Glioactive ATP controls BDNF recycling in cortical astrocytes

Beatrice Vignoli and Marco Canossa

Centre for Integrative Biology (CIBIO), University of Trento, Povo (TN), Italy; European Brain Research Institute (EBRI)—Rita Levi-Montalcini, Rome, Italy

ABSTRACT

We have recently reported that long-term memory retention requires synaptic glia for proBDNF uptake and recycling. Through the recycling course, glial cells release endocytic BDNF, a mechanism that is activated in response to glutamate via AMPA and mGluRI/II receptors. Cortical astrocytes express receptors for many different transmitters suggesting for a complex signaling controlling endocytic BDNF secretion. Here, we demonstrated that the extracellular nucleotide ATP, activating P2X and P2Y receptors, regulates endocytic BDNF secretion in cultured astrocytes. Our data indicate that distinct glioactive molecules can participate in BDNF glial recycling and suggest that cortical astrocytes contributing to neuronal plasticity can be influenced by neurotransmitters in tune with synaptic needs.

KEYWORDS

calcium; glial network; gliotransmission; neurotrophins; synaptic plasticity

Most studies focusing on astrocyte-synapse interaction point at identifying specific molecules and signaling pathways that unveil the impact of astrocytes on synaptic transmission and plasticity. In a recent publication, we provided clear evidence that long-term potentiation (LTP) requires synaptic glia for (pro)-brain-derived neurotrophic factor (proBDNF) clearance and subsequent recycling (Fig. 1). The neurotrophin-uptake receptor p75NTR is expressed on astrocytes, that clear excessive proBDNF released at the synapse following LTP-inducing electrical stimulation. Endocytic proBDNF is then released as mature protein, which provides a source of neurotrophin that is essential to sustain TrkB phosphorylation in postsynaptic neurons. Given BDNF glial recycling, neurotrophin signaling is preserved for both strengthening synaptic activity and consolidating memory.

Astrocytes are not electrically excitable cells; thus, receiving signal from neurons at synaptic cleft is the most expected mechanism for these cells to regulate BDNF recycling. This provides a model by which astrocytes, that express receptors sensing transmitter release from active synapses, transduce internal signals leading to endocytic BDNF secretion. Astrocytes express receptors for many different transmitters and modulators, including glutamate, ATP, adenosine, and guanosine and display calcium signaling in response to their stimulation. This raises the possibility that BDNF glial recycling is highly regulated, and suggests that complex activation of transmitter receptors on glial cells controls final neurotrophin availability. This idea, which proposes a novel mechanism in neuron-glia interaction, suggests that astrocytes are functional targets for multiple transmitters and they can respond to and, in turn, regulate plasticity in tune with synaptic needs. In support of this hypothesis, we provided clear evidence that in addition to glutamate the extracellular nucleotide ATP regulates endocytic BDNF secretion in cultured cortical astrocytes.

Recycling of BDNF for transmitter-induced secretion by astrocytes

To obtain insight on the triggering events responsible for endocytic BDNF secretion by astrocytes, we used astrocyte cultures from P1-2 rat cortices, in which basal and stimulated neurotrophin secretion were not detected. Astrocytes were incubated with a mixture of precursor and mature BDNF isoforms (mix) for 10 min. This timing was chosen, as it correlates with sufficient proBDNF internalization in these cells. Astrocytes were then placed in a perfusion chamber and the perfusate was collected at 5-min intervals. The quantity of BDNF in each fraction was determined by a 2-site immunoassay.

We initially analyzed the role of high-potassium (40 mM KCl) that was described previously to promote secretion of glioactive BDNF in cultured astrocytes.
This stimulation is also known to regulate the secretion of BDNF from neurons through a mechanism involving depolarization and calcium increase, but whether it could also cause endocytic BDNF secretion by astrocytes is unclear. Treating cells for 5 min with KCl increased BDNF immunoreactivity (Fig. 2). The release of BDNF augmented rapidly, with maximum levels in the perfusate being reached 5 min after the beginning of KCl administration. This indicates that depolarization regulates endocytic BDNF secretion in astrocytes.

Given high-potassium is known to increase intracellular calcium levels through the activation of voltage-gated calcium channels in cultured astrocytes, we next sought to investigate whether the exposure of cortical astrocytes to stimuli known to raise intracellular calcium could mimic the effect of high-potassium on BDNF release. In support of this hypothesis, glutamate (500 μM) applied for 5 min to BDNF (mix)-treated astrocytes showed to trigger endocytic BDNF secretion (Fig. 2). The extent of BDNF secretion elicited by glutamate was similar to that observed after treatment with KCl, and BDNF levels peaked in the same fraction. Thus, astrocytes can provide endocytic BDNF secretion by sensing transmitter-mediated depolarization. This suggests that in physiologic conditions glioactive molecules rather than voltage-dependent excitation induce astrocytes depolarization, and BDNF secretion. In line with this idea, we have previously demonstrated that while high frequency (50 Hz) electrical stimulation induces endocytic BDNF secretion in cultured neurons, the same stimulation in cultured astrocytes was not effective. Our data clearly indicate that electrical activity is not sufficient to induce endocytic BDNF secretion from glial cells.

**ATP-mediated release of endocytic BDNF in cortical astrocytes**

While secretion of endocytic BDNF from astrocytes uses glutamate as exogenous stimuli, this is not necessarily restricted to this type of transmitter; rather it is likely expected that similar secretion of endocytic BDNF can be triggered by different transmitters. This prompted us to investigate whether cultured astrocytes could eventually release endocytic BDNF upon depolarizing conditions triggered by the extracellular nucleotides ATP, a well-known signaling molecules participating in synaptic transmission.
To address whether ATP may serve as a ligand for glial purinergic receptors that trigger endocytic BDNF secretion, we incubated astrocyte cultures for 10 min with exogenous BDNF (mix) and subsequently stimulated with ATP (100 μM) for 5 min (Fig. 3). This elicited an increase in endocytic BDNF secretion that was comparable to that induced by glutamate administration (Fig. 2), providing evidence that both transmitters induce endocytic BDNF secretion with similar efficacy. To obtain further insight into the mechanism of ATP-induced BDNF secretion from astrocytes, we used the ATP analogs 2-methyl-Tio-ATP (50 μM) or α/β-methylene-ATP (50 μM), specific ligands for ionotropic P2X or metabotropic P2Y purinergic receptors, respectively. Addition of ATP-analogs to BDNF (mix)-treated astrocytes produced a significant increase of BDNF in the perfusates (Fig. 3). Our results indicate that P2X and P2Y receptors enable astrocytes to respond to ATP finally promoting BDNF release through calcium elevation, a mechanism that was previously observed in microglia. However, what intracellular signaling are involved in the release process are hotly debated issues that will require further investigations.

The extracellular nucleotide ATP is known to co-localize with conventional neurotransmitters at cortical synapses, from which it can be released upon presynaptic depolarizing stimuli or can be secreted from post-synaptic sites. Astrocytes get in direct contact with the released neurotransmitters, suggesting that secretion of endocytic BDNF from glial cells is potentially triggered by ATP. In the tripartite hypothesis of synaptic transmission, where astrocytes form a third effective component of the synapse, glutamate and ATP trafficking from neurons to astrocytes can play a complex regulatory signaling for maintaining sufficient levels of BDNF according to synaptic requirements (Fig. 4). As glial vesicular release of BDNF is regulated by neuronal activity, astrocytes may then couple neuronal network activity to the local synaptic need of the neurotrophin. Such an

**Figure 3.** ATP- and ATP analogs-mediated release of endocytic BDNF in cortical astrocytes (Left) Astrocytes previously exposed to BDNF (mix) for 10 min were perfused and stimulated with ATP (100 µM) for 5 min. ELISA quantification of BDNF secretion from perfusates showed levels of BDNF before (0 to 10 min) during (10 to 15 min) and after (15 to 30 min) ATP application. (Right) Increased BDNF secretion induced by ATP (100 µM), α/β-methylene-ATP (50 µM) or 2-methyl-Tio-ATP (50 µM). Data are means ± SEM (error bars). *P < 0.05.

**Figure 4.** Local and long-distance BDNF recycling from astrocytes (left) Glioactive glutamate and/or ATP promote local BDNF recycling at glial endfeet by increasing intracellular calcium (Ca2+). While glutamate can be released from pre-synaptic-terminals, ATP release could conceivably occur from both pre- and post-synaptic-terminals. (right) Glioactive ATP promotes local elevation of intracellular calcium that afterward spread to different endfeet of the same astrocyte and/or adjacent astrocytes. This raises the possibility that ATP enable glial cells to function as cellular network competent for BDNF glial recycling at nearby or long-distance synapses.
demand” supply of BDNF may serve to recruit active synapses at behaviorally relevant circuits; as those contributing to the recognition of learned objects in perirhinal cortex. Overall, our results indicate that neurons may regulate glial BDNF recycling via the release of distinct transmitters, suggesting that neuron-astrocyte interaction can play a much more intricate and functional role in information processing coupled to cognitive functions than previously expected.

Glia cells are also skilled of inducing a local elevation of intracellular calcium. Surprisingly, whereas the calcium signals in glial cells is assumed to be dependent on IP3-mediated release from internal stores, very recent data demonstrated that cortical astrocyte signals depend on calcium influx through ATP-gated P2×1R ion channels. This calcium elevation occurs in astrocyte fine processes, which lack IP3 receptors and in which sensory stimulation evokes rapid increases in astrocytic calcium that precede increases in blood flow. These new data hint at the possibility that the calcium-dependent signaling pathways in different endfeet of the same astrocyte, which enwrap multiple distinct synapses, may be recruited differently according to network activity. Moreover, local calcium afterword spread (calcium waves) to adjacent astrocytes. Calcium waves are thought to propagate as a result of dif fusible ATP that is required for distant calcium signaling. This raises the possibility that ATP enable glial cells to function as cellular network competent for information processing and storage, features that are commonly assigned to neurons. At the network level, the electrical stimulation of afferents is likely to activate several types of neurons and glial cells and, concomitantly, the release of ATP. Our results that ATP mediates endocytic BDNF secretion raise the possibility that glial network, with precise temporal and spatial regulation of BDNF release, actively participate in information processing and memory storage together with neuronal network (Fig. 4). Reciprocal interactions between glial and neuronal networks may thus strengthen the ability to organize memory, and considerably expand storage capabilities. Whether BDNF glial recycling could act, as signaling mechanism involved in neuronal to glial network communication in the cortex and vice-versa is an attractive hypothesis that remains to be elucidated.

Material and methods

Release experiments were performed as described previously; stimulations were obtained by high potassium (40 mM), glutamate (500 μM), ATP (100 μM) and the ATP analogs αβ -methylene-ATP (50 μM) or 2-metil-Tio-ATP (50 μM). Treatment started 10 min after the beginning of perfusion and was maintained over a 5-min period. Two-site ELISA was used to determine the amount of BDNF in each fraction.10–13,29

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We acknowledge Stefano Ferroni for his critical reading of the manuscript and Ranzi Barbara for laboratory management.

Funding

This work was supported by MURST under Grant PRIN-2012-2015; Italian Research Council under Grant Framework Agreement EBRI-CNR 2015–2017; Lazio Innova under grant FILAS 2015–2017.

References

[1] Vignoli B, Battistini G, Melani R, Blum R, Santi S, Berardi N, Canossa M. Peri-synaptic glia recycles brain-derived neurotrophic factor for LTP stabilization and memory retention. Neuron 2016; 92:873-87; PMID:27746130; http://dx.doi.org/10.1016/j.neuron.2016.09.031
[2] Zuccaro E, Bergami M, Vignoli B, Bony G, Pierchala BA, Santi S, Cancedda L, Canossa M. Polarized expression of p75NTR specifies axons during development and adult neurogenesis. Cell Rep 2014; 7:138-52; PMID:24685135; http://dx.doi.org/10.1016/j.celrep.2014.02.039
[3] Bergami M, Santi S, Formaggio E, Cagnoli G, Verderio C, Blum R, Berninger B, Matteoli M, Canossa M. Uptake and recycling of pro-BDNF for transmitter-induced secretion by cortical astrocytes. J Cell Biol 2008; 183:213-21; PMID:18852301; http://dx.doi.org/10.1083/jcb.200806137
[4] Bergami M, Rimondini R, Santi S, Blum R, Götz M, Canossa M. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc Natl Acad Sci USA 2008; 105:15570-5; PMID:18832146; http://dx.doi.org/10.1073/pnas.0803702105
[5] Bergami M, Vignoli B, Motori E, Pifferi S, Zuccaro E, Menini A, Canossa M. TrkB signaling directs the incorporation of newly generated periglomerular cells in the adult olfactory bulb. J Neurosci 2013; 33:11464-78; PMID:23843518; http://dx.doi.org/10.1523/JNEUROSCI.4812-12.2013
[6] Bergami M, Berninger B, Canossa M. Conditional deletion of TrkB alters adult hippocampal neurogenesis and anxiety-related behavior. Commun Integr Biol 2009; 2:14-6; PMID:19704856; http://dx.doi.org/10.4161/cib.2.1.7349
[7] Canossa M, Rovelli G, Shooter EM. Transphosphorylation of the neurotrophin Trk receptors. J Biol Chem 1996; 271:5812-8; PMID:8621450; http://dx.doi.org/10.1074/jbc.271.10.5812
[8] Rovelli G, Heller RA, Canossa M, Shooter EM. Chimeric tumor necrosis factor-TrkA receptors reveal that ligand-
dependent activation of the TrkA tyrosine kinase is sufficient for differentiation and survival of PC12 cells. Proc Natl Acad Sci USA 1993; 90:8717-21; PMID:7690970; http://dx.doi.org/10.1073/pnas.90.18.8717

[9] Agulhon C, Petravicz J, McMullen AB, Sweger EJ, Minton SK, Taves SR, Casper KB, Fiacco TA, McCarthy KD. What is the role of astrocyte calcium in neurophysiology? Neuron 2008; 59:932-46; PMID:18817732; http://dx.doi.org/10.1016/j.neuron.2008.09.004

[10] Canossa M, Griesbeck O, Berninger B, Campana G, Kolbeck R, Thoenen H. Neurotrophin release by neurotrophins: implications for activity-dependent neuronal plasticity. Proc Natl Acad Sci USA 1997; 94:13279-86; PMID:9371837; http://dx.doi.org/10.1073/pnas.94.24.13279

[11] Santi S, Cappello S, Riccio M, Bergami M, Aicardi G, Schenk U, Matteoli M, Canossa M. Hippocampal neurons recycle BDNF for activity-dependent secretion and LTP maintenance. EMBO J 2006; 25:4372-80; PMID:1718862; http://dx.doi.org/10.1002/emboj.200400504

[12] Canossa M, Gärnert A, Campana G, Inagaki N, Thoenen H. Regulated secretion of neurotrophins by metabotropic glutamate group I (mGlur1) and Trk receptor activation is mediated via phospholipase C signalling pathways. EMBO J 2001; 20:1640-50; PMID:11285228; http://dx.doi.org/10.1093/semboj.20.7.1640

[13] Aicardi G, Argilli E, Cappello S, Santi S, Riccio M, Thoenen H, Canossa M. Induction of long-term potentiation and depression is reflected by corresponding changes in secretion of endogenous brain-derived neurotrophic factor. Proc Natl Acad Sci USA 2004; 101:15788-92; PMID:15505222; http://dx.doi.org/10.1073/pnas.0406960101

[14] Rutledge EM, Kimelberg HK. Release of [3H]-D-aspartate from primary astrocyte cultures in response to raised external potassium. J Neurosci 1996; 16:7803-11. doi:10.1073/pnas.94.24.13279

[15] Yaguchi T, Nishizaki T. Extracellular high K+ stimulates vesicular glutamate release from astrocytes by activating voltage-dependent calcium channels. J Cell Physiol 2010; 225:512-8; PMID:20506270; http://dx.doi.org/10.1002/jcp.22231

[16] Bölch A, Thoenen H. Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. Eur J Neurosci 1995; 7:1220-8; PMID:7582095; http://dx.doi.org/10.1111/j.1460-9586.1995.tb01112.x

[17] Griesbeck O, Canossa M, Campana G, Gärnter A, Hoener MC, Nawa H, Kolbeck R, Thoenen H. Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc Res Tech 1991; 45:262-75; PMID:10383119; http://dx.doi.org/10.1002/(SICI)1097-0029(19990515/01)45:4%3c262::AID-JEMT1%3e3.0.CO;2-K

[18] Balkowiec A, Katz DM. Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J Neurosci 2002; 22:10399-407; PMID:12451139

[19] Wang X, Butowt R, Vasko MR, von Bartheld CS. Mechanisms of the release of anterogradely transported neurotrophin-3 from axon terminals. J Neurosci 2002; 22:931-45; PMID:11826122

[20] MacVicar BA, Hochman D, Delay MJ, Weiss S. Modulation of intracellular Ca2+ in cultured astrocytes by influx through voltage-activated Ca2+ channels. Glia 1991; 4:448-55; PMID:1718862; http://dx.doi.org/10.1002/glia.440040504

[21] Bezzi P, Volterra A. A neuron-glial signalling network in the active brain. Curr Opin Neurobiol 2001; 11:387-94; PMID:11399439; http://dx.doi.org/10.1016/S0959-4388(00)00223-3

[22] Trang T, Beggs S, Wan X, Salter MW. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38 mitogen-activated protein kinase activation. J Neurosci. 2009; 29(11):3518-28; http://dx.doi.org/10.1523/JNEUROSCI.5714-08.2009

[23] Illes P, Ribeiro JA. Neuronal P2 receptors of the central nervous system. Curr Top Med Chem 2004; 4:831-8; PMID:15078214; http://dx.doi.org/10.2174/1568026043451032

[24] Derouiche A, Anlauf E, Aumann G, Mühlstädt B, Lavialle M. Anatomical aspects of glia-synapse interaction: the perisynaptic glial sheath consists of a specialized astrocyte compartment. J Physiol Paris 2002; 96:177-82; PMID:12445894; http://dx.doi.org/10.1016/S0928-4257(02)00004-9

[25] Araque A, Sanzgiri RP, Parpura V, Hayden PG. Astrocyte-induced modulation of synaptic transmission. Curr Opin Neurobiol 2002; 99:871-21; http://dx.doi.org/10.1016/S1074-1743(02)00004-9

[26] MacVicar BA, Hochman D, Delay MJ, Weiss S. Modulation of intracellular Ca2+ in cultured astrocytes by influx through voltage-activated Ca2+ channels. Glia 1991; 4:448-55; PMID:1718862; http://dx.doi.org/10.1002/glia.440040504

[27] Derouiche A, Anlauf E, Aumann G, Mühlstädt B, Lavialle M. Anatomical aspects of glia-synapse interaction: the perisynaptic glial sheath consists of a specialized astrocyte compartment. J Physiol Paris 2002; 96:177-82; PMID:12445894; http://dx.doi.org/10.1016/S0928-4257(02)00004-9

[28] Perea G, Araque A. Synaptic information processing by astrocytes. Purin Signal 2006; 2:595-604; http://dx.doi.org/10.1111/j.1460-9586.1995.tb01112.x

[29] Griesbeck O, Canossa M, Campana G, Gärnter A, Hoener MC, Nawa H, Kolbeck R, Thoenen H. Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc Res Tech 1991; 45:262-75; PMID:10383119; http://dx.doi.org/10.1002/(SICI)1097-0029(19990515/01)45:4%3c262::AID-JEMT1%3e3.0.CO;2-K