

## DOES GLUTAMATE RELEASED BY ASTROCYTES CAUSE FOCAL EPILEPSY?

### An Astrocytic Basis of Epilepsy

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*Nat Med* 2005;11:973–981.

Hypersynchronous neuronal firing is a hallmark of epilepsy, but the mechanisms underlying simultaneous activation of multiple neurons remains unknown. Epileptic discharges are in part initiated by a local depolarization shift that drives groups of neurons into synchronous bursting. In an attempt to define the cellular basis for hypersynchronous bursting activity, we studied the occurrence of paroxysmal depolarization shifts after suppressing synaptic activity using tetrodotoxin (TTX) and voltage-gated  $\text{Ca}^{2+}$  channel blockers. Here we report that paroxysmal depolarization shifts

can be initiated by release of glutamate from extrasynaptic sources or by photolysis of caged  $\text{Ca}^{2+}$  in astrocytes. Two-photon imaging of live exposed cortex showed that several antiepileptic agents, including valproate, gabapentin, and phenytoin, reduced the ability of astrocytes to transmit  $\text{Ca}^{2+}$  signaling. Our results show an unanticipated key role for astrocytes in seizure activity. As such, these findings identify astrocytes as a proximal target for the treatment of epileptic disorders.

### COMMENTARY

Epilepsy investigators frequently have theorized that a dysfunction in glial cells—and not in neurons or synapses—may be the initiating cause of epilepsy. This suggestion has been based on several observations. Robust reactive gliosis was necessary to induce posttraumatic epilepsy (1). Astrocytes play a crucial role in ion homeostasis and, therefore, in neuronal excitability (2). Manipulation of glial cell volume and, thus, of extracellular volume affects neuronal hypersynchrony (3,4). Astrocytes have a direct role in the regulation of synaptic strength and neuronal excitability (5,6). Thus, evidence has been steadily accumulating that a dysfunction in the astrocytic compartment can lower seizure threshold and precipitate seizures. However, glial cells have never gained center-stage attention, probably because the predominant investigative technology available for most of the last century, electrophysiology, allowed the study of electrically excitable membranes but not of other forms of excitability.

Recent advances in the technology of live-cell imaging and light-excitation, however, have revealed that astrocytes, while lacking membranes that are excitable in classic terms, can generate oscillatory intracellular calcium waves, which can propagate through the astrocytic network (7) and release neuroactive transmitters, such as glutamate (8,9), even by means of  $\text{Ca}^{2+}$ -mediated exocytosis (10). It has been shown that astrocytic  $\text{Ca}^{2+}$  waves propagate via the extrusion of ATP into the extracellular

space. ATP then acts in a paracrine fashion, binding purinergic receptors expressed on nearby astrocytes (11,12). Therefore, the development of live-cell imaging technology has now revealed the existence of the astrocytic “excitable cytoplasm,” similarly to the venerable demonstration of the axonal excitable membrane by Hodgkin, Huxley, Cole and Curtis in the 1930s.

The discovery of excitable astrocytes begged the question of whether these cells could actively drive the abnormal neuronal changes underlying epilepsy. Indeed, astrocytic calcium waves have properties that could facilitate seizures. They can propagate spatially and result in long-range changes in excitation of synaptically unconnected neurons (6). In addition, astrocytic  $\text{Ca}^{2+}$  waves have a propagation velocity comparable to that of spreading depression, and it was found that  $\text{Ca}^{2+}$  waves often precede spreading depression, although a cause–effect relationship between the two phenomena was not demonstrated (13–15).

The recent work by Tian and colleagues significantly adds to this line of investigation by providing direct evidence that activation of a single astrocyte by photolytic uncaging of intracellular  $\text{Ca}^{2+}$  induces paroxysmal depolarizing shifts (PDSs), which can be a form of interictal epileptiform activity. To study the mechanisms underlying the generation of PDSs, the authors performed an impressive and complex series of experiments. First they exposed rat hippocampal slices to 4-aminopyridine (4-AP), a proconvulsant agent. Bath application of tetrodotoxin (TTX), a  $\text{Na}^+$ -channel blocker, fully abolished neuronal action potentials but decreased the occurrence PDSs induced by 4-AP by only  $\sim 30\%$ . This observation was confirmed in the presence of synaptic blockers, such as calcium channel blockers.

Conversely, glutamatergic antagonists abolished about 80% of the TTX-resistant PDSs, thereby confirming a previous report by Strowbridge that glutamate receptors mediate a good portion, but not all of the TTX-independent 4-AP-induced PDSs (16). Further pharmacological manipulations indicated that the glutamatergic receptor predominantly involved in the generation of PDSs is the *N*-methyl-D-aspartate (NMDA) receptor. The authors also determined that similar TTX-insensitive PDSs occurred by employing other well-known proepileptic paradigms, such as applications of penicillin, bicuculline, or low-magnesium/calcium media.

Because astrocytes can release glutamate in a  $\text{Ca}^{2+}$ -dependent manner, Tian et al. evaluated whether this mechanism was responsible for TTX-insensitive PDSs. Astrocytes cultured in the absence of neurons were treated with 4-AP, as well as with other paradigms of experimental seizures, which potentially initiated astrocytic  $\text{Ca}^{2+}$  oscillations, thus suggesting that possible proepileptic mechanisms targeted by these agents lie in the astrocytic and not necessarily in the neuronal compartment. By placing field electrodes close to the astrocytes ( $\sim 20 \mu\text{m}$ ), the authors next determined whether a temporal correlation existed between the oscillatory  $\text{Ca}^{2+}$  events in astrocytes and the occurrence of TTX-insensitive PDSs, which occurred with an average latency of 0.4 seconds from the beginning of the intracellular  $\text{Ca}^{2+}$  increase. Importantly, no PDS ever was observed to precede astrocytic  $\text{Ca}^{2+}$  waves. The authors then loaded astrocytes in brain slices with caged  $\text{Ca}^{2+}$  and selectively released  $\text{Ca}^{2+}$  into the astrocytic cytoplasm by photolysis induced by 2-photon excitation. This manipulation resulted in the crucial finding that increases in astrocytic  $\text{Ca}^{2+}$  were sufficient to induce local PDSs.

To test the theory that astrocytes release glutamate during epileptic seizures, the authors used a microdialysis probe implanted in the rat hippocampus in vivo and induced seizures with 4-AP. The observed increase in glutamate was considered to be consistent with astrocytic origin, because it was accompanied by the correct fingerprint of amino acids, such as taurine, glutamine, and serine. The authors then hypothesized that compounds that reduce astrocytic glutamate release would suppress TTX-resistant epileptiform activity. Indeed, benzoic acid, flufenamic acid, and anion channel blockers also known to block glutamate release from astrocytes, markedly, although incompletely, reduced the frequency and amplitude of TTX-insensitive PDSs, providing evidence that glutamate released from astrocytes may contribute to abnormal neuronal activity.

The authors, furthermore, investigated the importance of astrocytic activation in the generation of seizures by observing  $\text{Ca}^{2+}$  signaling in the mouse cortex in vivo during focal application of 4-AP. As observed in brain slices, 4-AP-induced epileptiform activity was preceded by astrocytic  $\text{Ca}^{2+}$  waves in two-thirds of the cases. Administration of valproate, gabapentin,

or phenytoin resulted in a profound reduction in both amplitude and frequency of neuronal discharges and astrocytic responses. Thus, commonly used antiepileptic drugs (AEDs) depressed astrocytic  $\text{Ca}^{2+}$  signaling triggered by 4-AP. To determine whether the AED directly decreased neuronal activity or instead targeted astrocytes, Tian et al. induced astrocytic  $\text{Ca}^{2+}$  waves by iontophoretic application of ATP. In control animals, ATP-induced astrocytic  $\text{Ca}^{2+}$  waves that propagated beyond the field of view. Conversely, various AEDs depressed ATP-induced  $\text{Ca}^{2+}$  signaling to different extents. The authors concluded that AEDs may target astrocytes directly.

The work by Tian and colleagues represents a step forward in the identification of glial mechanisms of epilepsy, because it further validates, by applying relatively new 2-photon imaging techniques, the older idea that pathophysiological changes in astrocytes could be novel targets for improved therapeutic treatments of epilepsy. The strength of the work lies in the elegant demonstration that the selective increase in intercellular  $\text{Ca}^{2+}$  in an astrocyte by 2-photon excitation results in a local PDS. The mechanisms responsible for this phenomenon are unclear, however. While it is possible, given the existence of  $\text{Ca}^{2+}$ -dependent astrocytic glutamate release, it remains to be demonstrated that these PDSs are the result of glutamate released by astrocytes. No data were provided to demonstrate that glutamatergic antagonists abolished PDSs induced by intracellular uncaging of  $\text{Ca}^{2+}$  in astrocytes. Although interesting, the use of anion channel blockers, benzoic acid and flufenamic acid, to interfere with the  $\text{Ca}^{2+}$ -dependent release of glutamate from astrocytes is not compelling because these drugs notoriously lack specificity. Furthermore, it remains unclear whether the sudden increase in cytoplasmic  $\text{Ca}^{2+}$  results in the release of sufficient glutamate to precipitate a PDS or whether other agents (e.g., potassium, cytokines, ATP, or D-serine—all known to induce excitation of neuronal membranes) possibly acting synergistically with glutamate, are necessary for the phenomenon to occur. For example, astrocytes are known to express  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels. Therefore, artificially induced intracellular  $\text{Ca}^{2+}$  spikes are likely to activate these  $\text{K}^+$  channels, which would result in an outflow of  $\text{K}^+$  from the astrocytes into the extracellular space. Indeed, extracellular  $\text{K}^+$  accumulation induced by astrocytic  $\text{Ca}^{2+}$ -wave has been directly observed (17). In addition, glial swelling alters glial uptake of neuroactive substances from the extracellular space and may release osmotically active molecules, such as taurine, to restore proper cell volume (18,19). Tian and coworkers' observation of the corelease of several of these molecules along with glutamate, in fact, strongly suggests the occurrence of astrocytic regulatory volume decrease. Furthermore, glial swelling induces shrinkage of the extracellular space, which promotes neuronal hypersynchronization through ephaptic interaction (3,4,20,21). Therefore, a  $\text{Ca}^{2+}$ -mediated increase in astrocytes' cell volume may result in neuronal hypersynchrony

and hyperexcitability induced by ephaptic interactions in concert with the release of osmolytes that are restoring cell volume. The authors' observation of bicuculline-induced TTX-resistant PDSs points to ionic and volume-based mechanisms of PDS generation, since bicuculline-induced PDSs, originating from an imbalance between glutamatergic excitation and GABA-mediated inhibition, should be fully blocked by TTX. Conversely, if the GABA<sub>A</sub> receptor targeted by bicuculline to induce PDS were on astrocytes, (22,23), the predominant effect of bicuculline would be impairment of astrocytic ion homeostasis and impaired cell volume regulation. Similarly, intriguing is the finding that NMDA antagonists were more effective in preventing TTX-independent PDSs than  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) antagonists. Because depolarization is required to relieve the Mg<sup>2+</sup>-mediated voltage-dependent block of NMDA receptors, glutamate is not likely to be solely responsible for TTX-insensitive PDSs, which is consistent with the hypothesis that something else is co-released by astrocytes to potentially depolarize neurons. Not surprisingly, Tian and coworkers identified that ~20% of the astrocytic Ca<sup>2+</sup>-photolysis-mediated PDSs were insensitive to glutamatergic antagonists. Taken together, these data suggest that astrocytic Ca<sup>2+</sup> waves may affect neuronal excitability but not solely through the release of glutamate. Further work will be needed to understand the mechanistic link between astrocytic cytoplasmic Ca<sup>2+</sup> spikes and the initiation of PDSs.

Another intriguing finding by Tian and coworkers is that AEDs limit cortical epileptiform discharge, while also limiting the spatial propagation of cortical astrocytic Ca<sup>2+</sup> waves induced by 4-AP *in vivo*. Thus, contrary to common belief, AEDs may not exert an antiepileptic effect solely on neurons but on astrocytes as well. While this exciting theory warrants further investigations, simpler explanations are at hand. First, no data were provided to demonstrate that the Ca<sup>2+</sup> waves, which were more poorly propagated in the presence of AEDs, were not simply hampered by AED-induced reduction of neuronal activity. Second, in decreasing neuronal epileptiform activity, classic AEDs simultaneously decrease tissue metabolic demand and astrocytic swelling, thus limiting the shrinkage of the extracellular space associated with seizures. Because the astrocytic Ca<sup>2+</sup> wave propagates by paracrine action of ATP, propagation likely will be slower in the larger extracellular space. The focal iontophoretic application of ATP employed in the study is sufficient to initiate the astrocytic Ca<sup>2+</sup> wave locally, but it does not affect the long-range recurrent paracrine release of ATP throughout the astrocytic network that is required for the spatial propagation of the Ca<sup>2+</sup> wave. Thus, there is no assurance that extracellular concentrations of ATP reached levels in an enlarged extracellular space that were sufficient to propagate the wave along the astrocytic network. Indeed, evidence exists that manipulations of extracellular compartment affects prop-

agation of astrocytic Ca<sup>2+</sup> waves (24). The dampening of the astrocytic Ca<sup>2+</sup> wave by expansion of extracellular space would be an effect of AEDs and not associated, as hypothesized by the authors, with the astrocytic cellular mechanisms responsible for Ca<sup>2+</sup>-wave generation, and the key therapeutic development that could stem from it would involve the enlargement of the extracellular space, as previously proposed (3,4,21,25–27).

An issue not discussed by Tian et al. is that there is a difference between interictal events and epileptic seizures. The data presented only demonstrate that a massive nonspecific increase in cytoplasmic Ca<sup>2+</sup> in astrocytes is sufficient to negatively affect the activity of surrounding neurons and induce a PDS, which could be considered the parenchymal correspondent of an interictal EEG spike. However, no data were provided to determine whether this astrocytic phenomenon is responsible for, or at least contributes to, the precipitation of seizures, which are the real hallmark of epilepsy. When activating single astrocytes, the authors did not observe seizures or spreading depression, possibly because these events would require the occurrence of intracellular Ca<sup>2+</sup> spikes in larger ensembles of astrocytes. However, it is not clear that synchronous intracellular Ca<sup>2+</sup> spikes could occur in a larger ensemble of astrocytes in clinical epilepsy, considering that it cannot be achieved experimentally by application of strong proconvulsants, such as 4-AP. Thus, the experiments performed do not prove a causal role for astrocytic Ca<sup>2+</sup> waves in seizure generation or in epilepsy, and the question remains whether astrocytic Ca<sup>2+</sup> waves only allow the occurrence of occasional PDSs or interictal spikes.

Another issue not discussed by the authors is that astrocytic Ca<sup>2+</sup> waves have a physiological role; in particular, the coupling of regional cerebral blood flow to neuronal activity (28,29). Therefore, the experimental use of photolytic uncaging of Ca<sup>2+</sup> or the application of proconvulsants, which both induce PDSs, are likely to result in cytoplasmic changes in the astrocytes that are very different from those underlying physiological Ca<sup>2+</sup> waves, which occur without disrupting astrocytic or neuronal function. Further work will be required to understand whether the difference between physiological and experimental astrocytic Ca<sup>2+</sup> spikes depend on the concentration of cytoplasmic Ca<sup>2+</sup>, on their subcellular compartmentalization, or on other factors and whether these experimental changes are relevant to the cellular pathophysiology underlying clinical epilepsy.

In conclusion, the work by Tian et al. elegantly proves that intracellular Ca<sup>2+</sup> spikes in single astrocytes can result in focal abnormal neuronal activity. However, the work does not conclusively demonstrate a role for astrocytes in epilepsy. While the evidence obtained supports a role for astrocytic glutamate in the generation of PDSs, the phenomenon appears to be dependent on the synergistic action of different agents coreleased with glutamate. This finding is not surprising, given that astrocytes play a major role in virtually every aspect of homeostatic control of

the extracellular space surrounding neurons, and a nonspecific sudden cytoplasmic increase in  $\text{Ca}^{2+}$ , as obtained by photolytic uncaging, is likely to affect astrocytic homeostatic mechanisms in a variety of dramatic ways. For almost a century, evidence has been accumulating that pathophysiological changes in astrocytes may be involved in epilepsy. While evidence favoring a causative role of astrocytes in epilepsy is still missing, Tian and coworkers show how 2-photon excitation, by allowing selective release of compounds at subcellular level, permits the manipulation of the astrocytic compartment independently from the neuronal one, offering new ways to investigate the mechanisms by which astrocytes could cause, or contribute to, epilepsy.

by Raimondo D'Ambrosio, PhD

## References

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