Astrocyte calcium signalling: the third wave

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The discovery that transient elevations of calcium concentration occur in astrocytes, and release "gliotransmitters" which act on neurons and vascular smooth muscle, led to the concept of astrocytes being powerful regulators of neuronal spiking, synaptic plasticity and brain blood flow. These findings were challenged by a second wave of reports that astrocyte calcium transients did not mediate functions attributed to gliotransmitters and were too slow to generate blood flow increases. Remarkably, the tide has now turned again: the most important calcium transients occur in fine astrocyte processes not resolved in earlier studies, and new mechanisms have been discovered by which astrocyte $[Ca^{2+}]_i$ is raised and has its effects. Here we review how this third wave of discoveries has changed our understanding of astrocyte calcium signalling and its consequences for neuronal function.

Few topics in neuroscience are as controversial as the idea that calcium concentration elevations in astrocytes release transmitters that regulate neuronal and vascular function. As an example, highly respected scientists in the field have stated on the one hand that[1] astrocytes show "Ca^{2+}-dependent quantal glutamate release...previously considered to be specific to synapses" and on the other hand that[2] "the case for regulated release of glutamate from astrocytes onto neurons...is not convincing" and[3] "it is very difficult to conclude that astrocytes possess the...machinery required for Ca^{2+}-dependent release of glutamatergic vesicles". How could these mutually incompatible views arise? In this Perspective we will review briefly the turbulent history of the field, survey recent data suggesting a way out of some of the reported contradictions, and speculate on the future direction of research in this area.

The first wave - astrocytes also process information

The discovery that glutamate evokes a calcium concentration rise in astrocytes[4-8] (in culture, in brain slices, in whole retina and *in vivo*), which can propagate along astrocyte processes and even between glial cells[4,5,7,9,10], raised the possibility that glial Ca^{2+} waves might constitute an extra-neuronal signalling system in the CNS[7]. The subsequent demonstration

that rises in astrocyte $[Ca^{2+}]_i$, in turn, induce a $[Ca^{2+}]_i$ rise in adjacent neurons[11,12] sparked a flurry of studies that generated the concept of "gliotransmission" from astrocytes to neurons.

Increases of astrocyte $[Ca^{2+}]_i$ evoked by receptor agonists such as glutamate and GABA, or by uncaging of Ca^{2+} or IP_3 (inositol trisphosphate), were reported to release gliotransmitters from astrocytes, including glutamate[11,13-18], ATP[19-21], D-serine[22-24], and GABA[25-28]. (It is unknown whether $[Ca^{2+}]_i$ also regulates the release of slower acting astrocyte-derived factors that regulate receptor expression at synapses, such as TNF-α (tumour necrosis factor-α), TGF-β1 (transforming growth factor-β1) and glypicans[29-31]). The release of these gliotransmitters has been reported to generate a wide range of effects on neurons (summarised in **Fig. 1** and described at length in ref. 32),. Glutamate release evokes an inward membrane current in neurons, mediated by NMDA receptors, that regulates excitability and synchronises action potential firing[15-17,33,34]. Release of glutamate and GABA, and of ATP which is converted to adenosine by extracellular ectoATPases, regulates synaptic vesicle release probability by activating presynaptic receptors[18,35-38]. The resulting effects on synaptic strength regulate whether synaptic plasticity can occur[39-41], as does the release of D-serine which controls the amount of NMDA receptor activation occurring when glutamate is released at synapses[22,24]. These changes of neuronal function will modify information processing in circuit-specific ways, but a major high-level function of gliotransmitter release was suggested to be modulation of sleep induction, produced by the accumulation of adenosine derived from astrocyte-released ATP[42].

In addition to altering neuronal information processing, calcium-evoked release of messengers from astrocytes was suggested to regulate the energy supply to the brain in three important ways. First, increases of astrocyte $[Ca^{2+}]_i$ lead to the release of arachidonic acid-derived messengers (such as prostaglandins, epoxyeicosatrienoic acids and 20-hydroxyeicosatetraenoic acid (20-HETE)) that modify the contraction of vascular smooth muscle[43-45]. This provides a mechanism by which the polarized morphology of astrocytes - with many processes around synapses, and endfeet apposed to blood vessels - could regulate

cerebral blood flow and energy supply[46] according to the activity of synapses, the main consumers of energy in the brain[47]. Second, glutamate-evoked rises of astrocyte $[Ca^{2+}]_i$ trigger the insertion of more glucose transporters into the cell membrane, facilitating glucose uptake from the blood when synapses are active[48]. Third, regulation of oxygen supply to the whole body may involve CO_2 acidifying brainstem astrocytes, which leads to a $[Ca^{2+}]_i$ rise and ATP release, which in turn increases breathing rate[49].

All this work led to the idea that astrocytes constitute a network of cells that process information and regulate brain energy supply in parallel with neurons. It culminated in the proposal that an increase in astrocyte size and complexity was crucial for the increase in central neural processing power that has occurred during hominid evolution[50]. This idea was reinforced by an increase in synaptic plasticity and learning seen in mice seeded with human astrocytes[51], which propagate calcium waves four-fold faster than do rodent astrocytes[50]. Astrocytes therefore seemed to have come of age as players in information processing, and it was anticipated that disruptions of astrocyte calcium signalling might be important in diseases including epilepsy[52], inflammation[53], Alzheimer's[54], Huntington's[55] and HIV infection[56].

The second wave - the controversies

Throughout these exciting developments, an increasing number of conceptual problems were arising with the mechanisms by which $[Ca^{2+}]_i$ is raised in astrocytes, the time course of that elevation, and the mechanisms by which gliotransmitters are released (**Fig. 2**). These problems and, as we shall see later, their resolution, arose in part from the methods used to study all of these phenomena. Demonstrating a role for gliotransmission in brain function requires the use of methods that manipulate astrocytes specifically. This has involved the use of drugs thought to alter $[Ca^{2+}]_i$ specifically in astrocytes (which is hindered by the fact that many receptors, while being expressed preferentially in astrocytes, are also expressed in neurons), the introduction into astrocytes of agents that manipulate signalling pathways (such as caged calcium or IP_3, and tetanus or botulinum toxin), genetic manipulations that are

astrocyte-specific, or the use of agents that disrupt astrocyte metabolism. It turns out that none of these provide a magic bullet to test the functional role of astrocyte $[Ca^{2+}]_i$ transients[32].

G_q-coupled metabotropic glutamate receptors (mGluRs, particularly mGluR5), which generate IP_3 that releases calcium from internal stores, were suggested to initiate astrocyte calcium signalling[6,13,15,43] in the developing brain, because antagonists for these receptors blocked astrocyte $[Ca^{2+}]_i$ transients but not neuronal $[Ca^{2+}]_i$ transients[43] or synaptic activity[6,57] evoked by afferent stimulation. However, neurons also express mGluR5 and astrocyte expression of mGluR5 decreases as astrocytes mature[58]. Furthermore, despite originally reporting that in adult mice astrocyte $[Ca^{2+}]_i$ transients are evoked by synaptically released glutamate activating mGluR5 and mGluR1[8,57], Nedergaard's group later reported that mRNA expression for these receptors in astrocytes was undetectable after three weeks postnatally[59] (see also ref. 60). Consequently, mGluR5 agonists did not raise $[Ca^{2+}]_i$ in adult astrocytes, and it was concluded[59] that synaptically released glutamate is insufficient to raise astrocyte $[Ca^{2+}]_i$. Caveats for this work[59] include the fact that older, more ramified, astrocytes may be less easily isolated with all their mRNA (some of which may be in their processes), that mRNA level may not predict protein level, that Ca^{2+} signals appear to have been studied in the cells' somata rather than their processes, and hence that some mGluR5 may be present in the processes of adult astrocytes[61].

The intracellular signalling pathway often supposed to translate increases of extracellular neurotransmitter concentration into astrocyte $[Ca^{2+}]_i$ transients, i.e. the release of calcium from intracellular stores downstream of mGluR5 (or other G_q-coupled receptors), has similarly been criticised. Astrocyte processes very close to synapses have been reported to lack intracellular calcium stores[62] (although this may depend on fixation conditions[63]), and so may be unable to respond to synaptic transmitter release with a $[Ca^{2+}]_i$ transient produced by store release. Furthermore, when the type 2 receptor for IP_3 (which is expressed far more in glia than in neurons[64]) was knocked out, although this greatly reduced the number of $[Ca^{2+}]_i$ transients occurring in astrocyte somata[65,66], it had no effect on neuronal excitability[66], synaptic currents[66], synaptic plasticity[67] (although ACh-evoked LTP [long term potentiation] was

abolished[68,69]), neurovascular coupling[70,71] or various behavioural assays[72]. Although it is hard to rule out developmental compensation in response to this lifelong deletion, these data challenged the notion that astrocyte $[Ca^{2+}]_i$ transients driven by IP_3-evoked Ca^{2+} release from internal stores release gliotransmitters which have a major influence on neuronal function.

Raising astrocyte $[Ca^{2+}]_i$, by expressing in astrocytes a non-mammalian G_q-coupled receptor that could be activated by an exogenous molecule (a Designer Receptor Exclusively Activated by a Designer Drug, DREADD)[65,70], had no effect on neuronal $[Ca^{2+}]_i$ or excitability and did not modulate excitatory synaptic currents or evoke vascular effects. This is hard to reconcile with experiments showing that dialysis of astrocytes (from a patch pipette) with the calcium buffer BAPTA suppresses effects attributed to gliotransmitters[18,35,37]. To further complicate matters, raising $[Ca^{2+}]_i$ with a G_q-coupled receptor can fail to evoke gliotransmitter release even when uncaging of calcium within astrocytes does[73], and different G_q-coupled receptors can have a very different efficacy for evoking gliotransmitter release[74], suggesting that the subcellular localization of receptors with respect to internal calcium stores may be a crucial determinant of gliotransmitter release.

The mechanism by which $[Ca^{2+}]_i$ is raised in astrocytes will have an important influence on the speed of the $[Ca^{2+}]_i$ rise, which has become a disputed issue when considering how astrocyte $[Ca^{2+}]_i$ changes regulate the vasculature. Although rises of astrocyte $[Ca^{2+}]_i$ dilate arterioles[43,45,57], it appears that astrocyte $[Ca^{2+}]_i$ rises may be too slow[71,75,76], or occur in too few astrocytes[77] , to produce the rapid increase of blood flow evoked by neuronal activity that generates BOLD fMRI signals.

Downstream of $[Ca^{2+}]_i$ transients, prolonged controversy has existed over whether the release of gliotransmitters is mediated exocytotically[1,37] (as reported in freshly dissociated and cultured astrocytes[78,79]) or by ion channels[27,73,80] (as has been reviewed in detail[32]). We will return to channel-mediated release below. Exocytosis, in the case of glutamate and GABA, requires the presence of vesicular glutamate and GABA transporters in astrocytes. Although these were detected for glutamate using immunocytochemistry and single cell PCR[1], this has been disputed on the basis of a lack of overlap of vesicular glutamate transporter (VGLUT)

labelling with astrocyte markers (for the plasma membrane glutamate transporter GLT-1, the Ca^{2+}-binding protein S100β and the water channel aquaporin 4)[81] and transcriptome data do not detect VGLUT1, VGLUT2, VGLUT3 or VGAT (the vesicular GABA transporter) in astrocytes[64]. RNA-seq data can exhibit false negatives, but these transporters *are* detected in neurons, suggesting that VGLUT expression is genuinely low in astrocytes, although it is hard to rule out a highly spatially localised expression in astrocyte compartments that do not express GLT-1, S100β or aquaporin 4.

Exocytosis also requires the formation of a SNARE (Soluble NSF Attachment Protein REceptor) complex which mediates Ca^{2+}-dependent release of transmitter-filled vesicles. Although transcriptome data[64] suggest that some SNARE complex proteins are relatively selectively expressed in astrocytes (SNAP-23, VAMP3) or in neurons (SNAP-25, SNAP-47, VAMP2, syntaxin), there is no exclusively astrocytic component that could be knocked out to prevent transmitter exocytosis from astrocytes. Similarly, the potential calcium sensor for vesicle release which has the highest expression[64] in astrocytes, synaptotagmin XI, is also expressed at a high level in neurons. An innovative approach to suppressing astrocyte vesicle release was provided by expressing part of the SNARE molecule VAMP2 in astrocytes, driven (in a doxycyclin-suppressible manner) by the GFAP (glial fibrillary acidic protein) promoter, to inhibit exocytotic release of transmitters from astrocytes[39]. This was found to alter synaptic transmission and plasticity[39], and to reduce the pressure to sleep[42], by suppressing the release from astrocytes of ATP, which is converted to adenosine by ecto-ATPases. The interpretation of this experiment depends crucially on expression of the transgene being specific to astrocytes, since VAMP2 is also involved in neuronal exocytosis. A paper re-examining this issue[82] suggested that the transgene was also expressed at a lower level (in a doxycyclin-suppressible manner) in some neurons (defined by PSA-NCAM expression), disagreeing with control experiments in the original reports[39,42], and raising the question of whether the effects seen were dominated by alterations of exocytosis in astrocytes or in neurons[83]. The vigorous response published with ref. 82

(http://www.jneurosci.org/content/34/50/16594/reply#jneuro_el;112097) shows that this debate is not over yet, and the new data need to be confirmed by an independent laboratory (ideally using further neuronal markers), nevertheless the data in ref. 82 emphasise the importance of checking that supposedly astrocyte-specific transgenic lines are truly astrocyte-specific[84]. An alternative approach, injection of SNARE-cleaving toxins into astrocytes has been shown to decrease gliotransmitter release[37,85], but it is hard to rule out the possibility that these agents also affect trafficking to the surface membrane of ion channels that mediate release (see below).

These controversies, over the receptors raising astrocyte $[Ca^{2+}]_i$ in response to neuronal activity, the involvement of internal calcium stores in producing $[Ca^{2+}]_i$ transients and the mechanism (if any!) by which astrocytes release transmitters in response to $[Ca^{2+}]_i$ rises, left the field in a prolonged state of uncertainty, with some dismissing all the evidence as unsatisfactory[83].

The third wave - partial resolution of the controversies

The first hint of a possible explanation for some of these controversies came with the realisation that, just like neurons, astrocytes must be considered as comprising many different subcellular compartments, and that (easily detected) calcium transients occurring in the soma may not be telling the experimenter very much about what is happening in the fine astrocyte processes near synapses. Indeed, different astrocyte processes generate $[Ca^{2+}]_i$ transients at different times[86], and spatially localised $[Ca^{2+}]_i$ transients in the cells' processes occur much more frequently than in the somata[87-89]. The advent of two-photon fluorescence imaging, with its improved vertical spatial resolution and decreased excitation light scatter and photon damage, combined with the presence of Ca^{2+}-sensing molecules in single astrocytes (achieved either by expressing genetically-encoded calcium indicator (GECI) proteins, or by dialysing cells from a patch pipette with Ca^{2+}-sensing dyes), resulted in the characterisation of different types of calcium transient in different parts of the astrocyte[86,90-93].

By targeting a GECI to the membranes of astrocytes, where changes in $[Ca^{2+}]_i$ would be most relevant to controlling the release of gliotransmitters, it was shown[90] that $[Ca^{2+}]_i$

transients can differ in the bulk cytoplasm from those occurring just under the cell membrane. Indeed some $[Ca^{2+}]_i$ transients occur mainly near the membrane, and were attributed to the spontaneous opening of TRPA1 (Transient Receptor Potential Ankyrin type 1) channels in the astrocyte membrane[91], consistent with the finding that some $[Ca^{2+}]_i$ transients in astrocyte processes are independent of neuronal activity[86]. The TRPA1 contribution to the resting $[Ca^{2+}]_i$ was found to promote the insertion of GABA transporters into the astrocyte membrane, and thus to regulate GABAergic inhibition[91]. Characterization of $[Ca^{2+}]_i$ transients at different locations along astrocyte processes using AM ester-[88] or pipette-loaded dyes[92,93] (which combine greater sensitivity with greater response speed than currently-available GECIs[94]) revealed $[Ca^{2+}]_i$ elevations occurring on different spatial and temporal scales: in hippocampal dentate gyrus astrocytes, spontaneous synaptic transmitter release produces brief duration (~0.7 s) spatially-localised (~4 μm) transients in astrocyte processes, while action potential driven release triggers larger, longer-lasting (~3 s) spatially broader (~12 μm) events[92]. $[Ca^{2+}]_i$ transients in astrocyte processes can sometimes propagate along the process[86,89], into the soma[89] and even between cells[88]. Both in dentate gyrus[92] and in hippocampal area CA1[93], introducing the Ca^{2+} chelator BAPTA into an astrocyte led to an increase in the rate of synaptic failures (but see ref. 95 where a stronger stimulation strength was used; although the BAPTA concentration was high - 75 mM - in one of these studies[92], 10 mM BAPTA had the same effect[79,93]). Since these studies did not add any Ca^{2+} to the internal solution, the resting $[Ca^{2+}]_i$ is ill defined (contrast with ref. 24), and it is not possible to say whether the change of synaptic activity reflects BAPTA lowering the resting $[Ca^{2+}]_i$ or buffering synaptically-evoked $[Ca^{2+}]_i$ rises in the astrocyte. Despite this problem, and the fact that these studies attributed the $[Ca^{2+}]_i$ rises in astrocytes and the subsequent effects on neurons to different neuro- and glio-transmitters (ATP and glutamate[92], or glutamate and ATP or adenosine[93], respectively), this work was a crucial step forward in the recognition that compartmentation of astrocyte $[Ca^{2+}]_i$ rises may explain some of the discrepancies in the literature.

The best example of the importance of astrocyte Ca^{2+} compartmentation concerns the effect of knocking out IP_3R2 receptors. Whereas the earlier reports[65-67,70-72] discussed above seemed to imply that most astrocyte $[Ca^{2+}]_i$ transients were abolished when IP_3R2 was knocked out, it turns out that loss of this store release receptor has much less effect in the fine processes of astrocytes than in the soma[89,96]. While IP_3R2 knock-out abolished all but ~10% of somatic $[Ca^{2+}]_i$ transients, it spared ~40% of the $[Ca^{2+}]_i$ transients occurring in the cells' processes and, for the sites of $[Ca^{2+}]_i$ rise that remained, there was little change in the frequency of transients[96]. Furthermore, while removal of extracellular Ca^{2+} had little effect on $[Ca^{2+}]_i$ transients at the soma, in nearly half of the locations in processes where transients were generated it reduced the frequency of the transients by 50-75%. This suggests that a release of Ca^{2+} from internal stores is the main source of $[Ca^{2+}]_i$ transients at the soma, while in the astrocyte processes transmembrane entry of Ca^{2+}, presumably through endogenously active channels like TRPA1[91] or receptor-gated Ca^{2+}-permeable ion channels, generates 30-40% of $[Ca^{2+}]_i$ elevations[96]. Interestingly, when IP_3R2 receptors were knocked out, $[Ca^{2+}]_i$ rises evoked in astrocytes by endothelin were greatly reduced in the somata of the cells but much less affected in the processes, suggesting that Ca^{2+} signalling evoked by this agonist may be mediated by release from internal stores in the soma but by direct coupling to a plasma membrane ion channel in the processes. Thus, there are major differences between Ca^{2+} signalling in the somata and processes of astrocytes, and overall there are at least eight-fold more Ca^{2+} transients in processes than in somata[96,89]. Consequently, previous conclusions[65-67,70-72], based on knocking out IP_3R2 receptors and assuming (on the basis of a lack of somatic Ca^{2+} responses) that astrocyte Ca^{2+} signalling then has little functional effect on neurons, require re-examination.

The importance for understanding function, of characterising $[Ca^{2+}]_i$ rises in the processes of astrocytes, is illustrated by papers examining whether astrocyte $[Ca^{2+}]_i$ rises are fast enough to drive the local increases of blood flow evoked by neuronal activity. While $[Ca^{2+}]_i$ rises in astrocyte somata may be too slow[71,75,76] to generate rapid blood flow increases, examination of $[Ca^{2+}]_i$ transients in astrocyte processes suggests that they are faster than in

somata[97], and occur before or with a similar time course to the increase of blood flow[88,98,99].The lack of effect of IP_3R2 receptor knockout on blood flow responses[70,71], together with data showing that 30-40% of astrocyte process $[Ca^{2+}]_i$ rises are initiated by transmembrane Ca^{2+} entry[96], suggests that active neurons release agents that gate Ca^{2+}-permeable ion channels in astrocyte processes (for example AMPA[98,100], NMDA[100,101] or P2X[100-102] receptors, or TRPA1[91] channels). This raises the question of how $[Ca^{2+}]_i$ rises evoked in astrocyte processes around synapses lead to the release of vasoactive messengers at the spatially-distant endfeet made by astrocytes onto vessels. The lack of effect on blood flow responses of knocking out IP_3R2 receptors rules out the possibility that a $[Ca^{2+}]_i$ wave produced by regenerative IP_3 generation and Ca^{2+} release from endoplasmic reticulum stores propagates through the cell. Instead, either vasoactive messengers (NO, arachidonic acid derivatives) generated in astrocyte processes near synapses may diffuse to the nearest vessel, or a $[Ca^{2+}]_i$ wave generated by Ca^{2+}-induced Ca^{2+} release from stores mediated by ryanodine receptors[103] or by Ca^{2+}-induced ATP release and activation of ATP receptors[104] may transmit the signal from the astrocyte's synapse-wrapping processes to its endfeet.

Other novel pathways that can raise $[Ca^{2+}]_i$ in astrocytes have also been suggested. For glutamate, the canonical $G_{\alpha i}$ signalling pathway activated by mGluR2 and mGluR3 receptors inhibits cAMP production, however activation of these receptors (the expression of which, unlike mGluR5, has not been questioned in the adult[59]) has been suggested to raise $[Ca^{2+}]_i$ in the processes of astrocytes[105]. This may involve activation of Ca^{2+} release from stores or entry to the cell mediated by the G protein's $\beta\gamma$ subunits. In addition, glutamate[75] and GABA[106] transporters have been suggested to raise astrocyte $[Ca^{2+}]_i$ in response to neuronal activity. This may be mediated by reversed operation of Na^+/Ca^{2+} exchangers[106], following an uptake-evoked rise of $[Na^+]_i$. Astrocyte $[Ca^{2+}]_i$ is also regulated by the modulatory neurotransmitters noradrenaline[107] (via α_1 receptors) and acetylcholine[108] (via muscarinic receptors), which are released from wide ranging axons with somata in the locus coeruleus (a region involved in arousal, attention and memory) and the nucleus basalis of Meynert (an area

involved in arousal, learning and reward), respectively. These responses are developmentally regulated in the opposite direction to those generated by mGluR5, being barely detectable before the end of the first post-natal week[108]. Recent *in vivo* work has revealed that locomotion or an aroused state evoke a large noradrenaline-mediated astrocyte $[Ca^{2+}]_i$ elevation over a broad spatial area[109,110], while stimulation of the nucleus basalis of Meynert evokes similar acetylcholine-mediated $[Ca^{2+}]_i$ elevations[69,111]. Importantly, the noradrenaline results were obtained in unanaesthetized animals, and so were not compromised by the suppressive effect of anaesthetics on astrocyte Ca^{2+} transients[112]. The significance of these results is discussed below.

As indicated above, in our view the jury is still out on whether gliotransmitters are exocytosed from astrocytes, but new developments make that issue less crucial. First, recent work has provided molecular candidates for ion channels that have been suggested[80] to release neurotransmitters from astrocytes: both GABA and glutamate can be released from astrocytes via Ca^{2+}-activated bestrophin-1 anion channels (despite the apparently low expression of mRNA for this channel in astrocytes[58,64]) and, surprisingly, normally K^+-selective TREK (Tandem of P-domains in a Weakly Inward rectifying K^+ channel - related K^+ channel) two-pore domain channels[27,113,114]. Furthermore, raising the proton concentration within cerebellar astrocytes (Bergmann glia) using channelrhodopsin evokes glutamate release via an as yet unidentified H^+-gated mechanism (cf. ref. 49 on brainstem astrocytes) and this mechanism contributes to glutamate release during ischaemia[115]. Second, there is increasing awareness that changes of astrocyte $[Ca^{2+}]_i$ can have effects on neurons not by releasing substances but via changes in the activity of transporters in the astrocyte membrane. Astrocyte $[Ca^{2+}]_i$ rises via TRPA1 channels increase the insertion of GABA transporters into the astrocyte membrane and thus regulate GABAergic effects on neurons without a need for the astrocyte to release GABA[91]. Similarly, G_q-coupled mGluRs increase glutamate uptake currents[116] and membrane-insertion of GLAST glutamate transporters[117], and thus decrease glutamate effects on nearby neurons[117] (but see ref. 118), and conceivably the surface mobility of glutamate transporters[119] could be altered by $[Ca^{2+}]_i$ changes. Alterations of astrocyte

morphology driven by $[Ca^{2+}]_i$ changes[120,121] will also contribute to altering the efficiency of astrocyte transporters in regulating neurotransmitter effects on neurons. Finally, the increase of Na^+/Ca^{2+} exchange activity that is generated by increases of astrocyte $[Ca^{2+}]_i$ following transmitter-induced calcium release from internal stores has been reported to raise $[Na^+]_i$ sufficiently to increase sodium pump activity, leading to a decrease of $[K^+]_o$ (or less increase during neuronal activity) and local hyperpolarization of neurons[122]. This reduced spontaneous excitatory (but not inhibitory) synaptic activity, but decreased failures of action potential evoked excitatory synaptic transmission[122]. The discovery of these mechanisms for modulating neuronal synaptic currents by altering astrocyte transmitter uptake or sodium pump activity implies that future studies wishing to attribute the effects of changes of astrocyte $[Ca^{2+}]_i$ to altered gliotransmitter release will first need to rule out (as in ref. 79) changes in the activity of a range of astrocyte membrane transporters.

Outlook for the future

With the discovery of a plethora of astrocyte $[Ca^{2+}]_i$ transients with different spatial locations and temporal dynamics, new ways in which $[Ca^{2+}]_i$ can be raised, and novel mechanisms by which astrocytes can modulate neuronal function downstream of the $[Ca^{2+}]_i$ rises, the field is on a more secure footing than it was 10 years ago (**Fig. 3**). In this section we will highlight what we see as the most important questions to be resolved in the future. Methodological advances needed for this progress have been dealt with in three recent reviews[123-125].

How do locally and globally-evoked $[Ca^{2+}]_i$ changes interact? In brain slices, astrocyte $[Ca^{2+}]_i$ transients generated locally either spontaneously[86,91] or by local release of transmitters[41,92,93] such as glutamate, GABA and ATP, are readily detected. *In vivo*, however, these can be greatly outweighed by noradrenaline- and ACh-mediated $[Ca^{2+}]_i$ transients evoked by movement or attention changes (or nucleus basalis stimulation)[69,109,110], which potentiate the $[Ca^{2+}]_i$ rise produced by the locally released transmitters. These $[Ca^{2+}]_i$ transients evoke a long-term potentiation of local neuronal responses (which depends on store-mediated release of Ca^{2+} in astrocytes, and subsequent D-serine and ATP

release)[69,103,111]. The locus coeruleus and nucleus basalis of Meynert comprise a relatively small number of cells (~35,000 for the locus coeruleus in humans), with widely ranging axons, which cannot mediate very specific modulation of the neural circuitry, and so their activation evokes an astrocyte $[Ca^{2+}]_i$ rise more or less globally, in many brain areas[109]. It therefore seems likely that these pathways serve to prime astrocytes to globally modulate neuronal function in certain behavioural states. The underlying mechanisms, and how they are modulated in disorders of noradrenergic or cholinergic function, are important questions for the future.

How are astrocyte $[Ca^{2+}]_i$ transients decoded? In other cell types it has been shown that the frequency and time course of $[Ca^{2+}]_i$ transients determines their downstream effect[126]. At present we have no information on how the variety of $[Ca^{2+}]_i$ transients seen in astrocytes, with different spatial and temporal characteristics[86,88-93], are decoded into functional effects that are short term (e.g. modulation of synaptic transmission) and long term (e.g. modulation of synaptic existence by release of factors like glypicans or regulation of synaptic pruning). Is it the mean $[Ca^{2+}]_i$ that matters, the amplitude of $[Ca^{2+}]_i$ transients, their duration, or all of these? As noted above, it is common for studies probing the role of astrocyte $[Ca^{2+}]_i$ transients to introduce BAPTA into astrocytes with a solution lacking added Ca^{2+}, producing a reduction of baseline $[Ca^{2+}]_i$ in addition to an increase of buffering power. Future work will need to determine which of these has the dominant effect on the release of gliotransmitters or the activity of proteins in the astrocyte membrane. A related issue is that, conceivably, some BAPTA could leak out of astrocytes via gap junctional hemichannels and have an effect by lowering the local $[Ca^{2+}]_o$ and suppressing synaptic transmission (although dyes of a similar molecular weight do not appear to leak out of the cells): this could be tested by conducting such experiments in the presence of hemichannel blockers.

Which other major factors regulate astrocyte $[Ca^{2+}]_i$? We have restricted our review to the most well known neurotransmitters that may regulate astrocyte $[Ca^{2+}]_i$. However, new modulators of astrocyte $[Ca^{2+}]_i$ are likely to be discovered, and a case has already been made for protons[49], cannabinoids[127,128], polyphosphate[129] and endothelin[130] in this regard. Although

it has been widely assumed that the majority of neuronally-evoked astrocyte $[Ca^{2+}]_i$ transients reflect release of neurotransmitters at presynaptic terminals, neuronal depolarization may instead raise astrocyte $[Ca^{2+}]_i$ by action potential evoked release of substances from postsynaptic dendrites, including glutamate, ATP and cannabinoids[128,131]. Astrocyte Ca^{2+} signalling and its effects are also, as for neuronal signalling, likely to show plasticity themselves[95].

Which is more important: release of gliotransmitters or alteration of astrocyte membrane proteins? A gliotransmitter-sceptic might assert that many of the phenomena attributed to gliotransmitter release can in fact be explained by astrocyte-intrinsic effects, specifically changes in the activity or expression of membrane transporters. Thus, increases in the failure rate of action potential evoked synaptic transmission seen when astrocyte $[Ca^{2+}]_i$ is buffered might theoretically reflect a reduction of the astrocyte Ca^{2+}-evoked decrease of $[K^+]_o$ produced by increased sodium pump activity[122], while changes in the amplitude of synaptic currents might reflect Ca^{2+}-driven changes of neurotransmitter transporter level in the surface membrane of astrocytes[91,116,117]. Although occlusion experiments, showing that signalling to transmitter receptors on neurons is downstream of astrocyte $[Ca^{2+}]_i$ changes, can argue against the former idea, we believe that future research will increasingly need to consider astrocyte-intrinsic explanations of changes in neuronal function induced by astrocyte $[Ca^{2+}]_i$ transients. Astrocytes may also release factors other than small molecule transmitters, including the calcium-binding protein S100β[132].

Does cyclic nucleotide signalling have similar effects to Ca^{2+}? Most research is done on astrocyte Ca^{2+} signalling because of the easy availability of indicators to monitor $[Ca^{2+}]_i$. Yet many astrocyte neurotransmitter receptors evoke changes of cAMP (cyclic AMP) concentration rather than $[Ca^{2+}]_i$. There is almost certainly a whole world of cAMP-mediated effects in astrocytes, influencing membrane proteins, astrocyte morphology and thus neuronal function, which is waiting to be discovered.

Does all this happen in other glia? While more than a decade of research has now been carried out on astrocyte Ca^{2+} signalling, this area of research has barely begun for

oligodendrocytes and microglia. The lessons learnt from the pioneering studies of astrocyte $[Ca^{2+}]_i$ transients will surely be helpful to those studying whether Ca^{2+} signalling is involved in regulating myelination or immune cell function.

Conclusion

From our perspective, the astrocyte Ca^{2+} signalling field seems in robust good health. Controversies remain, but if one examines fields of neuronal physiology that are at a similar developmental stage (e.g. the role of neuronal oscillations in brain function, determining what fMRI really measures, assessing how the cortical canonical circuit works, defining what the cerebellum does) they seem to show similar growing pains to the astrocyte Ca^{2+} field. Constructively handled disagreements are a useful stimulus to further research. We look forward to the fourth wave!

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Figure Legends

Figure 1. The first wave. Elevations of astrocyte $[Ca^{2+}]_i$ evoked by G protein coupled receptors activated by glutamate, GABA and ATP (or ADP) have been reported to evoke the release of the gliotransmitters ATP, glutamate, D-serine and GABA. These can modulate neuronal activity postsynaptically, notably by glutamate and D-serine inducing NMDA receptor mediated currents. They can also modulate transmitter release probability by acting on presynaptic receptors. Astrocyte P2X and NMDA receptors represent other channel mediated sources of $[Ca^{2+}]_i$ elevation in astrocytes. Ca^{2+} waves can spread through the astrocyte's processes to the soma, and to its vascular endfeet where vasoactive messengers are released (PG, prostaglandin; 20-HETE, 20-hydroxyeicosatetraenoic acid; EETs, epoxyeicosatrienoic acids). Astrocyte morphology has been distorted to define the location of signalling processes.

Figure 2. The second wave controversies. 1. mGluR5 is absent in adult astrocytes, but they still show glutamate-evoked $[Ca^{2+}]_i$ transients. **2.** Knock-out of the gene for IP_3R2 receptors on astrocyte calcium stores suppresses calcium transients at the soma, but does not affect many functions attributed to $[Ca^{2+}]_i$-driven transmitter release from astrocytes. **3.** Raising astrocyte $[Ca^{2+}]_i$ using a DREADD did not evoke functions attributed to $[Ca^{2+}]_i$-driven transmitter release from astrocytes. **4.** $[Ca^{2+}]_i$ transients seem too slow to account for rapid blood flow increases. **5.** There is debate about whether astrocytes express the VGLUTs and VGAT needed to package glutamate and GABA into vesicles for exocytosis. **6.** Inhibiting SNARE-driven exocytotic release from astrocytes with a dominant negative construct (dnSNARE) suppresses effects attributed to gliotransmitter (GlioT) release (**6a**), but the dnSNARE may also be expressed in neurons and suppress their release of neurotransmitter (NeuroT) (**6b**).

Figure 3. The third wave and the future. 1. $[Ca^{2+}]_i$ transients in the processes of astrocytes (**1a**) differ from those in the soma (**1b**) in terms of frequency, kinetics and spatial spread. **2.** $[Ca^{2+}]_i$ transients in the processes of astrocytes (**2a**) depend roughly equally on Ca^{2+} entry from the extracellular space via ion channels (40%) and on Ca^{2+} release from intracellular stores (60%), while those in the soma (**2b**) depend largely (90%) on Ca^{2+} release from the intracellular stores. **3.** $[Ca^{2+}]_i$ transients can be generated by Ca^{2+} entry through spontaneously

opening TRPA1 channels or neurotransmitter-gated channels (**3a**), by mGluR2 or mGluR3 (**3b**), and by neurotransmitter uptake raising $[Na^+]_i$ and reversing Na^+/Ca^{2+} exchange (**3c**). **4.** $[Ca^{2+}]_i$ rises may release transmitters via ion channels like Best-1 as well as via exocytosis. **5.** $[Ca^{2+}]_i$ rises alter the surface expression of neurotransmitter transporters. **6.** Activation of Na^+/Ca^{2+} exchange by a $[Ca^{2+}]_i$ rise can raise $[Na^+]_i$ and activate the sodium pump, lowering $[K^+]_o$ and hyperpolarizing nearby neurons. This increases the release probability (Prelease) for action potential driven vesicle release, and thus decreases synaptic failure rate. **7.** ATP released by a $[Ca^{2+}]_i$ rise may act on P2X or P2Y receptors to raise $[Ca^{2+}]_i$ further along the cell, propagating a Ca^{2+} wave along the cell (**7a**), or be converted to adenosine which acts on presynaptic receptors to increase (A_{2A}) or decrease (A_1) transmitter release (**7b**). **8.** Noradrenaline released from locus coeruleus neurons, and ACh released from nucleus basalis neurons produce large $[Ca^{2+}]_i$ rises in astrocytes.

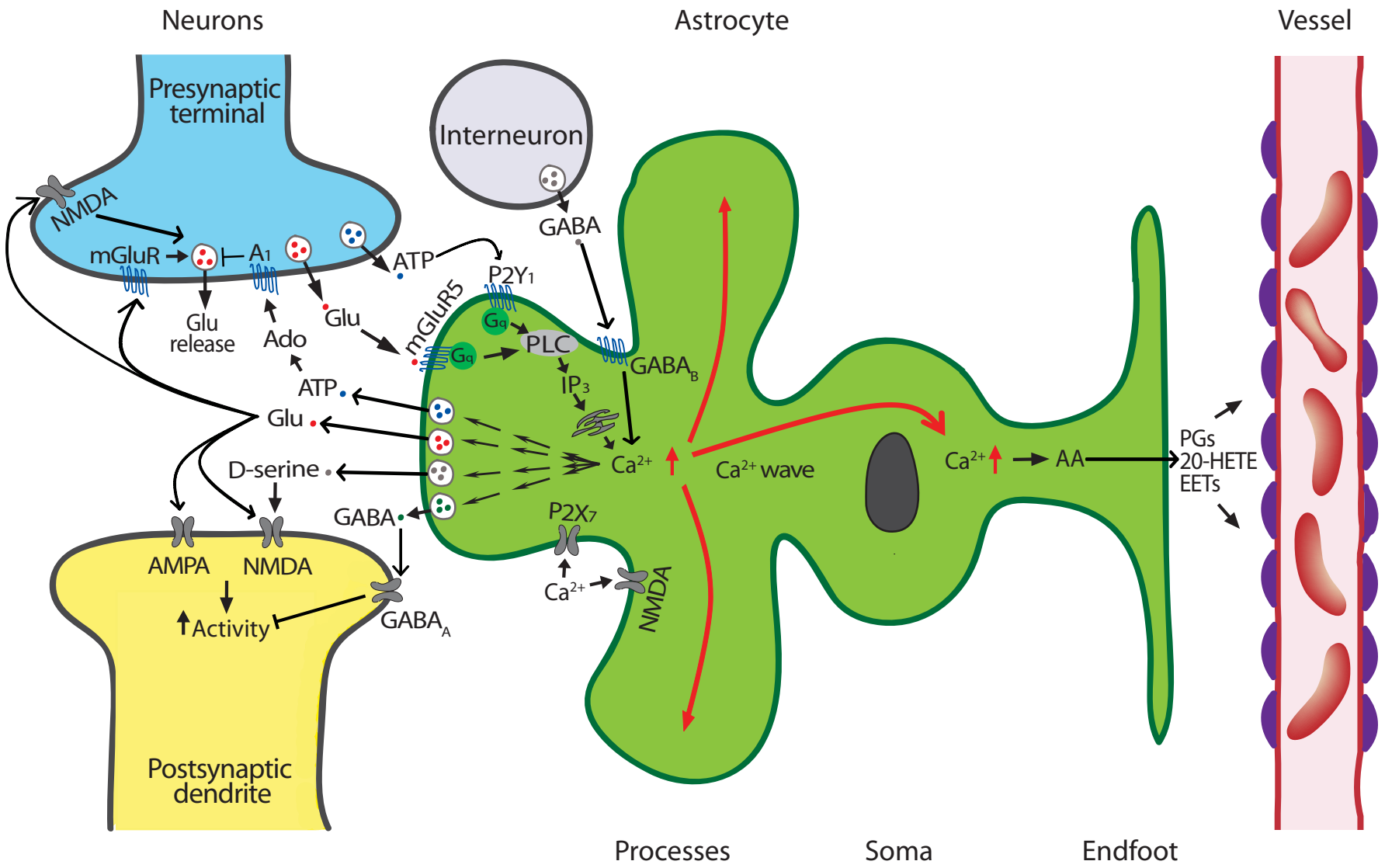
Neurons
Astrocyte
Vessel
Presynaptic terminal
Interneuron
NMDA
mGluR
A1
ATP
P2Y1
GABA
Glu release
Ado
Glu
mGluR5
Gq
PLC
IP3
GABAB
ATP
Glu
D-serine
Ca2+
Ca2+ wave
Ca2+
AA
PGs
20-HETE
EETs
GABA
P2X7
AMPA
NMDA
Activity
GABAA
Ca2+
NMDA
Postsynaptic dendrite
Processes
Soma
Endfoot

Fig 1

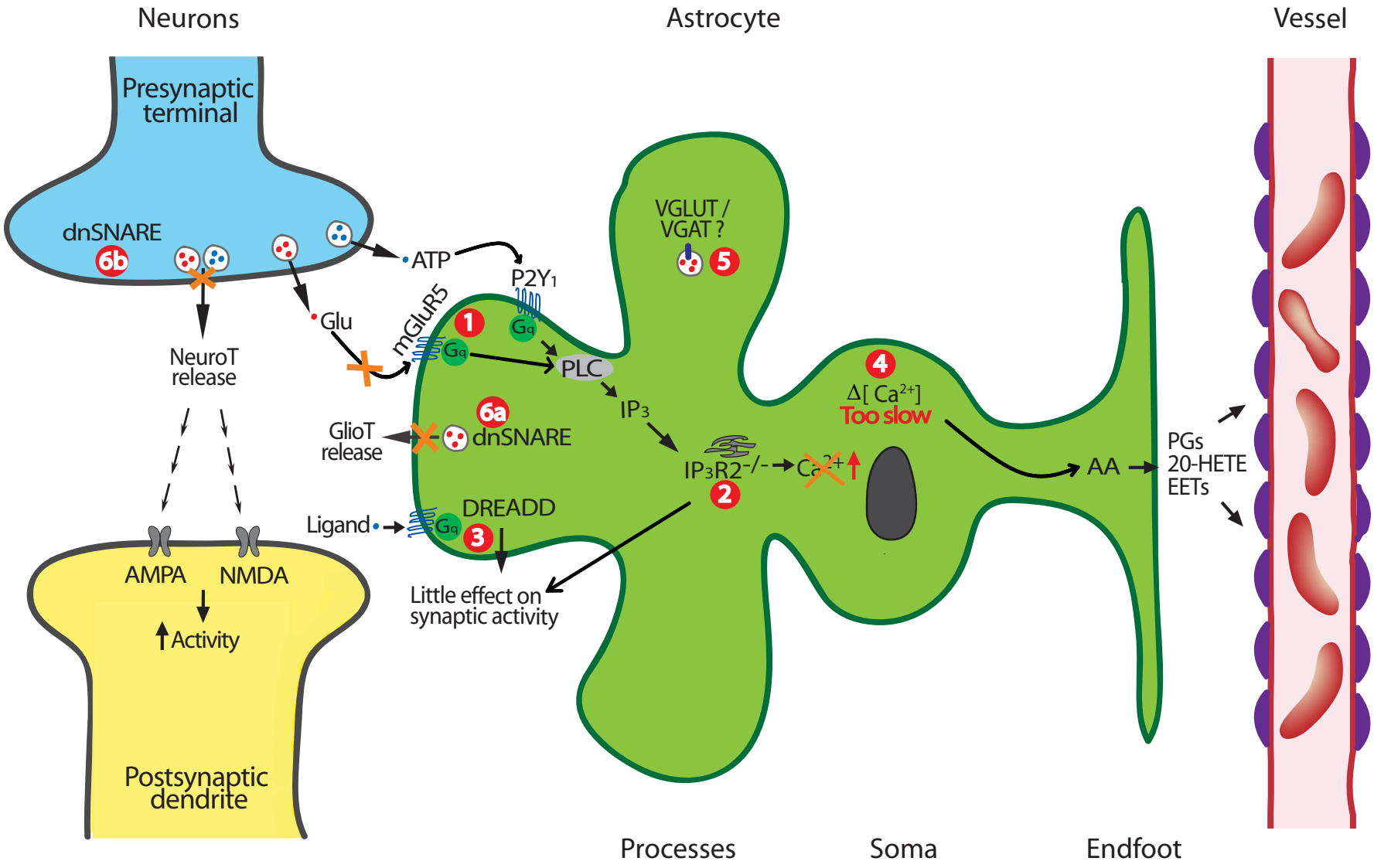
Neurons
Astrocyte
Vessel
Presynaptic terminal
dnSNARE
6b
ATP
P2Y1
Glu
mGluR5
1
Gq
NeuroT release
PLC
IP3
VGLUT / VGAT ?
5
4
Δ[Ca2+]
Too slow
6a
GlioT release
dnSNARE
IP3R2-/-
Ca2+
2
AA
PGs
20-HETE
EETs
Ligand
Gq
DREADD
3
AMPA
NMDA
Activity
Little effect on synaptic activity
Postsynaptic dendrite
Processes
Soma
Endfoot

Fig 2

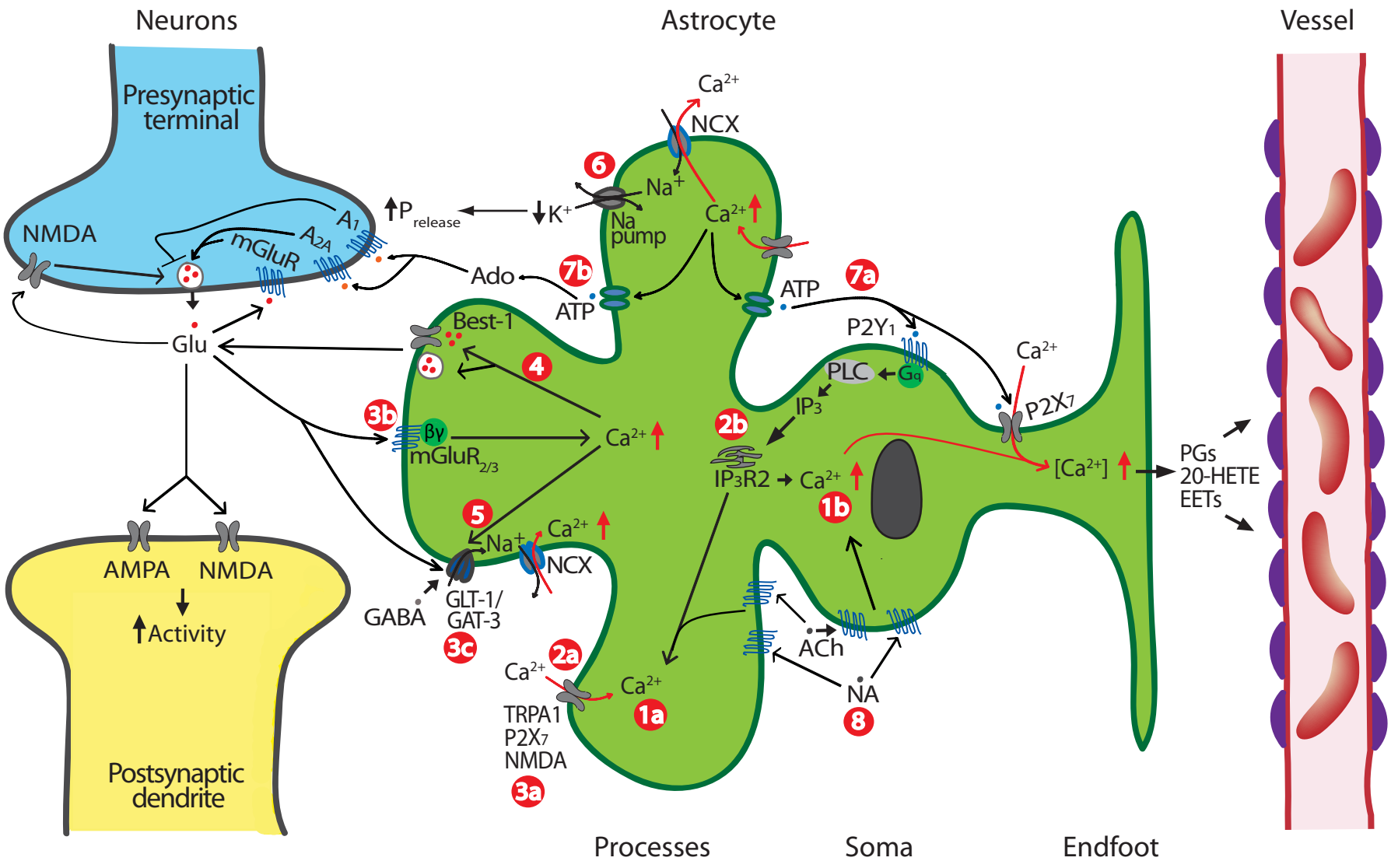
Neurons
Astrocyte
Vessel
Presynaptic terminal
NMDA
A1
A2A
mGluR
Glu
Ado
ATP
Best-1
Ca2+
NCX
Na+
Na pump
K+
P release
6
7b
7a
4
3b
βγ
mGluR2/3
2b
IP3R2
IP3
PLC
Gq
P2Y1
P2X7
[Ca2+]
1b
PGs
20-HETE
EETs
5
GABA
GLT-1/
GAT-3
3c
2a
TRPA1
P2X7
NMDA
3a
1a
ACh
NA
8
AMPA
NMDA
Activity
Postsynaptic dendrite
Processes
Soma
Endfoot

Fig 3