adjacent astrocytes despite propagating throughout the entire stimulated cell (Fiacco and McCarthy, 2004). There is one noteworthy exception to lack of evidence for astrocyte Ca^{2+} waves, from studies in the hindbrain. In the intact cerebellum, Nimmerjahn et al. (2009) and Hoogland et al. (2009) observed radially expanding wave-like Ca^{2+} activity that propagated between Bergmann glia, a specialized astrocyte subtype. These Ca^{2+} waves depended on Ca^{2+} release from internal stores, and wave propagation was blocked by P2Y purinergic receptor antagonists (Hoogland et al., 2009). Similar Ca^{2+} waves, however, have not been reported in other brain areas.

Similar to observations made in cultured astroglia, propagating Ca^{2+} waves can also occur between reactive astrocytes in diseased tissue. Kuchibhotla et al. (2009) found Ca^{2+} waves propagating between astrocytes in Alzheimer's disease mice, but not in the tissue sections from control mice. Reactive astrocytes in diseased or damaged tissue are very different compared with healthy astrocytes, altering their expression profile of many genes (Zamanian et al., 2012). In an environment that includes secretion of inflammatory mediators, such as $TNF\alpha$ and prostaglandins, reactive astrocytes in many brain areas may become competent for gliotransmission (Bezzi et al., 1998; Domercq et al., 2006; Santello et al., 2011; Agulhon et al., 2012; Habbas et al., 2015). This may also explain why gliotransmission is clearly observed *in vitro*, as cultured astroglia represent an immature or reactive phenotype (Cahoy et al., 2008; Hamby et al., 2012) that express key vesicular proteins (Wilhelm et al., 2004). Unlike cultured astroglia, evi-

dence suggests that astrocytes in healthy or intact tissue do not express Ca^{2+}-sensitive vesicular release machinery for the commonly described gliotransmitters (Li et al., 2013; Zhang et al., 2014; Chai et al., 2017). This provides an explanation for why, in cultured astroglia, GPCR-linked Ca^{2+} elevations consistently result in gliotransmission, whereas those in astrocytes in intact tissue do not. In summary, the absence of propagating intercellular Ca^{2+} waves between forebrain astrocytes *in situ* and *in vivo* provides strong evidence that forebrain astrocytes do not release the gliotransmitter ATP or participate in gliotransmission.

Recent studies on functional hyperemia challenge the validity of the tools used to demonstrate gliotransmission

Functional hyperemia refers to the process whereby increases in neuronal activity lead to regionally restricted increases in blood flow (Vargová et al., 2001). Although functional hyperemia is not gliotransmission per se, the proposed involvement of astrocytes, astrocytic GPCRs, and Ca^{2+} is similar, and results vary greatly depending on use of pharmacological versus genetic approaches to stimulate astrocyte Ca^{2+} elevations. Investigators in this area have long thought that astrocytes play a role in functional hyperemia given that astrocyte processes cover >99% of brain vascular elements (Prokopová-Kubinová et al., 2001; Mathiisen et al., 2010). Findings from a large number of studies using acutely isolated brain slices demonstrated that manipulating astrocyte Ca^{2+} using a variety of pharmacological approaches affects arteriole diameter (Woerly et al., 1998; Syková et al., 2000; Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Straub et al., 2006; Gordon et al., 2008; He et al., 2012). These findings led to the generally accepted hypothesis that neurovascular coupling results from neuronal activation of astrocytic GPCRs, IP3-mediated astrocyte Ca^{2+} elevations, and release of vasoactive molecules from the astrocyte to regulate arteriole diameter and blood flow.

While studies using pharmacological approaches in brain slices generally support this hypothesis, a number of *in vivo* studies do not (Prokopová et al., 1997; Nizar et al., 2013; Bonder and McCarthy, 2014). Bonder and McCarthy (2014) used *in vivo* imaging and selective genetic approaches to determine whether either increasing or inhibiting Ca^{2+} activity in astrocyte endfeet that wrap arterioles in the visual cortex affected visually stimulated functional hyperemia: the answer was no. In one set of experiments, IP3R2 KO mice were used to significantly reduce GPCR-mediated Ca^{2+} increases selectively in astrocytes; functional hyperemia was unaffected in these mice. In a second set of experiments, mice that express an engineered Gq-GPCR (Gq-designer receptor exclusively activated by designer drug [DREADD]) (Nichols and Roth, 2009) selectively in astrocytes were used to assess the role of GPCR-mediated Ca^{2+} increases in functional hyperemia. The activation of Gq-DREADD led to Ca^{2+} increases in astrocyte endfeet-wrapping arterioles but failed to affect arteriole diameter or blood flow (Bonder and McCarthy, 2014). It should be noted that increasing astrocyte Ca^{2+} through Gq-DREADD is far more physiological than methods typically used to study functional hyperemia, such as uncaging Ca^{2+} or IP3. A recent study out of Eric Newman's laboratory demonstrated that astrocytes modulate capillary, but not arteriole diameter, when using more physiological methods to stimulate astrocyte Ca^{2+} (Biesecker et al., 2016). Overall, similar to data on gliotransmission, studies in this area demonstrate that, although it is possible to use nonphysiological tools to drive astrocyte modulation of arteriole blood flow, this does not appear to occur when more physiological approaches are used.

Gliotransmitter release and use of dn-SNARE transgenic mice

Early work suggested that the Ca^{2+}-dependent glutamate release observed in cultured astroglia occurred via SNARE-dependent vesicular exocytosis (Araque et al., 2000; Montana et al., 2004; Zhang et al., 2004). These observations provided the rationale for the generation of a transgenic mouse line expressing a dominant-negative (dn) mutation of synaptobrevin 2 designed to block SNARE-dependent vesicular release from astrocytes (Pascual et al., 2005); these mice are referred to as dn-SNARE mice. In the making of dn-SNARE mice, the dn-SNARE was not directly tagged with a reporter construct. Therefore, to determine the cellular expression of dn-SNARE *in vivo*, two additional reporter constructs were coinjected with dn-SNARE into fertilized eggs before implantation into developing blasts. The advantage of this approach is that the binding of dn-SNARE to the SNARE complex would not be affected by directly tagging it with a reporter. The disadvantage is that it is not possible to directly visualize dn-SNARE. Although this method was not uncommon at the time, it is rarely used today due to difficulties in directly tracking the expression of the transgene. dn-SNARE mice have been used by a large number of investigators to demonstrate that expression of dn-SNARE in astrocytes interferes with synaptic transmission and plasticity.

Over the past several years, a number of issues have been raised that question the validity of using dn-SNARE mice to demonstrate gliotransmission. First, Maiken Nedergaard's group reported that the dn-SNARE peptide was widely expressed in neurons (although at low levels), thereby directly inhibiting neuronal vesicular exocytosis (Fujita et al., 2014). Second, in the case of dn-SNARE, there is an absence of published evidence (although unpublished data very likely exist) that expression of dn-SNARE blocks glutamate release from cultured astroglia. This is surprising because evidence suggests that cultured astroglia express the necessary SNARE components to perform vesicular-dependent gliotransmitter exocytosis (Wilhelm et al., 2004). Lack of evidence that dn-SNARE inhibits glutamate release from purified astroglia is somewhat alarming and raises the specter that actions of dn-SNARE in intact tissue are not due to its action in astrocytes, as recently reported (Fujita et al., 2014). Third, even if the dn-SNARE peptide were only expressed in astrocytes, it could be exerting its effects by interfering with trafficking of membrane proteins (such as glutamate transporters) to influence synaptic transmission (Ropert et al., 2016). This possibility is supported by a recent transcriptome analysis indicating that striatal and hippocampal astrocytes express membrane traffic-related genes but show little evidence for minimal requirements for Ca^{2+}-dependent glutamate exocytosis (Chai et al., 2017).

Recent data using newer technologies indicate that D-serine is not a gliotransmitter

The three molecules most widely cited to be gliotransmitters are glutamate, ATP, and D-serine (Perea et al., 2009; Araque et al., 2014; Hollborn et al., 2015). It is difficult to selectively manipulate cellular levels of glutamate or ATP due to their role in metabolic pathways. However, this is not the case for D-serine, whose role is largely restricted to that of a required coagonist at NMDA receptors (Traynelis et al., 2010; Mothet et al., 2000). The synthesis of D-serine requires serine racemase (SR) to convert L-serine into D-serine. A large number of studies have reported that the release of D-serine as a gliotransmitter from astrocytes plays a necessary role in synaptic plasticity (Yang et al., 2003; Oliet and Mothet, 2006; Panatier et al., 2006; Oliet and Mothet, 2009; Henneberger et al., 2010; Berk et al., 2015; Pankratov and Lalo, 2015;

Sherwood et al., 2017). The rationale for thinking that D-serine might serve as a gliotransmitter largely stemmed from early reports indicating that D-serine and SR were localized to astrocytes (Schell et al., 1995; Wolosker et al., 1999; Berk et al., 2015), and that D-serine was released from cultured astroglia via a Ca^{2+}-dependent mechanism (Yang et al., 2003; Mothet et al., 2005; Zhuang et al., 2010). A series of recent studies using more advanced technologies clearly demonstrate that neurons, rather than astrocytes, synthesize and release D-serine and that it is the release of D-serine from neurons, not astrocytes, that acts as a coagonist with glutamate to regulate synaptic plasticity (Wolosker et al., 2016). The advance in this area has stemmed largely from the development of SR KO (Miya et al., 2008) and conditional KO (cKO) (Benneyworth et al., 2012) mice. These mice enable the development of highly specific immunocytochemical localization of SR and D-serine as well as electrophysiological and behavioral experiments where SR has been selectively deleted from either neurons or astrocytes. The primary findings clearly demonstrating that D-serine is not a gliotransmitter include the following: (1) using $SR^{-/-}$ mice as well as improved antibodies to define cellular specificity, SR protein (Kartvelishvily et al., 2006; Miya et al., 2008) and mRNA (Yoshikawa et al., 2007) are localized *in vivo* to neurons, not astrocytes; similar neuronal localization of D-serine and SR has been reported in human brain (Voigt et al., 2015); (2) using the SR transcriptional unit to drive GFP expression in mice leads to the exclusive expression of GFP in neurons, not astrocytes (Kartvelishvily et al., 2006); (3) a cKO of neuronal SR, but not astrocytic SR, significantly reduced LTP at the Schaffer collateral-CA1 synapse (Benneyworth et al., 2012); and (4) a cKO of neuronal SR, but not astrocytic SR, decreased dendritic spine complexity, an indicator of neuronal plasticity (Flo et al., 2004). These findings clearly demonstrate that neuronal D-serine is the NMDA receptor coagonist that participates in synaptic plasticity. It is worth noting that all of the experimental approaches being used to argue that glutamate and/or ATP are gliotransmitters are used to argue that D-serine is a gliotransmitter, including the following: (1) d/n SNARE mice; (2) toxins blocking vesicular release; (3) increasing astrocyte Ca^{2+}; (4) buffering intracellular Ca^{2+}; (5) decreasing extracellular Ca^{2+}; (6) blocking release from intracellular stores; (7) blocking vesicular ATPase; and (8) isolation of synaptic vesicles containing gliotransmitters. Given the strong evidence that neurons, not astrocytes, make and release D-serine, it is again worth questioning the methods that continue to be used to stimulate Ca^{2+} and gliotransmission from astrocytes. The D-serine saga appears to be another example where glial biologists reported evidence for gliotransmission that was strongly invalidated with improved technology.

Gliotransmission, or regulation of astrocyte transporters, metabolic activity, gap junction proteins, or ion channels?

Astrocytes modulate neuronal function in a number of ways, including secretion of factors regulating synaptogenesis and pruning during development (Ullian et al., 2004; Chia et al., 2011; Chung et al., 2015), uptake and buffering of extracellular potassium (Djukic et al., 2007; Sanz et al., 2009), regulation of gap junctional coupling or metabolic activity (Hertz et al., 1999; Rouach et al., 2008; Wagnerova et al., 2009; Brown and Ransom, 2015), and intracellular transport of glutamate during synaptic transmission (Tanaka et al., 1997; Bergles et al., 1999). It is becoming increasingly evident that many of these astrocyte functions can be modified directly or through activation of astrocytic GPCR-signaling pathways in an activity-dependent manner. For example, astrocytic Gq-GPCRs,

Ca^{2+}, and protein kinase C acutely regulate glutamate and potassium uptake (Wang et al., 2012; Devaraju et al., 2013). Alterations in K^+ uptake can modify neuronal excitability in an extracellular K^+ concentration-dependent manner (Wang et al., 2012) and reduce synaptic responses to repetitive stimulation and post-tetanic potentiation (Sibille et al., 2014). Armbruster et al. (2016) found slowing of glutamate uptake following bursts of neuronal activity ≥30 Hz and that these changes affected the neuronal response to released glutamate on an acute timescale. Murphy-Royal et al. (2015) demonstrated that neuronal activity-dependent surface diffusion of the astrocyte glutamate transporter GLT-1 (EAAT2) can shape subsequent synaptic transmission. Activity-dependent regulation of astrocytic connexin 43 can allow intercellular trafficking of glucose and its metabolites through astroglial networks (Rouach et al., 2008) and modulate network "up" states and neuronal firing rates (Roux et al., 2015). Astrocyte Ca^{2+} microdomains driven by synergistic interaction between IP3R2 and mitochondria may facilitate ATP production by enhancing glycogenolysis in an activity-dependent manner (Agarwal et al., 2017).

Methods used with the intent of stimulating astrocyte Ca^{2+} elevations can also affect astrocyte functions. Strong astrocytic depolarization using a patch pipette or optogenetic stimulation of astrocytic ion channels can lead to changes in ionic driving forces with subsequent reduction or even reversal of transporter activity, producing effects on excitability of adjacent neurons independent of gliotransmission. Intracellular Na^+ accumulation in astrocytes generated by glutamate uptake (or possibly as a result of stimulation of channelrhodopsins) has been proposed as an energy currency and mediator of metabolic signals in neuron-glia interactions (Chatton et al., 2016). In summary, there are many mechanisms through which astrocytes modulate neurons in an activity-dependent manner through regulation of astrocyte transporters, metabolic activity, gap junction proteins, or ion channels. It will be important in future studies to carefully consider alternative possibilities, such as these, before settling on gliotransmission as the mechanism by which astrocytes modulate neuronal activity. Discovery of valuable new information about the role of astrocytes in brain function may otherwise be overlooked.

A recent study on astrocyte heterogeneity finds no evidence to support Ca^{2+}-dependent glutamate exocytosis from astrocytes

Recently, the Bal Khakh laboratory at the University of California–Los Angeles comprehensively evaluated the transcriptomic, proteomic, morphological, and functional profiles of striatal and hippocampal astrocytes (Chai et al., 2017). Neither striatal nor hippocampal astrocytes expressed significant RNA for vesicular glutamate transporters or Ca^{2+}-sensitive synaptotagmins. Furthermore, although vesicles were readily observed in 138 striatal and 139 hippocampal synapses, no astrocyte processes contained structures akin to neurotransmitter vesicles at the same synapses. Stimulation of the Gq-GPCR hM3D DREADD always increased astrocyte Ca^{2+} levels but resulted in no change in signal of the coexpressed glutamate sensor iGluSnFR, whereas the positive controls of exogenous glutamate, electrical field stimulation, and inhibition of astrocyte glutamate uptake all resulted in significant glutamate detection by iGluSnFR. Stimulation of hM3D DREADD also failed to evoke NMDA receptor-dependent slow inward currents in striatal medium spiny neurons or hipoocampal pyramidal neurons. In summary, although considerable differences between the two astrocyte subtypes were found, neither subtype

was capable of GPCR- or Ca^{2+}-dependent release of glutamate (Chai et al., 2017). This recent study provides further strong evidence that astrocytes do not participate in gliotransmission.

In conclusion, there is little doubt that neuronal activity and the consequent activation of astrocytic GPCRs affect processes important for maintaining and modulating normal brain function. However, evidence that neuronal activity leads to Ca^{2+}-dependent gliotransmitter release in intact brain tissue to actively control neuronal plasticity and synaptic transmission is being challenged by the findings from many laboratories using advanced technologies. Absence of propagating Ca^{2+} waves between forebrain astrocytes provides evidence against astrocytic release of the gliotransmitter ATP in sufficient quantity to affect activity of adjacent neurons or astrocytes. Recent evidence convincingly demonstrating neuronal synthesis and release of D-serine has led to the demise of D-serine as a gliotransmitter (Wolosker et al., 2016). Use of complementary genetic approaches that are specific to astrocytes and that recapitulate or inhibit endogenous activity-driven astrocyte Ca^{2+} release mechanisms provide strong evidence against gliotransmission. Specifically, stimulation of receptors selectively expressed or enriched in astrocytes results in IP3 receptor-dependent Ca^{2+} elevations exhibiting the spatiotemporal dynamics suggested to be important for gliotransmission, while producing no effect on neuronal synaptic transmission or plasticity. Removal of the predominant source of local microdomain and sensory- or startle-evoked astrocyte Ca^{2+} responses exhibiting the spatiotemporal dynamics recently suggested to be important for gliotransmission has no effect on synaptic transmission or plasticity, modulation of arteriole diameter, or behavior. Use of traditional pharmacological approaches to stimulate astrocyte Ca^{2+} elevations, most often recorded in the astrocyte soma even in recent publications supporting gliotransmission (e.g., Pankratov and Lalo, 2015; Martín et al., 2015), do not universally evoke astrocyte Ca^{2+} elevations displaying the delicate temporal or spatial requirements recently proposed to be essential for gliotransmission. On the contrary, gliotransmission can be caused to occur using a variety of very nonspecific, nonphysiological approaches to elicit astrocyte Ca^{2+} elevations. Together, the weight of the evidence strongly argues that, under physiological conditions, Ca^{2+}-dependent release of neurotransmitters is the function of neurons, not astrocytes.

Response from Dual Perspectives Companion Authors–Iaroslav Savtchouk and Andrea Volterra

We thank Fiacco and McCarthy for presenting their view on gliotransmission. They conclude that gliotransmission does not occur under physiological conditions but do not explain what "physiological conditions" means for them. For instance, they obtained key "negative" data using brain slices and whole-cell patched cells (Fiacco et al., 2007; Agulhon et al., 2010). Are these fully physiological conditions? Do their genetic models (MrgA1 and IP3R2ko) fully mimic/disrupt astrocyte physiology? Do not Fiacco and McCarthy overinterpret and generalize their negative data while, in parallel, omitting positive data by others? Are their negative IP3R2ko data truly negative? Overall, in our view, the gliotransmission theory is well alive and

supported by evidence, but the general picture remains incomplete, making experimental results prone to discordant interpretations.

Indisputably, the idea behind the MrgA1 transgenic model is elegant. However, experimental data do not convincingly support the view that MrgA1 stimulation mimics astrocyte physiology. Fiacco and McCarthy claim physiological validity of the model (and of the resulting negative data), arguing that MrgA1 stimulation produces the same pattern of Ca^{2+} elevations as agonist stimulation of endogenous GPCRs. However, neither treatment reproduces the native astrocytic Ca^{2+} activity observed *in vivo* in the awake mouse (Bindocci et al., 2017). This is mostly fast, asynchronous, peripheral (in gliapil and fine processes), and local, like the activity evoked by local axonal stimulation. In contrast, MrgA1-evoked activity is long-lasting and spatially spread (Fiacco et al., 2007). Similarly unconvincing are the complementary negative data with the IP3R2ko model. Here, knocking down IP3R2 does not abolish all the astrocytic Ca^{2+} activity, notably the peripheral activity (Kanemaru et al., 2014; Srinivasan et al., 2015; Rungta et al., 2016; Agarwal et al., 2017), possibly reflecting local interactions with synapses (Bindocci et al., 2017). This activity is best revealed by genetically encoded Ca^{2+} indicators, not used in the key negative MrgA1 and IP3R2ko studies. Therefore, those studies are insufficient to exclude a physiological role of gliotransmission in synaptic plasticity (and vascular control).

Moreover, Fiacco and McCarthy neglect important evidence not matching their view. To mention only a few examples, the "positive" blockade of cholinergic LTP in IP3R2ko mice (Navarrete et al., 2012), apparently at odds with their negative LTP data (Agulhon et al., 2010), or the "positive" evidence for vesicular glutamate release in astrocytes (Bezzi et al., 2004) (see our Fig. 1), obtained with more sensitive methods than those producing the negative data they emphasize. By endorsing and strongly highlighting the statement that astrocytes "show little evidence for minimal requirements for Ca^{2+}-dependent glutamate exocytosis" (Chai et al., 2017), Fiacco and McCarthy disregard the many studies reporting expression by astrocytes *in situ* of multiple synaptotagmin (Mittelsteadt et al., 2009), SNARE, and S/M isoforms known to support Ca^{2+}-dependent exocytosis (for review, see Bohmbach et al., 2017). Furthermore, by proposing that gliotransmission occurs exclusively under pathological (inflammatory) conditions just because TNFα and prostaglandins control the phenomenon, they dismiss the many physiological functions of these agents (e.g., Santello and Volterra, 2012) and specifically the differential effect exerted by TNFα on gliotransmission at physiological (Santello et al., 2011) and pathological (Bezzi et al., 2001; Habbas et al., 2015) concentrations. Finally, the logic behind their argument implies that "positive" gliotransmission studies used "pathological slices," whereas "negative" ones used "physiological slices." Can they really suggest this?

In conclusion, the controversy about gliotransmission is not resolved by the arguments of either party here.

Resolution requires improved models and deeper investigations. Meanwhile, it is best to keep an open mind!

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