

Loss of IP₃ Receptor-Dependent Ca²⁺ Increases in Hippocampal Astrocytes Does Not Affect Baseline CA1 Pyramidal Neuron Synaptic Activity

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Astrocytes in the hippocampus release calcium (Ca²⁺) from intracellular stores intrinsically and in response to activation of G_q-linked G-protein-coupled receptors (GPCRs) through the binding of inositol 1,4,5-trisphosphate (IP₃) to its receptor (IP₃R). Astrocyte Ca²⁺ has been deemed necessary and sufficient to trigger the release of gliotransmitters, such as ATP and glutamate, from astrocytes to modulate neuronal activity. Several lines of evidence suggest that IP₃R type 2 (IP₃R2) is the primary IP₃R expressed by astrocytes. To determine whether IP₃R2 is the primary functional IP₃R responsible for astrocytic Ca²⁺ increases, we conducted experiments using an IP₃R2 knock-out mouse model (IP₃R2 KO). We show, for the first time, that lack of IP₃R2 blocks both spontaneous and G_q-linked GPCR-mediated increases in astrocyte Ca²⁺. Furthermore, neuronal G_q-linked GPCR Ca²⁺ increases remain intact, suggesting that IP₃R2 does not play a major functional role in neuronal calcium store release or may not be expressed in neurons. Additionally, we show that lack of IP₃R2 in the hippocampus does not affect baseline excitatory neuronal synaptic activity as measured by spontaneous EPSC recordings from CA1 pyramidal neurons. Whole-cell recordings of the tonic NMDA receptor-mediated current indicates that ambient glutamate levels are also unaffected in the IP₃R2 KO. These data show that IP₃R2 is the key functional IP₃R driving G_q-linked GPCR-mediated Ca²⁺ increases in hippocampal astrocytes and that removal of astrocyte Ca²⁺ increases does not significantly affect excitatory neuronal synaptic activity or ambient glutamate levels.

Key words: astrocyte; calcium; inositol 1,4,5-trisphosphate; IP₃ receptor; hippocampus; gliotransmitter

Introduction

Astrocytes have been reported recently to have a functional role in neuronal excitability (Araque et al., 1998a; Haydon and Carmignoto, 2006), heterosynaptic depression (Pascual et al., 2005; Serrano et al., 2006; Andersson et al., 2007), cerebrovascular dynamics (Zonta et al., 2003; Straub and Nelson, 2007), and pathological states such as epilepsy (Kang et al., 2005; Tian et al., 2005; Fellin et al., 2006a). One unifying feature of these findings is that astrocyte modulation of these processes occurs via a Ca²⁺-dependent release of “gliotransmitters,” including ATP (which is converted to adenosine by ectonucleotidases) and glutamate (Montana et al., 2006). Astrocytes primarily use spatially and temporally encoded increases in Ca²⁺ as an intracellular signaling mechanism (Cornell-Bell et al., 1990; Jensen and Chiu, 1990; Scemes and Giaume, 2006). Astrocytes display Ca²⁺ increases both spontaneously (Parri et al., 2001; Nett et al., 2002; Hirase et al., 2004) and in response to neuronal stimulation (Porter and McCarthy, 1996; Aguado et al., 2002; Perea and Araque, 2005)

and have been reported to modulate synaptic transmission through activation of metabotropic glutamate receptors (mGluRs), ionotropic glutamate receptors (iGluRs), and adenosine receptors (Haydon and Carmignoto, 2006).

Calcium increases in astrocytes are elicited predominantly by G_q-linked G-protein-coupled receptor (GPCR) activation, driving the production of IP₃ and the activation of IP₃ receptors (IP₃Rs) coupled to endoplasmic reticulum (ER) Ca²⁺ stores. IP₃Rs are a family of genes expressing three isoforms (types 1–3) of an ER Ca²⁺ release channel that are found in nearly every cell type (Foskett et al., 2007). Immunohistochemical studies aimed at identifying the expression profile of IP₃Rs in the brain suggest that hippocampal astrocytes express primarily IP₃R2. Evidence for IP₃R2 expression in neurons is inconclusive (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007). These data point to IP₃R2 as a potential key mediator of astrocyte intracellular Ca²⁺ release and Ca²⁺-dependent signaling cascades, but functional evidence for IP₃R2 in astrocytes is limited and has not been demonstrated *in situ* (Sheppard et al., 1997; Weerth et al., 2007).

We used an IP₃R2 knock-out (KO) mouse model to determine whether IP₃R2 has a functional role in Ca²⁺ increases of hippocampal astrocytes and neurons. We present the novel finding that genetic deletion of IP₃R2 results in complete loss of spontaneous and agonist-evoked IP₃R-dependent Ca²⁺ increases in astrocytes but leaves intact agonist-evoked IP₃R-dependent Ca²⁺

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increases in neurons. These data indicate that IP₃R2 is the primary functional IP₃R in astrocytes, and that IP₃R2 does not play a demonstrated role in CA1 pyramidal neurons. We performed electrophysiological recordings of CA1 pyramidal neuron spontaneous EPSCs (sEPSCs) to determine the effect of eliminating astrocytic Ca²⁺ responses on baseline neuronal excitatory synaptic activity. No significant changes were found in any of the AMPA receptor (AMPA) and NMDA receptor (NMDAR) sEPSC parameters of mice lacking astrocytic Ca²⁺ responses compared with littermate controls. Our results indicate that astrocytic Ca²⁺ responses are not important in modulating basal neuronal excitatory synaptic activity, contrary to the current state of the literature. We also found no differences in activation of NMDARs by ambient glutamate, providing additional evidence that ambient glutamate of glial origin is not released in a Ca²⁺-dependent manner (Jaubaudon et al., 1999; Cavelier and Attwell, 2005). This study provides the first functional evidence that IP₃R2 is the only IP₃R isoform expressed by astrocytes, IP₃R2 is not required for neuronal G_q GPCR-mediated Ca²⁺ elevations, and removal of astrocyte Ca²⁺ increases has no effect on basal neuronal excitatory activity.

Materials and Methods

Generation of IP₃R2 KO mice. IP₃R2 KO mice were generated as described previously (Li et al., 2005). Briefly, a 539 bp fragment of exon 3 of IP₃R2 (116 bp) was inserted into a targeting vector between two loxP sites. Mice were bred to heterozygosity for the floxed allele (IP₃R2^{+/*fl*ox}) and crossed to Pro-Cre mice. Pro-Cre, IP₃R2^{+/*fl*ox} were crossed to generate germline heterozygous null mutant offspring (IP₃R2^{+/-}), which were interbred to generate homozygous full mutant mice (IP₃R2^{-/-}) and littermate controls (IP₃R2^{+/+} and IP₃R2^{+/-}). Mice were genotyped by PCR analysis using genomic DNA with IP₃R2 wild-type (WT) and mutant allele-specific primers as given in the study by Li et al. (2005).

Hippocampal slice preparation. All procedures followed the guidelines of the Institutional Animal Care and Use Committee of University of North Carolina at Chapel Hill. Littermate control and IP₃R2 knock-out mice 10–16 d of age [postnatal day 10 (P10) to P16] were anesthetized by isoflurane inhalation. The brains were rapidly removed after decapitation and submerged into 4°C slicing buffer containing the following (in mM): 125 NaCl, 10 glucose, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.5 KCl, 3.8 MgCl₂, and 0.1 kynurenic acid and bubbled with 95% O₂ and 5% CO₂. Brains were cut sagittally at a thickness of 300 μm on a Leica (Bannockburn, IL) vibratome. During sectioning, brains were kept submerged in 4°C oxygenated slicing buffer. Hippocampi were dissected out of each brain slice and incubated 45 min in artificial CSF (ACSF) warmed to 35–37°C and bubbled continuously with 95% O₂ and 5% CO₂. The ACSF contained the following (in mM): 125 NaCl, 10 glucose, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.5 KCl, 2.5 CaCl₂, and 1.3 MgCl₂.

Calcium imaging. Astrocytes were bulk loaded with either the Ca²⁺ indicator Calcium Green-1 A.M. or Fluo-4 A.M. as described previously (Nett et al., 2002). Slices were incubated for 45 min at 35–37°C in oxygenated ACSF that included either 16 μM Fluo-4 A.M. or 11 μM Calcium Green 1-AM ester dye and 0.07% pluronic acid (final DMSO concentration, 0.4%). For measuring neuronal Ca²⁺ increases, CA1 pyramidal neurons were patch clamped with 200 μM Alexa 568 and 400 μM Fluo-4 calcium indicator dye made up in neuronal internal solution (see below). The pipette was then removed, and the neuron was allowed to recover for 10 min. Regions of interest were placed over the cell bodies of astrocytes and over the cell body and primary dendrite of CA1 pyramidal neurons. Increases in average fluorescence in regions of interest indicate an increase in Ca²⁺ concentration. Fold increase over baseline was calculated for each trace and reported as ΔF/F₀.

Neuronal patch-clamp recordings. For AMPAR sEPSC recordings, CA1 pyramidal neurons were patch clamped using pipettes (4.0–6.0 MΩ resistance), and a gap-free recording was performed for 10 min in ACSF as described previously (Fiacco and McCarthy, 2004). For NMDAR sEPSCs, neurons were voltage clamped at -70 mV and superfused with

ACSF containing 5 μM Mg²⁺ and 10 μM CNQX to block AMPA responses. For the ambient glutamate recordings, neurons were patch clamped at a holding potential of +40 mV in normal ACSF with an internal solution containing the following (in mM): 100 cesium methanesulfonate (CH₃CSO₃S), 10 tetraethylammonium-Cl, 4 NaCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 5 phosphocreatine, 2 ATP, 0.3 GTP, pH adjusted to 7.3 with CsOH. Ambient glutamate current was recorded in ACSF containing 1 μM tetrodotoxin (TTX) and 100 μM picrotoxin.

Data collection and analysis. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Traces were analyzed for sEPSCs in Clampfit 10.2 software (Molecular Devices) using a template constructed from four to six sEPSCs intrinsic to each recording. The event statistics were taken for each individual event and then averaged. The averaged event statistics from each cell were then averaged together and reported as mean ± SEM. For tonic NMDA currents from ambient glutamate recordings, membrane currents were normalized to the amplitude of the currents blocked by a saturating concentration of D-AP-5. Statistical differences between two samples were evaluated using Student's *t* test performed using Prism 4 software (Graphpad, San Diego, CA).

Histology. Littermate control and IP₃R2 KO brains were fixed with formalin and embedded sagittally in paraffin. Brains were sectioned at 6 μm thickness with a Leica (Nussloch, Germany) microtome from P30 littermate controls and IP₃R2 KOs. Sections were stained with hematoxylin and eosin and imaged using a Zeiss (Oberkochen, Germany) Axio-scope light microscope.

Reagents. D-AP-5, TTX, CNQX, (RS)-3,5-dihydroxyphenylglycine (DHPG), histamine, carbachol, thapsigargin, DL-threo-β-benzyloxyaspartic acid (DL-TBOA), and picrotoxin were obtained from Tocris Bioscience (Bristol, UK). Calcium Green-1 A.M., Fluo-4, Fluo-4 A.M., and Alexa 568 were obtained from Invitrogen (Carlsbad, CA).

Results

Histological analysis of IP₃R2 KO mouse brains

It has been reported previously that mice homozygous for the IP₃R2 KO allele are viable and fertile and that the mice display no overt behavioral abnormalities (Li et al., 2005). We performed histological staining of paraffin-embedded sections from adult IP₃R2 KO mice to determine whether there were any obvious abnormalities in brain cytoarchitecture indicative of improper proliferation or neurite outgrowth during development. Hematoxylin and eosin staining of P30 littermate control (*n* = 3) and IP₃R2 KO (*n* = 3) brains revealed that lack of IP₃R2 does not significantly affect brain cytoarchitecture. The hippocampus, cortex, and cerebellum of IP₃R2 KO mice were examined and did not show any obvious structural abnormalities (Fig. 1A). These findings suggest that IP₃R2-mediated release of Ca²⁺ is not critical to the overall development of the brain or that alternate IP₃Rs may be expressed in astrocytes during development.

IP₃R2 KO hippocampal astrocytes lack spontaneous and Gq-linked GPCR Ca²⁺ increases

Astrocytes are known to respond to a wide variety of Gq GPCR agonists with Ca²⁺ elevations (Verkhatsky and Kettenmann, 1996). To address the functional role of IP₃R2 in hippocampal astrocytes, we performed Ca²⁺ imaging experiments on bulk-loaded slices from littermate control and IP₃R2 KO mice. Bath application of a G_q-linked GPCR agonist cocktail (DHPG, histamine, carbachol; 10 μM each) to hippocampal slices taken from littermate controls elicited robust increases in astrocyte intracellular Ca²⁺ (65% of 144 cells from 17 slices; five animals total) (Fig. 2A,B). In striking contrast, the GPCR cocktail failed to elicit Ca²⁺ increases in IP₃R2 KO hippocampal astrocytes (0% of 104 cells from 11 slices; five animals total) (Fig. 2A,B). Similar results were obtained in experiments using 100 μM ATP, with 80% of 40 astrocytes from littermate controls (four slices total from one

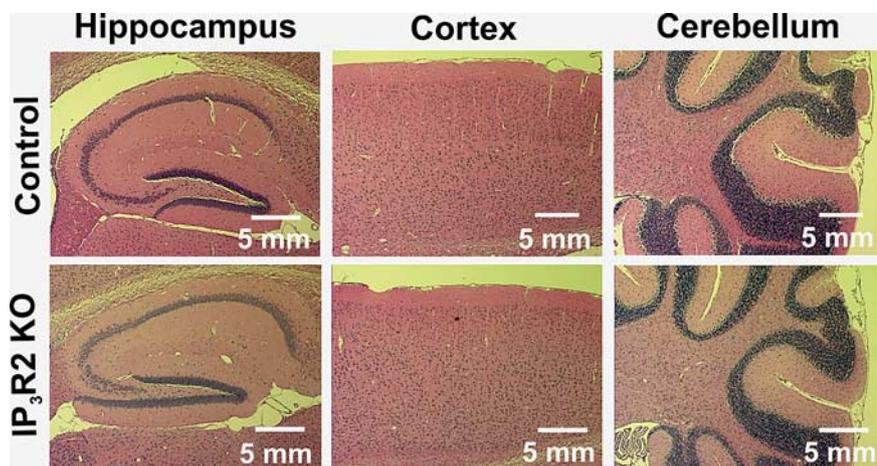


Figure 1. Histological analysis of IP₃R2 KO brains reveals no obvious abnormalities. Histological staining of brain sections were taken from littermate control ($n = 3$) and IP₃R2 KO ($n = 3$) mice. Six-micrometer-thick paraffin-embedded sections were cut and stained with hematoxylin and eosin to visualize brain cytoarchitecture. No difference in the gross overall morphology or in the general cell layering was apparent between the IP₃R2 KO mice and littermate controls in any brain region; data from hippocampus, cortex, and cerebellum are shown.

animal) and 0% of 38 astrocytes from IP₃R2 KO mice (three slices total from one animal) responding with Ca²⁺ increases (Fig. 2*A, B*). In experiments where thapsigargin (2 μM) was applied as a control for Ca²⁺ increases, both littermate control (45% of 40 cells; four slices total from one mouse) and IP₃R2 KO (45% of 38 cells, three slices total from one mouse) astrocytes responded to thapsigargin with increases in intracellular Ca²⁺, indicating that the Ca²⁺ stores themselves were intact (Fig. 2*A, B*).

Astrocytes have also been reported to exhibit spontaneous Ca²⁺ oscillations in the absence of neuronal activity (Nett et al., 2002). To determine whether IP₃R2 is necessary for spontaneous Ca²⁺ oscillations, Ca²⁺ measurements were analyzed in astrocytes in the absence of agonist application. In bulk-loaded slices from littermate controls, spontaneous Ca²⁺ increases were observed in 21% of 144 total astrocytes (17 slices from five animals) but were absent in IP₃R2 KO astrocytes (0% of 104 total cells, 11 slices from five animals). Together, these data indicate that astrocytes lacking IP₃R2 are incapable of releasing Ca²⁺ from internal stores either spontaneously or in response to agonists to G_q-linked GPCRs.

Neuronal Ca²⁺ increases are intact in IP₃R2 KO CA1 pyramidal neurons

To address the question of a potential functional role of IP₃R2 in hippocampal neurons, we conducted Ca²⁺ imaging experiments on CA1 pyramidal neurons loaded with Ca²⁺ indicator via a patch pipette. Application of a G_q-linked GPCR agonist cocktail (in μM: 50 DHPG, 10 histamine, 10 carbachol) in 1 μM TTX to block action potentials elicited Ca²⁺ transients in IP₃R2 KO neurons (six cells from six slices, five animals total) not significantly different in amplitude (Fig. 2*D, left*) ($p = 0.39$) or duration (Fig. 2*D, right*) ($p = 0.08$) from those measured in littermate controls (eight cells from eight slices, five animals total). These results indicate that lack of IP₃R2 does not significantly alter IP₃R-dependent Ca²⁺ signaling in CA1 pyramidal neurons.

Lack of spontaneous Ca²⁺ oscillations in astrocytes does not affect CA1 pyramidal neuron sEPSCs

It has been reported that spontaneous and evoked astrocyte Ca²⁺ elevations lead to gliotransmitter release, which modulates basal

neuronal excitatory and inhibitory synaptic activity via the activation of neuronal mGluRs and iGluRs (Hassinger et al., 1995; Araque et al., 1998b; Kang et al., 1998; Parri et al., 2001; Fiocco and McCarthy, 2004; Liu et al., 2004a,b). The effect of blocking astrocyte Ca²⁺ elevations and therefore Ca²⁺-dependent gliotransmitter release on basal neuronal excitatory activity has not been addressed thoroughly. Therefore, we performed whole-cell patch-clamp experiments on CA1 pyramidal neurons and found that basic neuronal properties such as resting membrane potential (control, -61.7 ± 1.1 mV; IP₃R2 KO, -60.3 ± 0.8 mV; $p = 0.33$), membrane resistance (control, 274.5 ± 26.2 MΩ; IP₃R2 KO, 249.1 ± 15.5 MΩ; $p = 0.40$), and membrane capacitance (control, 89.6 ± 5.5 pF; IP₃R2 KO, 96.4 ± 4.8 pF; $p = 0.36$) were not significantly different between littermate control and IP₃R2 KO neurons (control, 21 cells from 20 slices, 10 animals total; IP₃R2 KO,

22 cells from 17 slices, 10 animals total). In addition, there were no significant differences found between littermate control and IP₃R2 KO AMPAR sEPSC peak amplitude ($p = 0.62$), 10–90% rise time ($p = 0.94$), decay tau ($p = 0.66$), and event frequency ($p = 0.90$) (Fig. 3*B*) (control, 15 cells from 14 slices, nine animals total; IP₃R2 KO, 16 cells from 11 slices, nine animals total). Additionally, no significant differences were found in NMDAR-mediated sEPSCs in peak amplitude ($p = 0.39$), 10–90% rise time ($p = 0.92$), decay tau ($p = 0.94$), and event frequency ($p = 0.70$) (Fig. 3*D*) (control, 15 cells from 13 slices, six animals total; IP₃R2 KO, nine cells from eight slices, three animals total). Together, these results indicate that lack of IP₃R-dependent Ca²⁺ increases in astrocytes has no significant effect on either spontaneous AMPAR- or NMDAR-mediated excitatory synaptic currents in CA1 pyramidal neurons.

Ambient glutamate of astrocyte origin is not released in a Ca²⁺-dependent manner

Ambient glutamate levels in the hippocampus have been the focus of several recent studies (Herman and Jahr, 2007; Le Meur et al., 2007). Findings in this area suggest that the majority of ambient glutamate present in the hippocampus is of glial origin and might be released in a Ca²⁺-independent manner (Jabaudon et al., 1999; Cavelier and Attwell, 2005). To address whether ambient glutamate release occurs in a Ca²⁺-independent manner, whole-cell currents were recorded from CA1 pyramidal neurons held at +40 mV in ACSF containing 1 μM TTX and 100 μM picrotoxin to isolate NMDAR-mediated currents. Similar to previous reports (Herman and Jahr, 2007; Le Meur et al., 2007), application of the NMDAR antagonist D-AP-5 (50 μM) revealed a tonic NMDAR current of 33.7 ± 6.0 pA in littermate control neurons (six cells from six slices, four animals total) (Fig. 4*A, B*). Recordings done in IP₃R2 KO neurons found a D-AP-5-sensitive current that was not significantly different from that found in littermate control neurons (35.7 ± 9.2 pA; $p = 0.8$; five cells from five slices, three animals total) (Fig. 4*A, B*).

To determine whether removal of astrocytic Ca²⁺ increases affects ambient glutamate accumulation during elevated extracellular glutamate, glutamate transporters were blocked using 100 μM TBOA. Application of TBOA caused a 7.0 ± 1.3 -fold

change in the tonic NMDAR current of littermate control neurons (six cells from six slices, four animals total) and a similar fold change in KO neurons of 8.6 ± 1.1 (five cells from five slices, three animals total; $p = 0.4$) (Fig. 4C,D). Additionally, there was no significant difference in the level of synaptic noise during TBOA application in the IP₃R2 KOs (55.4 ± 4.1 pA; six cells from six animals; four slices total) versus littermate controls (66.8 ± 5.4 pA; $p = 0.14$; five cells from five slices; three animals total). Overall, these data indicate that astrocyte Ca²⁺ elevations do not play a significant role in regulating the ambient extracellular concentration of glutamate.

Discussion

Astrocytes have been reported to display both spontaneous and evoked intracellular Ca²⁺ increases using a variety of stimulation protocols (Montana et al., 2006). Astrocyte Ca²⁺ increases are caused by the release of Ca²⁺ from internal stores after activation of IP₃Rs (Sheppard et al., 1997; Scemes, 2000). In this communication, we show that knock-out of IP₃R2 abolishes both spontaneous and G_q-linked GPCR agonist evoked IP₃R-dependent Ca²⁺ increases in astrocytes. To our knowledge, the findings presented here are the first demonstration that astrocyte Ca²⁺ release *in situ* is functionally reliant on IP₃R2. In contrast, IP₃R-dependent Ca²⁺ increases in CA1 pyramidal neurons remain intact, indicating that IP₃R2 is not necessary for neuronal IP₃R-mediated Ca²⁺ increases, or that IP₃R2 may not be expressed by CA1 pyramidal neurons. This is supported by immunostaining data showing that IP₃R2 is not expressed in neurons (Sharp et al., 1999; Hertle and Yeckel, 2007). It is somewhat surprising that in astrocytes, in which IP₃R-dependent intracellular Ca²⁺ signals are thought to modulate an increasingly large number of key processes in brain (neuronal excitability, synaptic plasticity, and cerebrovascular control), that deletion of IP₃R2 should result in the complete loss of Ca²⁺ activity without any apparent form of compensation. It is even more surprising that these mice: (1) are not embryonic lethal, (2) do not show early mortality, and (3) do not exhibit any obvious histological or behavioral abnormalities. They appear healthy, breed well, and live normal lifespans. This is in stark contrast to the IP₃R1 KO mouse model, which displays tonic-clonic seizures, ataxia, and either die *in utero* or by weaning age (P21) (Matsumoto et al., 1996). Additional behavioral testing of the IP₃R2 KO mouse model will provide valuable insight into the role of astrocyte Ca²⁺-dependent signaling in specific animal behaviors such as learning and memory.

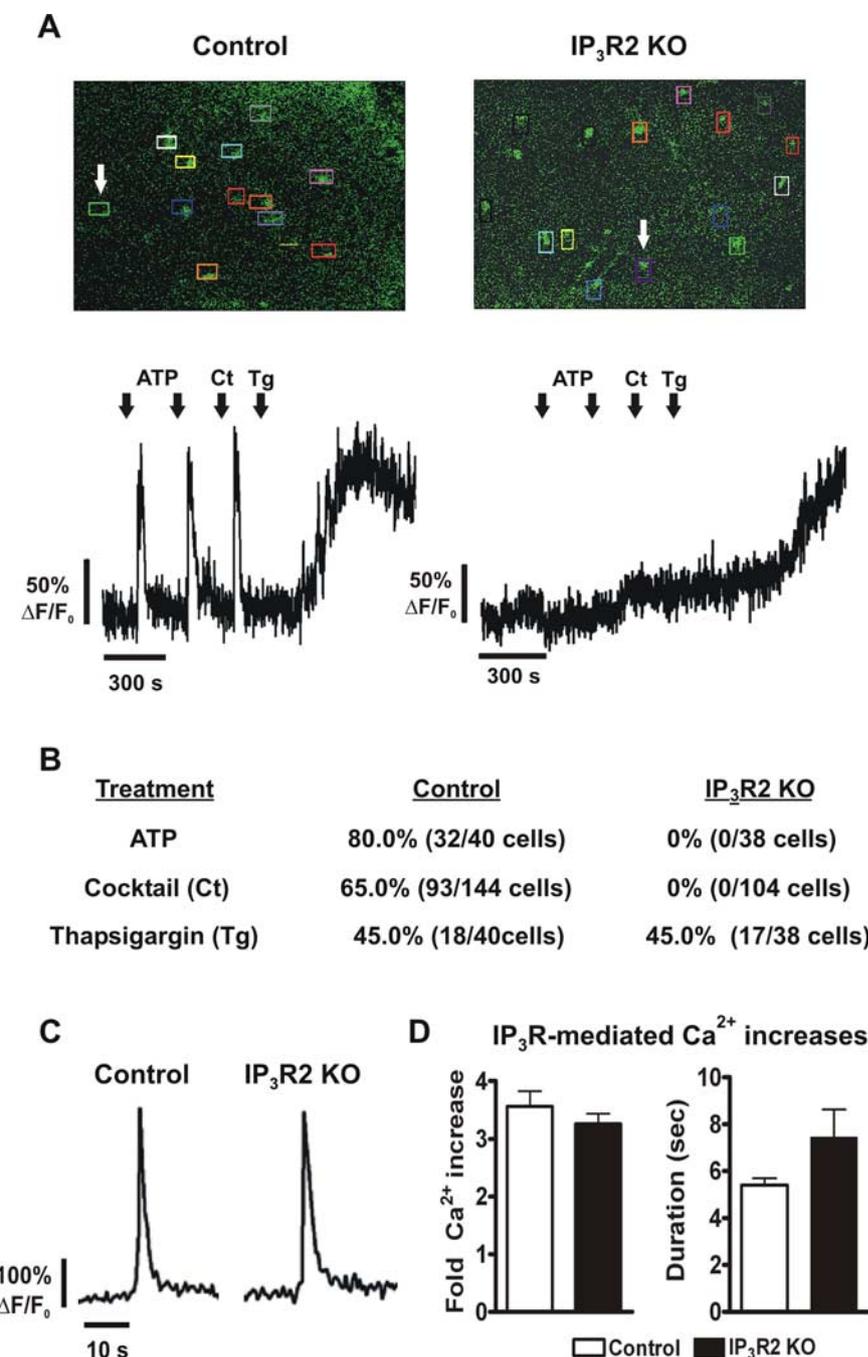


Figure 2. Knock-out of IP₃R2 affects astrocyte but not neuronal GPCR-mediated Ca²⁺ increases. **A**, Representative Ca²⁺ traces from astrocytes of Calcium Green AM-loaded hippocampal slices. Regions of interest were placed over the cell bodies of bulk-loaded hippocampal astrocytes to measure Ca²⁺ increases in response to agonist application (top). Application of ATP (100 μM) or a G_q-linked GPCR agonist cocktail (Ct, 10 μM DHPG, 10 μM histamine, and 10 μM carbachol) elicited Ca²⁺ responses in astrocytes from littermate control but not IP₃R2 KO hippocampal slices. The arrows indicate the astrocyte Ca²⁺ traces shown in the bottom panels. Thapsigargin (Tg; 2 μM) was used as a control and increased Ca²⁺ in astrocytes of both littermate control and IP₃R2 KOs. Data are presented as fold increases over baseline. **B**, Percentage of astrocytes responding to application of ATP or the G_q-linked GPCR agonist cocktail from all experiments. **C**, Representative Ca²⁺ traces from CA1 pyramidal neurons patch clamped with internal solution containing Fluo-4 Ca²⁺ indicator in response to application of a G_q-linked GPCR cocktail (50 μM DHPG, 10 μM histamine, 10 μM carbachol) in the presence of 1 μM TTX to block action potentials. **D**, Amplitude and duration of IP₃R-mediated Ca²⁺ responses in CA1 pyramidal neurons (control, $n = 8$; IP₃R2 KO, $n = 6$). There were no significant differences for amplitude (left; $p = 0.39$) or duration (right; $p = 0.08$). Error bars indicate SEM.

Astrocytes have been implicated as a major source of ambient glutamate in the hippocampus (Jabaudon et al., 1999; Cavalier and Attwell, 2005; Herman and Jahr, 2007; Le Meur et al., 2007). In the present study, removal of astrocyte Ca²⁺ increases has no

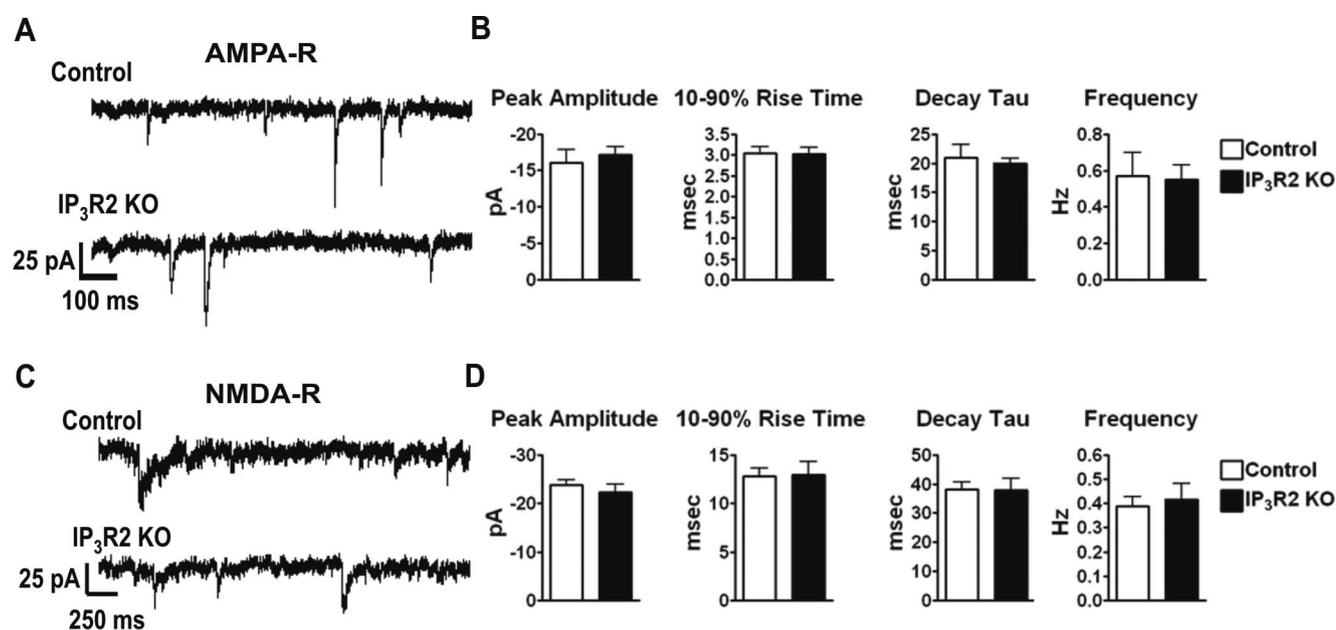


Figure 3. Spontaneous EPSCs from CA1 pyramidal neurons are unchanged in IP₃R2 KO mice. **A**, Representative AMPAR sEPSC traces from littermate control ($n = 15$) and IP₃R2 KO ($n = 16$) CA1 pyramidal neurons. **B**, AMPAR sEPSC peak amplitude ($p = 0.62$), 10–90% rise times ($p = 0.94$), decay tau ($p = 0.66$), and event frequency ($p = 0.90$) were not significantly different between littermate control and IP₃R2 KO CA1 pyramidal neurons as determined by Student's t test. **C**, Representative NMDAR sEPSC traces from littermate control ($n = 15$) and IP₃R2 KO ($n = 9$) CA1 pyramidal neurons. **D**, NMDAR sEPSC peak amplitude ($p = 0.39$), 10–90% rise times ($p = 0.92$), decay tau ($p = 0.94$), and event frequency ($p = 0.70$) were not significantly different between littermate control and IP₃R2 KO CA1 pyramidal neurons as determined by Student's t test. Error bars indicate SEM.

effect on the amplitude of the tonic NMDA-R mediated current activated by ambient glutamate. Furthermore, use of TBOA to block glutamate transporters revealed that the extent to which ambient glutamate accumulates during transporter block is unaffected by removal of IP₃R2 in astrocytes (Fig. 4B). These findings are in agreement with the hypothesis that ambient glutamate release from astrocytes occurs in a Ca²⁺-independent manner, possibly through nonvesicular release mechanisms such as connexin hemichannels, P2X channels or anion channels (Cavelier and Attwell, 2005; Malarkey and Parpura, 2008).

A substantial literature has developed in the field of astrocyte biology concerning the role of Ca²⁺-dependent release of gliotransmitters such as ATP (which is converted to adenosine by ectonucleotidases) and glutamate on neuronal activity (for review, see Carmignoto and Fellin, 2006; Fellin et al., 2006b; Fiacco and McCarthy, 2006). These findings led to the development of the tripartite synapse model, in which astrocytes are active participants in synaptic transmission through Ca²⁺-dependent gliotransmitter release (Araque et al., 1999). Although the majority of studies have focused on the outcome of pharmacologically evoking astrocyte Ca²⁺, very few studies have directly described the effect of blocking IP₃R-dependent Ca²⁺ release on basal excitatory neuronal activity. Furthermore, it has been reported that spontaneous Ca²⁺ increases and subsequent glutamate release from astrocytes directly evoke NMDAR-mediated currents in neurons (Parri et al., 2001). Additionally, astrocyte Ca²⁺ increases have been associated with neuronal Ca²⁺ increases mediated by iGluRs (Hassinger et al., 1995; Pasti et al., 2001; Fellin et al., 2004). A mathematical model incorporating data from numerous studies, including our own (Fiacco and McCarthy, 2004), on the role of astrocytes at the tripartite synapse predicts that astrocytes enhance synaptic release (Nadkarni and Jung, 2007). This is reflected by an increase in spontaneous postsynaptic events during and immediately after astrocyte Ca²⁺ elevations that trigger astrocytic release of glutamate compared with syn-

apses lacking an associated astrocyte (Nadkarni and Jung, 2007). According to this model, a lack of astrocyte Ca²⁺ increases would produce a reduced event frequency, reflecting reduced synaptic release. It has also been reported that heterosynaptic depression caused by Ca²⁺-dependent ATP release from astrocytes suppresses glutamate release at CA3–CA1 synapses (Pascual et al., 2005). Based on these findings, it is reasonable to speculate that abolishment of Ca²⁺ increases in IP₃R2 KO astrocytes would lead to significant changes in basal excitatory neuronal activity and perhaps long-term changes in brain activity and behavior. In recordings of sEPSCs from IP₃R2 KO and littermate control CA1 pyramidal neurons, we found no significant differences in peak amplitude, 10–90% rise time, and decay tau of both AMPAR- and NMDAR-mediated synaptic currents. Furthermore, we found no change in the frequency of AMPAR- and NMDAR-mediated sEPSCs, suggesting that lack of Ca²⁺ increases and Ca²⁺-dependent gliotransmitter release may not significantly affect baseline release probability from neuronal synaptic terminals.

The IP₃R2 KO mouse model offers a compliment to another mouse model developed in our laboratory that enables selective stimulation of G_q-GPCR signaling cascades in astrocytes (Fiacco et al., 2007). The findings presented here support our recent discovery using the Mrg1A transgenic mice that selective, widespread astrocyte Ca²⁺ elevations have no effect on baseline neuronal excitatory synaptic activity. It has been reported previously by a number of labs (including our own) that mechanical stimulation or uncaging Ca²⁺ or IP₃ in astrocytes leads to gliotransmitter release and changes in neuronal excitatory activity (Parpura and Haydon, 2000; Fiacco and McCarthy, 2004), neuronal inhibitory activity (Kang et al., 1998; Liu et al., 2004a,b), and cerebrovascular tone (Zonta et al., 2003; Straub et al., 2006). Although use of these pharmacological tools may elicit such responses, they may represent a nonphysiological level of stimulation that does not occur *in vivo* and therefore does not accurately

recapitulate endogenous IP₃ generating signaling pathways. There may be important regulatory mechanisms activated in GPCR signaling that work downstream of Ca²⁺ to inhibit vesicular release of glutamate by astrocytes. The IP₃R2 KO and MrgA1 mouse models together fully corroborate the concept that stimulation of astrocytic G_q GPCRs and spontaneous astrocyte Ca²⁺ activity are not sufficient to cause vesicular release of glutamate from astrocytes, and that astrocyte Ca²⁺ elevations are not necessary for normal neuronal excitatory synaptic activity in hippocampal CA1 pyramidal neurons.

The IP₃R2 mouse model also affords significant improvement over previously used techniques to examine the necessity of evoked IP₃R-dependent Ca²⁺ increases to changes in neuronal synaptic activity. Calcium chelators such as BAPTA or the bulk loadable BAPTA-AM have been used to block astrocyte Ca²⁺ increases. There are technical issues with the use of BAPTA and Ca²⁺ chelators that are eliminated by the use of the IP₃R2 KO mouse model. In the IP₃R2 KO, release of Ca²⁺ from astrocyte internal stores is removed specifically, without causing a global change in the resting cytoplasmic Ca²⁺ concentration.

In conclusion, the IP₃R2 KO mouse model represents a significant step forward in our ability to study the astrocyte Ca²⁺ contribution to key physiological processes. Use of this model has the potential to clarify and further define the role of astrocytes in physiology and pathology without the use of pharmacological manipulations to block astrocyte Ca²⁺ increases. This model can be used to identify Ca²⁺-dependent and Ca²⁺-independent mechanisms and their influence on astrocyte–neuronal communication, as well as re-evaluate the many important brain functions to which Ca²⁺-dependent gliotransmitter release has been reported to play a significant role.

References

- Aguado F, Espinosa-Parrilla JF, Carmona MA, Soriano E (2002) Neuronal activity regulates correlated network properties of spontaneous calcium transients in astrocytes *in situ*. *J Neurosci* 22:9430–9444.
- Andersson M, Blomstrand F, Hansén E (2007) Astrocytes play a critical role in transient heterosynaptic depression in the rat hippocampal CA1 region. *J Physiol (Lond)* 585:843–852.
- Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998a) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci* 10:2129–2142.
- Araque A, Sanzgiri RP, Parpura V, Haydon PG (1998b) Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci* 18:6822–6829.
- Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci* 22:208–215.
- Carmignoto G, Fellin T (2006) Glutamate release from astrocytes as a non-synaptic mechanism for neuronal synchronization in the hippocampus. *J Physiol (Paris)* 99:98–102.
- Cavelier P, Attwell D (2005) Tonic release of glutamate by a DIDS-sensitive mechanism in rat hippocampal slices. *J Physiol (Lond)* 564:397–410.

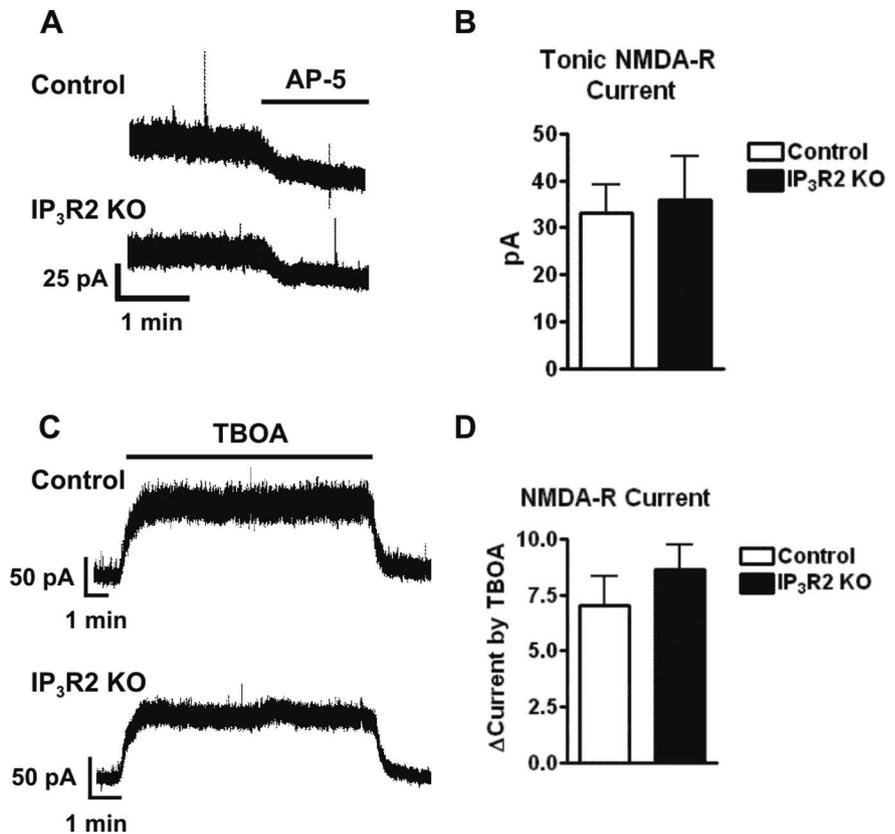


Figure 4. Ambient glutamate is unaffected by the lack of Ca²⁺ increases in astrocytes. **A**, Bath application of 50 μM D-AP-5 blocked a tonic NMDAR current in CA1 pyramidal neurons held at +40 mV in ACSF from both littermate control and IP₃R2 KOs. **B**, The amplitude of the D-AP-5-sensitive NMDAR current was not significantly different ($p = 0.8$) in IP₃R2 KO neurons ($n = 5$) versus littermate controls ($n = 6$). **C**, Representative traces showing that bath application of 100 μM TBOA induced a large fold increase of the tonic current in CA1 pyramidal neurons held at +40 mV from both littermate control and IP₃R2 KOs. **D**, The fold increase over baseline induced by TBOA is not significantly different ($p = 0.4$) between IP₃R2 ($n = 5$ cells) and littermate controls ($n = 6$ cells). Error bars indicate SEM.

- Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* 247:470–473.
- Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G (2004) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron* 43:729–743.
- Fellin T, Gomez-Gonzalo M, Gobbo S, Carmignoto G, Haydon PG (2006a) Astrocytic glutamate is not necessary for the generation of epileptiform neuronal activity in hippocampal slices. *J Neurosci* 26:9312–9322.
- Fellin T, Sul JY, D’Ascenzo M, Takano H, Pascual O, Haydon PG (2006b) Bidirectional astrocyte–neuron communication: the many roles of glutamate and ATP. *Novartis Found Symp* 276:208–217; discussion 217–221, 233–237, 275–281.
- Fiacco TA, McCarthy KD (2004) Intracellular astrocyte calcium waves *in situ* increase the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons. *J Neurosci* 24:722–732.
- Fiacco TA, McCarthy KD (2006) Astrocyte calcium elevations: properties, propagation, and effects on brain signaling. *Glia* 54:676–690.
- Fiacco TA, Agulhon C, Taves SR, Petraevic J, Casper KB, Dong X, Chen J, McCarthy KD (2007) Selective stimulation of astrocyte calcium *in situ* does not affect neuronal excitatory synaptic activity. *Neuron* 54:611–626.
- Foskett JK, White C, Cheung KH, Mak DO (2007) Inositol trisphosphate receptor Ca²⁺ release channels. *Physiol Rev* 87:593–658.
- Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Kossel AH, Kater SB (1995) Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J Neurobiol* 28:159–170.
- Haydon PG, Carmignoto G (2006) Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev* 86:1009–1031.
- Herman MA, Jahr CE (2007) Extracellular glutamate concentration in hippocampal slice. *J Neurosci* 27:9736–9741.

- Hertle DN, Yeckel MF (2007) Distribution of inositol-1,4,5-trisphosphate receptor isoforms and ryanodine receptor isoforms during maturation of the rat hippocampus. *Neuroscience* 150:625–638.
- Hirase H, Qian L, Bartho P, Buzsaki G (2004) Calcium dynamics of cortical astrocytic networks in vivo. *PLoS Biol* 2:E96.
- Holtzclaw LA, Pandhit S, Bare DJ, Mignery GA, Russell JT (2002) Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. *Glia* 39:69–84.
- Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gahwiler BH, Gerber U (1999) Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Proc Natl Acad Sci USA* 96:8733–8738.
- Jensen AM, Chiu SY (1990) Fluorescence measurement of changes in intracellular calcium induced by excitatory amino acids in cultured cortical astrocytes. *J Neurosci* 10:1165–1175.
- Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat Neurosci* 1:683–692.
- Kang N, Xu J, Xu Q, Nedergaard M, Kang J (2005) Astrocytic glutamate release-induced transient depolarization and epileptiform discharges in hippocampal CA1 pyramidal neurons. *J Neurophysiol* 94:4121–4130.
- Le Meur K, Galante M, Angulo MC, Audinat E (2007) Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus. *J Physiol (Lond)* 580:373–383.
- Li X, Zima AV, Sheikh F, Blatter LA, Chen J (2005) Endothelin-1-induced arrhythmogenic Ca²⁺ signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ Res* 96:1274–1281.
- Liu QS, Xu Q, Kang J, Nedergaard M (2004a) Astrocyte activation of presynaptic metabotropic glutamate receptors modulates hippocampal inhibitory synaptic transmission. *Neuron Glia Biol* 1:307–316.
- Liu QS, Xu Q, Arcuino G, Kang J, Nedergaard M (2004b) Astrocyte-mediated activation of neuronal kainate receptors. *Proc Natl Acad Sci USA* 101:3172–3177.
- Malarkey EB, Parpura V (2008) Mechanisms of glutamate release from astrocytes. *Neurochem Int* 52:142–154.
- Matsumoto M, Nakagawa T, Inoue T, Nagata E, Tanaka K, Takano H, Minowa O, Kuno J, Sakakibara S, Yamada M, Yoneshima H, Miyawaki A, Fukuuchi Y, Furuichi T, Okano H, Mikoshiba K, Noda T (1996) Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. *Nature* 379:168–171.
- Montana V, Malarkey EB, Verderio C, Matteoli M, Parpura V (2006) Vesicular transmitter release from astrocytes. *Glia* 54:700–715.
- Nadkarni S, Jung P (2007) Modeling synaptic transmission of the tripartite synapse. *Phys Biol* 4:1–9.
- Nett WJ, Oloff SH, McCarthy KD (2002) Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity. *J Neurophysiol* 87:528–537.
- Parpura V, Haydon PG (2000) Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. *Proc Natl Acad Sci USA* 97:8629–8634.
- Parri HR, Gould TM, Crunelli V (2001) Spontaneous astrocytic Ca²⁺ oscillations in situ drive NMDAR-mediated neuronal excitation. *Nat Neurosci* 4:803–812.
- Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310:113–116.
- Pasti L, Zonta M, Pozzan T, Vicini S, Carmignoto G (2001) Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. *J Neurosci* 21:477–484.
- Perea G, Araque A (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. *J Neurosci* 25:2192–2203.
- Porter JT, McCarthy KD (1996) Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J Neurosci* 16:5073–5081.
- Scemes E (2000) Components of astrocytic intercellular calcium signaling. *Mol Neurobiol* 22:167–179.
- Scemes E, Giaume C (2006) Astrocyte calcium waves: what they are and what they do. *Glia* 54:716–725.
- Serrano A, Haddjeri N, Lacaillie JC, Robitaille R (2006) GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. *J Neurosci* 26:5370–5382.
- Sharp AH, Nucifora Jr FC, Blondel O, Sheppard CA, Zhang C, Snyder SH, Russell JT, Ryugo DK, Ross CA (1999) Differential cellular expression of isoforms of inositol 1,4,5-trisphosphate receptors in neurons and glia in brain. *J Comp Neurol* 406:207–220.
- Sheppard CA, Simpson PB, Sharp AH, Nucifora FC, Ross CA, Lange GD, Russell JT (1997) Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca²⁺ release sites that support Ca²⁺ waves in cultured astrocytes. *J Neurochem* 68:2317–2327.
- Straub SV, Nelson MT (2007) Astrocytic calcium signaling: the information currency coupling neuronal activity to the cerebral microcirculation. *Trends Cardiovasc Med* 17:183–190.
- Straub SV, Bonev AD, Wilkerson MK, Nelson MT (2006) Dynamic inositol trisphosphate-mediated calcium signals within astrocytic endfeet underlie vasodilation of cerebral arterioles. *J Gen Physiol* 128:659–669.
- Tian GF, Azmi H, Takano T, Xu Q, Peng W, Lin J, Oberheim N, Lou N, Wang X, Zielke HR, Kang J, Nedergaard M (2005) An astrocytic basis of epilepsy. *Nat Med* 11:973–981.
- Verkhatsky A, Kettenmann H (1996) Calcium signalling in glial cells. *Trends Neurosci* 19:346–352.
- Weerth SH, Holtzclaw LA, Russell JT (2007) Signaling proteins in raft-like microdomains are essential for Ca²⁺ wave propagation in glial cells. *Cell Calcium* 41:155–167.
- Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6:43–50.