

Perspective

Light of the stars: calcium signals light up astrocytes in the presence of autoreactive CD4⁺ T cells

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Introduction: Astrocytes play important roles in the central nervous system (CNS) to support and regulate CNS function. They are abundant type of glia that form a meshwork of interconnected cells almost completely tiling the CNS. Each astrocyte extends densely ramified processes that establish close contacts and interaction with other astrocytes, neurons and blood vessels, placing these cells in an ideal position to control extracellular milieu and to exert global effects on CNS physiology. Astrocytes are dynamic cells that continuously sense and respond to the physiological and pathological cues within their local environment. This responsiveness of astrocytes and their intercellular communication with neighboring cells in the CNS principally relies on increase in intracellular Ca²⁺ and release of neuroactive compounds such as adenosine 5'-triphosphate (ATP), that are critical for maintaining proper CNS function (Butt, 2011; Bazargani and Attwell, 2016). Intracellular Ca²⁺ level in astrocytes is under the control of diverse ionotropic and metabotropic receptors that upon activation promote Ca²⁺ entry into the cell or its liberation from cellular Ca²⁺ stores. This promotes rise of Ca²⁺ signals in astrocytes that spread information between astrocytes themselves and toward neurons to regulate CNS function. When CNS environment becomes disturbed following injury or in disease, astrocytes generally respond with an augmentation of Ca²⁺ signaling within their network that has deleterious effects and drive pathological processes (Shigetomi et al., 2019). Thus, Ca²⁺ signaling acts as a powerful and highly adaptable system for astrocyte communication with neighboring cells in the CNS and enables astrocytes to detect and respond to changes in their local environment in both, physiological and pathological conditions. In our recent paper (Bijelić et al., 2020) we show that astrocyte Ca²⁺ signals controlled by purinergic P2X7 ionotropic

receptors are also important for astrocytic communication with specific immune cells that infiltrate into the CNS in experimental autoimmune encephalomyelitis, a commonly used animal model of multiple sclerosis. We show that astrocytes respond to the nearby autoreactive CD4⁺ T cells with an increase in their intracellular Ca²⁺ (Figure 1), hinting at the previously unrecognized involvement of astrocytic Ca²⁺ signaling in the nervous system-immune system communication in an autoimmune disease of CNS.

CNS-infiltrated immune cells induce Ca²⁺ increase in astrocytes via P2X7 receptor activation:

In chronic demyelinating disease such as multiple sclerosis astrocyte interactions with CNS resident microglia and non-resident autoreactive immune cells promote loss of astrocytic supportive functions and fuel disease progression (Brambilla, 2019). The most studied astrocyte-immune cell interactions are those that are mediated by inflammatory signals to which astrocytes respond and in turn release to signal back to the immune cells. Yet, the abundance and broad coverage of astrocytes in both gray and white matter of the CNS, suggest that in multiple sclerosis astrocytes may frequently establish contacts with CNS-infiltrated immune cells. If so, could such cell-cell contact trigger intracellular Ca²⁺ increase in astrocytes? In our latest study, we pursued the answer to this question, and found that astrocyte interaction with the autoreactive immune cells displays properties remarkably similar to the fundamental Ca²⁺ dependent signaling astrocytes utilize to communicate with their resident CNS neighbors (Bijelić et al., 2020). In order to unambiguously study the role of Ca²⁺ signals in astrocyte interaction with immune cells, we have used naïve spinal cord astrocytes in culture and isolates of two populations of immune cells: CNS-infiltrated immune cells

isolated from the spinal cords of rats displaying symptoms of experimental autoimmune encephalomyelitis, and peripheral immune cells isolated from the cervical lymph nodes of the same animals. We imaged Ca²⁺ signals in astrocytes using Fluo-4 Ca²⁺ indicator and monitored astrocyte Ca²⁺ dynamics upon short bath application of CNS-infiltrated or peripheral immune cells. In our experimental system, astrocytes consistently responded to the brief application of CNS-infiltrated immune cells with vigorous intracellular Ca²⁺ increase, but astrocyte Ca²⁺ signaling remained silent when we applied peripheral immune cells (Figure 1). Remarkably, removal of CNS-infiltrated immune cells and application of their medium conditioned by released soluble factors, evoked Ca²⁺ response in minority of astrocytes and of lower magnitude. These results indicate that a contact between astrocytes and CNS-infiltrated immune cells triggers the major part of astrocytic Ca²⁺ response in our experimental system. Moreover, our results indicate that in addition to the immune cell-driven long-lasting phenotypic changes of astrocytes in pathological sequelae of multiple sclerosis (Brambilla, 2019), astrocytes may rapidly, within seconds, elevate their Ca²⁺ to respond to the presence of immune cells infiltrated into the CNS.

This breakthrough lead us further to identify ATP-dependent link and to conclusively demonstrate that ionotropic purinergic P2X7 receptor type controls increase in astrocyte Ca²⁺ in the presence of CNS-infiltrated immune cells, as shown by selective pharmacological targeting of different astrocytic receptors and ion channels. Using pharmacological antagonists, we have excluded contribution of astrocytic metabotropic group I receptors (mGluR1 and 5) for glutamate by applying CPCCOEt and MPEP blockers, nonselective transient receptor potential A1 cation channels by using A967079 inhibitor, and metabotropic purinergic P2Y1 receptors by blocking them with MRS 2179, as a possible routes for intracellular Ca²⁺ increase in astrocytes upon encountering autoreactive immune cells. Specific pharmacological inhibition of P2X7 receptors with A438079, however, substantially (by 72%) decreased the proportion of astrocytes that responded to the CNS-infiltrated immune cells, and Ca²⁺ change detected in a few cells was markedly reduced. In addition, link to P2X7 receptors was further strengthened by evidence that activation of this purinergic receptor by specific agonist BzATP evokes Ca²⁺ increase in astrocytes similar to that induced by immune cells. This finding is particularly important as P2X7Rs are associated with astrocyte response to the inflammatory conditions in multiple sclerosis in human CNS (Amadio et al., 2017). Considering that activation of P2X7 receptors in astrocytes is linked to the cytokine, glutamate and reactive oxygen species release from these glial cells (Burnstock and Knight, 2018), our data point toward a pathway by which astrocyte-immune cell interaction may fuel neurodegenerative processes in CNS autoimmunity.

Interaction between astrocytes and CNS-infiltrated immune cells requires astrocyte-derived ATP: A hot debate in the astrocyte field is how and when astrocytes release neuroactive substances to communicate with neighboring cells. Data from our paper provide new fuel to this debate. We show, surprisingly, that astrocytes rapidly release ATP when they encounter CNS-infiltrated immune cells

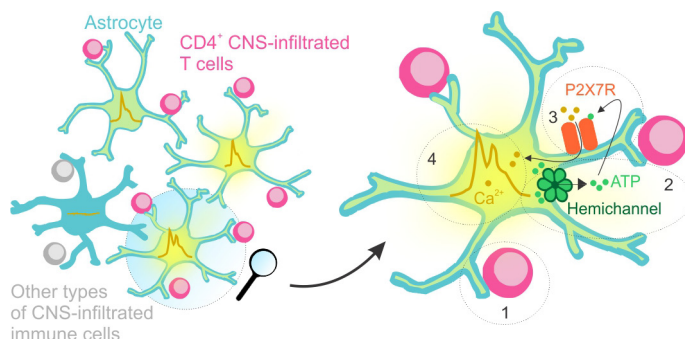


Figure 1 | Schematic depicting rapid astrocyte-immune cell interaction mediated by astrocytic Ca²⁺ signals.

(Left) Brief application of the CNS-infiltrated CD4⁺ T cells isolated from rat with experimental autoimmune encephalomyelitis induces a rapid increase in intracellular Ca²⁺ (yellow traces) in cultured naïve astrocytes. Other types of CNS-infiltrated immune cells do not change astrocytic baseline Ca²⁺ level. (dark yellow trace). (Right) Sequence of events showing that CD4⁺ T cell in close proximity to astrocyte (1) promotes hemichannel-dependent release of ATP (2), activation of P2X7Rs (3) and entry of Ca²⁺ that triggers vigorous transient Ca²⁺ increase in astrocyte (4). ATP: Adenosine 5'-triphosphate; CNS: central nervous system.

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in their close proximity. Astrocyte-derived ATP then activates P2X7 receptors on these glial cells (**Figure 1**). Moreover, we find that hemichannel-dependent mechanism of ATP release and not vesicular ATP is the main determinant of astrocyte Ca^{2+} increase during interaction with immune cells. However, some questions as to the initial step of the described signaling pathway remain unanswered in our paper. Indeed, the initial trigger of ATP release from astrocytic hemichannels remains undefined and it would be interesting, as we suggested, examining a role of integrins in the initiation of signaling cascade that mediates cellular interaction between astrocytes and CNS-infiltrated immune cells. Integrins are adhesion molecules that mediate cell-cell interaction, they are expressed both, in astrocytes and immune cells, and they are implicated in pathology of multiple sclerosis and experimental autoimmune encephalomyelitis (Archelos et al., 1999). Moreover, $\alpha_5\beta_3$ integrin has been linked to the P2X7 receptor activation by the hemichannel-derived ATP (Alvarez et al., 2016). Therefore, it would be interesting in future studies to examine the role of $\alpha_5\beta_3$ integrin in astrocyte interaction with immune cells in autoimmune disease of the CNS. In any case, astroglial responses mediated by P2 purinergic receptor-dependent signaling are involved in the initiation and maintenance of astrogliosis (Franke et al., 2012). This universal glial defense reaction to many CNS pathologies exerts both, beneficial and detrimental effects, and is also a prominent feature of experimental autoimmune encephalomyelitis (Brambilla, 2019). Our results showing that astrocytes respond to the presence of autoreactive immune cells by releasing ATP, suggest that ATP as a universal danger signal may be astrocytic alarm that CNS homeostasis has been compromised.

Astrocytes display Ca^{2+} response selectivity to CNS-infiltrated immune cells: Throughout the course of experimental autoimmune encephalomyelitis different types of immune cells infiltrate into the CNS and include predominantly T cells, while in a lower percentages macrophages, monocytes, granulocytes, and natural killer cells (Miljković et al., 2011). Among T cells, CD4^+ T lymphocytes recruited from the periphery are the largest population of immune cells that infiltrate into the CNS, and they are recognized to be crucial players in the early steps and perpetuation of experimental autoimmune encephalomyelitis. Our results indicate that astrocytes elevate Ca^{2+} specifically in response to the application of CD4^+ T cells, while the remaining pool of isolated CNS-infiltrated immune cells containing CD4^- population do not induce a change in astrocytic Ca^{2+} (**Figure 1**). Yet, further experiments need to be performed to analyze the effect of CNS-infiltrated CD4^+ T immune cells on astrocyte Ca^{2+} signals. The study of such immune cell type-dependent astrocyte Ca^{2+} signals might be valuable to gain further insight into pathogenic mechanism of multiple sclerosis. By using proximity analysis of immunolabeled astrocytes and CD4^+ T cells in the spinal cord of rat with experimental autoimmune encephalomyelitis, we show that more than 60% of infiltrated CD4^+ T cells interact with astrocytes in the inflamed CNS. Indeed, CD4^+ T cells scatter deeply in the spinal cord parenchyma and are closely neighbored by the meshwork of astrocytes, indicating that physical contact and interaction between these

two cell types is inevitable and frequent in the CNS autoimmunity. Our findings presented in (Bijelić et al., 2020) may significantly shift the perspective on the pathology of multiple sclerosis regarding interaction between the nervous and immune systems. Indeed, rapid and frequent *in situ* astrocyte-autoreactive CD4^+ T cell interactions in a contact-dependent manner may be critically important in formation of inflammatory environment in CNS autoimmunity.

Conclusions: The significance and surprise of the results presented in our paper (Bijelić et al., 2020) is the discovery that astrocytes use Ca^{2+} signals to communicate with CNS-infiltrated immune cells (**Figure 1**). These Ca^{2+} signals represent a separate astrocyte-immune cell communication system that involves contact-dependent mechanism of interaction. Increase in astrocyte Ca^{2+} occurs specifically due to the physical contact with autoreactive CD4^+ T cells. This striking new layer of astrocyte-immune cell communication offers new conceptual framework to further our understanding on the pathology of CNS autoimmune disease. The framework emerged from *in vitro* approach in our research would be strengthened by investigating astrocyte Ca^{2+} dynamics *in vivo* using animal models of multiple sclerosis, and by taking into account inputs of other CNS-resident cells such as microglia and neurons that can shape astrocyte Ca^{2+} signals. From our perspective it would be also important in future research to examine if astrocytic Ca^{2+} signals are involved in regulating immune cell function in the CNS autoimmunity. Notably, astrocyte-derived ATP may not only regulate Ca^{2+} signals in astrocytes, but also may be a feedback signal in modulating activity of immune cells. Furthermore, it is of importance to understand repercussions of immune-cell induced astrocyte Ca^{2+} signaling and ATP release on neuronal activity. Indeed, activation of Ca^{2+} signaling in astrocytes promotes release of glutamate which can increase neuronal activity, but ATP release from astrocytes can lead to the formation of adenosine which can dampen neuronal activity (Fellin et al., 2006). Thus, effect of astrocyte-immune cell interaction on excitation and inhibition of neuronal networks in CNS autoimmunity is an important question for the future waiting to be answered. We believe that upcoming research will increasingly consider astrocyte-immune cell interaction mediated by Ca^{2+} signals as an important component of pathological processes in multiple sclerosis and may be crucial avenue for the next generation of therapies to treat this disease. Although much remains to be discovered about the role of such astrocyte-immune cell interaction in CNS autoimmunity, it is clear that Ca^{2+} signals can light up a star-shape glia of the CNS universe near specific immune cells.

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