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REVIEW ARTICLE

EAAT expression by macrophages and microglia: still more questions than answers

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Abstract Glutamate is the main excitatory amino acid, but its presence in the extracellular milieu has deleterious consequences. It may induce excitotoxicity and also compete with cystine for the use of the cystine–glutamate exchanger, blocking glutathione neosynthesis and inducing an oxidative stress-induced cell death. Both mechanisms are critical in the brain where up to 20% of total body oxygen consumption occurs. In normal conditions, the astrocytes ensure that extracellular concentration of glutamate is kept in the micromolar range, thanks to their coexpression of high-affinity glutamate transporters (EAATs) and glutamine synthetase (GS). Their protective function is nevertheless sensitive to situations such as oxidative stress or inflammatory processes. On the other hand, macrophages and microglia do not express EAATs and GS in physiological conditions and are the principal effector cells of brain inflammation. Since the late 1990s, a number of studies have now shown that both microglia and macrophages display inducible EAAT and GS expression, but the precise significance of this still remains poorly understood. Brain macrophages and microglia are sister cells but yet display differences. Both are highly sensitive to their microenvironment and can perform a variety of functions that may oppose each other. However, in the very particular environment of the healthy brain, they are maintained in a repressed state. The aim of this review is to present the current state of knowledge on brain macrophages and microglial cells activation, in order to help clarify their role in the regulation of glutamate under pathological conditions as well as its outcome.

Keywords Microglia · Macrophage · EAAT · xCT · Glutamate · Glutathione · Neuroinflammation

Abbreviations

AD	Alzheimer's disease
AEG	Astrocyte elevated gene
BBB	Blood–brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
CCR	Chemokine (C-C motif) receptor
CX_3CL	Chemokine (C-X_3-C motif) ligand
CX_3CR	Chemokine (C-X_3-C motif) receptor
EAAT	Excitatory amino acid transporter
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FIZZ1	Found in Inflammatory Zone 1, a marker of alternative activation in murine macrophages
GS	Glutamine synthetase
GSH	L-γ-Glutamyl-L-cysteinyl-glycine (glutathione)
GSSG	Oxidised form of glutathione
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibition motif
MDM	Monocyte-derived macrophages
mGluR	Metabotropic glutamate receptors
MHC	Major histocompatibility complex
NF-κB	Nuclear factor-κB.
PDGF	Platelet-derived growth factor
PG	Prostaglandin
SIRPα	Signal-regulatory protein α

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SIV	Simian immunodeficiency virus
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
VGLUT	Vesicular glutamate transporter
xCT	Light chain subunit of the x_c^- cystine/glutamate exchanger
Ym1	A heparin-binding lectin, a marker of alternative activation in murine macrophages

Introduction

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) (Fonnum 1984) and is accordingly critical to many functions of the brain. Nevertheless, high or sustained glutamate receptor activation may induce neuronal death (Rothman 1984, 1985) via calcium and/or sodium deregulation, a mechanism called excitotoxicity. For further details on glutamate functions and toxicity in the brain, see the review by Had-Aissouni (2011) and references therein. Glutamate concentration in the extracellular milieu must thus be kept in the micromolar range (Lehmann et al. 1983), whereas it is in the millimolar range in glial cell cytoplasm and reaches 200 mM in synaptic vesicles (Ottersen et al. 1992). Glutamate concentration thus needs a tight control that requires an energy supply to maintain its gradient.

Extracellular glutamate is cleared by a family of transporter proteins called excitatory amino acid transporters (EAATs) that includes five cloned subtypes (Arriza et al. 1997; Fairman et al. 1995; Kanai and Hediger 1992; Pines et al. 1992; Storck et al. 1992; Tanaka 1993). EAAT1 and EAAT2 were primarily observed in astrocytes, EAAT3 is a neuronal transporter with a somatodendritic location (Rothstein et al. 1994), EAAT4 is expressed in the cerebellum (Fairman et al. 1995) and EAAT5 in the retina (Arriza et al. 1997). EAATs depend on the Na^+ and K^+ electrochemical gradients to take up extracellular glutamate and ensure a several thousand-fold concentration gradient, leading to an extracellular concentration below 1 μM (for review, see Gegelashvili and Schousboe 1997). For further details on EAAT functioning, see the review by Had-Aissouni (2011) and references therein. EAAT gene knockout experiments in mice show that the astroglial transporters EAAT1 and EAAT2 are essential for protection against excitotoxicity, by clearing extracellular glutamate, whereas the neuronal transporter EAAT3 is not (Rothstein et al. 1996; Tanaka et al. 1997). In contrast to EAAT1 and EAAT2 that localize at glutamatergic synapses, EAAT3 is localized throughout the neuronal cell body (Rothstein et al. 1994). Its deficiency induces decreases in cell glutathione (GSH) content and oxidative stress in vitro and in vivo (Aoyama et al. 2006; Himi et al. 2003), in line with its probable involvement in the regulation of GSH synthesis rather than glutamate clearance.

Astrocytes mediate the glutamate–glutamine cycle in the normal CNS

Astrocytes are the main glutamate scavenging cells in the normal CNS, because they are the only cells that express EAAT1 and EAAT2 as well as the critical enzyme glutamine synthetase (GS). The extracellular glutamate level is proportional to intracellular glutamate concentration (for review, see Attwell et al. 1993) and glutamate metabolism within glutamate-scavenging cells is indeed vital to prevent excitotoxicity. In astrocytes, glutamate is rapidly converted into glutamine by GS (Martinez-Hernandez et al. 1977; Riepe and Norenburg 1977) localized in the vicinity of glutamatergic synapses and EAATs (Derouiche and Frotscher 1991; Derouiche et al. 1996; Derouiche and Rauen 1995; Norenberg 1979; Norenberg and Martinez-Hernandez 1979). This subcellular colocalization of GS and the EAATs at the glutamatergic synapse allows immediate conversion of transported glutamate into glutamine, thus allowing astrocytes to provide glutamine to neurons later on. This also avoids any interference of intracellular glutamate accumulation on EAAT transport efficacy.

Glutamine is then secreted by astrocytes through different transport systems (Broer et al. 1999; Chaudhry et al. 1999; Gu et al. 2000) and taken up into neurons by the system A transport system (Varoqui et al. 2000). This coupling of glutamine synthesis and glutamine traffic from glia to neurons permits glutamate passage in the extracellular milieu in a non-neuroactive form (glutamine) thus avoiding toxicity (for review, see Broer and Brookes 2001; Fig. 1). Neurons then hydrolyse glutamine into glutamate and ammonia via the mitochondrial phosphate-dependent glutaminase (for review, see Daikhin and Yudkoff 2000) and store glutamate in vesicles by using the VGLUT transporters (Fremeau et al. 2002; Takamori et al. 2000).

EAAT expression and function are finely regulated in normal and pathological conditions

In the normal CNS, only astrocytes coexpress EAATs and GS and, accordingly, are the cellular support of the

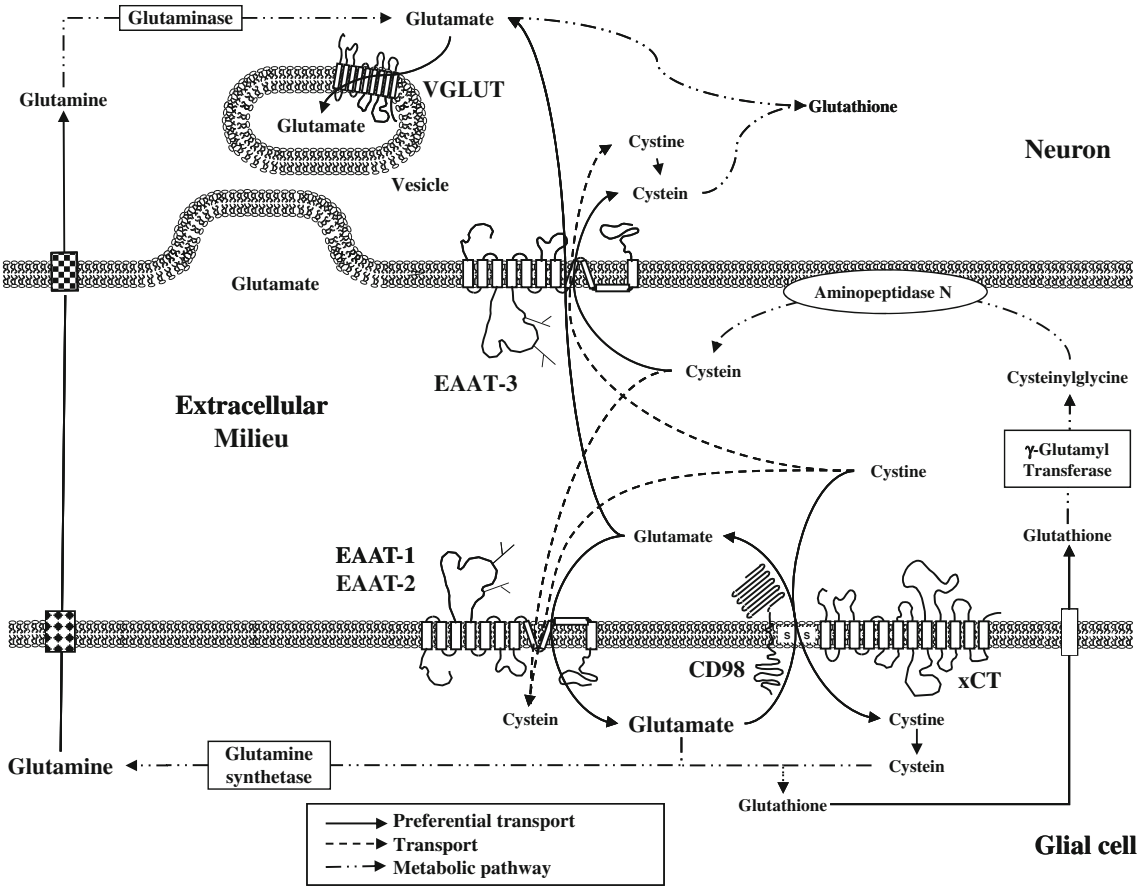

Fig. 1 Glutamate–glutamine cycle and relations with cysteine and glutathione metabolism in the brain. The glutamate–glutamine cycle (*left*) is based on the capture of neuron-released glutamate by glial cells, its metabolism into the non neuroactive amino acid glutamine, and the supply of glutamine to neurons thus avoiding excitotoxicity. Glutamine is then used by neurons for glutamate synthesis and its storage into presynaptic vesicles. The glutamate concentration gradient ensured by glial EAATs drives cystine uptake into glial cells through the CD98-xCT antiporter and thus regulates glutathione synthesis (*lower right*). Secreted glutathione serves as a source of cysteine in the neuron vicinity, where it is taken up by EAAT-3 for neuronal synthesis of glutathione (*upper right*)

glutamate–glutamine cycle. Moreover, Na^+-dependent transport of glutamate into astrocytes upregulates their glucose utilization as well as the production of lactate, coupling energetic metabolism and trophic function to the synaptic activity (Voutsinos-Porche et al. 2003). Likewise, EAAT functioning is itself tuned by glutamatergic synapse activity through mGluR5a on reactive astrocytes (Vermeiren et al. 2005). The physiological activity of astrocytes as the main glutamate scavenging cell type in the CNS is thus finely controlled and regulated by glutamatergic transmission itself. Nevertheless, several in vitro studies have shown that EAAT expression and function in astrocytes are reduced in a variety of pathological conditions. The main proinflammatory cytokine, TNF-α, inhibits glutamate clearance capacity (Fine et al. 1996) and decreases EAAT2 expression in astrocytes (Sitcheran et al. 2005), maybe under the influence of AEG-1 (Kang et al. 2005). Likewise, endothelins reduce astrocyte capacity to clear extracellular glutamate (Rozyczka et al. 2004). Apart from immune mediators, viruses and viral proteins can also directly impair astrocyte ability to clear glutamate like for instance shown for HIV and its coat protein gp120 (Dreyer and Lipton 1995; Kort 1998; Patton et al. 2000; Vesce et al. 1997). On the other hand, trophic or suppressive factors such as EGF, TGFα, FGF-2, and PDGF may increase Na^+-dependent glutamate transport by astrocytes (Figiel et al. 2003; Rozyczka et al. 2004). The suppressive molecule prostaglandin E2 indeed increases EAAT function, an effect that is amplified by IL-1β and inhibited by IL-6 (Okada et al. 2005). A second order of regulation thus appears for glutamate transport by astrocytes, which responds to inflammatory mediators beside its physiological one by glutamatergic transmission, adding complexity to an already scarcely understood scheme. In vivo too, astrocytes appear impaired in a variety of pathological conditions such as SIV encephalitis (Meisner et al. 2008),

controlled cortical impact (van Landeghem et al. 2001) or facial nerve axotomy (Lopez-Redondo et al. 2000).

Microglial cells and macrophages inducibly express EAATs and GS

In these pathological situations, a number of studies have now shown that microglia and/or brain macrophages do express the glial glutamate transporters EAAT1 and EAAT2 as well as GS (Chretien et al. 2002; Lopez-Redondo et al. 2000; van Landeghem et al. 2001). So do they in other human diseases too (Chretien et al. 2004; Gras et al. 2003; Vallat-Decouvelaere et al. 2003), indicating that regulation mechanisms in astrocytes and mononucleated phagocytes of the brain may be totally different with constitutive expression of EAATs and GS in astrocytes and an inducible pattern of expression in the latter.

This inducible expression of EAATs and GS by mononucleated phagocytes may correspond to a compensation of the altered astrocyte functioning, conferring to microglia and macrophages the very same protective and trophic abilities described for astrocytes. It can also correspond to an adaptation of microglia and macrophages to particular states of activation during which they utilize extracellular glutamate for enhancing their ability to produce the antioxidant tripeptide GSH through the coexpression of EAATs and of the cystine/glutamate antiporter (Gras et al. 2006; Persson et al. 2006, 2007; Rimaniol et al. 2001). The cystine/glutamate antiporter is a heterodimeric transporter protein including the CD98/4F2 heavy chain and the xCT light chain, the latter conferring substrate specificity. It exchanges extracellular cystine for intracellular glutamate (Bannai 1986), and is the main transporter allowing cystine uptake in macrophages (Rimaniol et al. 2001). In this context, EAAT-mediated glutamate uptake maintains a high glutamate concentration gradient over the cell membrane even if extracellular glutamate concentration rises. This gradient stimulates cystine uptake and GSH synthesis, even though competition for uptake occurs. This mechanism has been demonstrated in both Müller cells (Reichelt et al. 1997) and macrophages (Rimaniol et al. 2001; Fig. 1).

These two possible physiological functions for microglia/macrophage expressed EAATs are not mutually exclusive, and each might have its own importance depending on the context and the precise location of the EAAT-expressing cells. Indeed, EAAT regulation not only differs between astrocytes and microglia or macrophages. Further differences also exist between macrophages and microglia although some common features are striking. This may be linked to the particular nature of microglia among other cells of the mononuclear phagocyte system.

The origin of microglia: still a debate

During embryonic life myeloid cells migrate from the yolk sac to the developing CNS where they proliferate and establish microglia (Alliot et al. 1999). Beside, numerous studies have shown that during postnatal life, an afflux of monocytes continuously seeds the microglia through the blood–brain barrier (BBB) (Perry et al. 1985). The precise contribution of monocyte emigration (Geissmann et al. 2003) versus local proliferation (Lawson et al. 1992) to the microglial cell population is still not clear even though both perivascular macrophages (Kida et al. 1993) and parenchymal microglia (Lawson et al. 1992) now appear to be long-lived cell populations. Although they are indeed the macrophages of the CNS, microglia do differ in many aspects from other peripheral organ macrophages. Their morphological and functional specificities respond to cell–cell contacts and secreted factors provided by surrounding astrocytes and neurons, as well as strict separation from blood thanks to the BBB (reviewed in Ransohoff and Perry 2009).

Microglia are subjected to constant repression by their microenvironment

Many of the macrophage expressed antigens such as, CD45, CD4 or major histocompatibility complex (MHC) molecules are absent from basal microglia repertoire or expressed at very low levels. This may be a consequence of specificities of the CNS microenvironment that are still under investigation.

First, the BBB isolates microglia from serum proteins, some of which are potent activators (Adams et al. 2007). Then, suppressor factors such as TGFβ and PGE2 are present at higher concentrations in the CNS than in the periphery. Of note, PGE2 increases EAAT2 gene expression level by 15-fold and that of xCT by fivefold in human macrophages (Porcheray et al. 2006). Astrocytes actively contribute to the peculiar microglial phenotype: they provide signals that contribute to NF-κB and class II MHC down modulation, and they suffice in inducing a shift in macrophage phenotype and function toward microglia-like ones (Leone et al. 2006; Rosenstiel et al. 2001; Schmidtmayer et al. 1994; Sievers et al. 1994). Nevertheless, neuron-specific signals also appear to be major players in microglia repression. Even though

it is not necessary for monocyte migration toward the CNS, neuron-expressed fractalkine (CX_3CL1) contributes to repression (Cardona et al. 2006), concordant with the proposal that microglia precursor in the blood may be the $CCR2^-/CX_3CR1^+$ monocyte subpopulation (Geissmann et al. 2003; Tacke and Randolph 2006). Neuronal CD200 and other receptor–ligand pairs such as SIRPα/CD47 also signal microglia inhibition through ITIM bearing receptors (reviewed in van Beek et al. 2005).

Despite this repression microglia settle in an activated surveillant state in healthy CNS

In vivo imaging studies recently demonstrated that the fine processes of microglia continually palpate and monitor their local microenvironment (Davalos et al. 2005; Nimmerjahn et al. 2005; Raivich 2005), which led to change the previous "resting microglia" concept for the better adapted "surveillant microglia" (reviewed in Hanisch and Kettenmann 2007; Ransohoff and Perry 2009). Consistent with this view is the recent demonstration by Ponomarev et al. (2007) that steady-state microglia produce IL4 and express the alternative activation markers Ym1 and FIZZ1. These authors also showed that Ym1 expression is dependent upon local IL4 expression in vivo. Surveillant microglia may nevertheless not strictly correspond to alternatively activated macrophages. The mannose receptor CD206 as well as CD163, typical markers for type 2a activation by IL4/IL13 (Stein et al. 1992) and type 2c activation by IL10 or glucocorticoid (Buechler et al. 2000; Hogger et al. 1998; Kodelja and Goerdt 1994), respectively, are indeed not expressed by parenchymal microglia although present on perivascular macrophages (Fabriek et al. 2005; Galea et al. 2005). Of note, this difference is probably due to the specific CNS microenvironment, since in vitro, the microglial cell line BV2 as well as primary mouse microglia do express the mannose receptor together with arginase and Ym1 upon IL4/IL13 activation (Colton et al. 2006). This emphasizes not only the specificity of CNS tissue conditions, but also the fact that microglia activation may be very plastic as shown for macrophages (Porcheray et al. 2005; see Fig. 2).

Beside their basal IL4 activation, adult parenchymal microglia express steroid hormone receptors (Sierra et al. 2008) and they may recognize lipid-rich tissue fragments and apoptotic cells. In vitro, the phagocytosis of apoptotic neurons induces the same anti-inflammatory activation process in microglia as in macrophages (De Simone et al. 2003, 2004; Liu et al. 2006; Magnus et al. 2001), leading to type 2c activation as well as the expression of nerve growth factor (De Simone et al. 2003). The receptors needed for recognition and phagocytosis of apoptotic bodies are thus expressed by parenchymal microglia, including TREM2, a critical mediator of this anti-inflammatory programme (Takahashi et al. 2007).

Microglia undergo activation programmes that not only resemble those of macrophages but also respond to CNS specific stimuli

It has been for long known that microglia can enter classical activation pathways in different pathologies (reviewed in Hanisch and Kettenmann 2007; Ransohoff and Perry 2009) and express typical proinflammatory factors. Effectors of anti-inflammatory activation programmes are nevertheless also found induced in a variety of conditions. For example in EAE, microglial expression of IL4 and Ym1 is increased (Ponomarev et al. 2007). Likewise, in Alzheimer's disease (AD) as well as in different transgenic models of AD, alternative activation markers are found expressed in response to amyloid plaques (Colton et al. 2006; Jimenez et al. 2008). The precise knowledge of the spatial and temporal mechanisms that underlie microglial activation in CNS diseases and their outcome in term of neuron survival will be critical for the future. In addition to their macrophage-like functions, microglia are part of the glia and they are accordingly sensitive to neurotransmitters such as glutamate through their expression of a variety of neurotransmitter receptors (reviewed in Pocock and Kettenmann 2007). They express different classes of glutamate receptors and respond to fluxes in extracellular glutamate leading to either neurotoxic or neuroprotective outcomes (Byrnes et al. 2009; Kaushal and Schlichter 2008; Loane et al. 2009; Taylor et al. 2003). Likewise, microglia are sensitive to nucleotides/nucleosides such as ATP or ADP released by neurons. The metabotropic purine receptor P2Y12 signals microglial process movements in response to injury (Nimmerjahn et al. 2005; Raivich 2005), while P2Y6 plays a role in phagocytosis (Koizumi et al. 2007). These specific features of microglial cells compared to macrophages may explain why EAAT expression and function may also differ in these two cell types.

Glutamate metabolism in microglia and macrophages are different although they share common features

TNF-α induces EAAT function in differentiating monocytes (Rimaniol et al. 2000). On the other hand, TNF-α, induced in murine microglia by LPS (Jacobsson et al. 2006; O'Shea et al. 2006; Persson et al. 2005) increases EAAT2 expression and function, leading to neuronal protection in vitro. Likewise, LPS and TNF-α also increase EAAT expression and function in human monocyte-derived

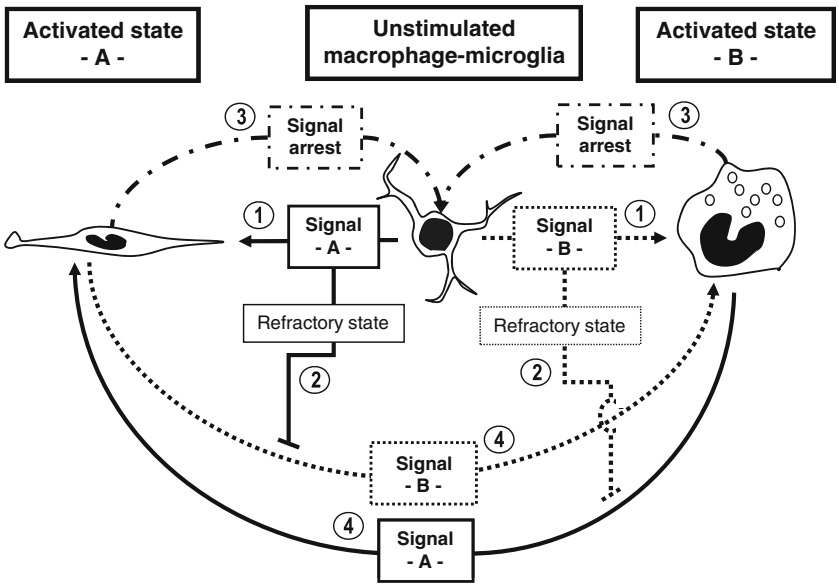

Fig. 2 Macrophage–microglia activation plasticity. Upon specific stimulation, unstimulated macrophage–microglia acquire specific activated phenotype and functions (*1*), and this stimulation entails a refractory state to other differential activators (*2*). On signal arrest, activated macrophages rapidly revert to their initial state (*3*), and even become sensitive to "counter" stimulation for a rapid and efficient activation switching (*4*)

macrophages (MDM) (Porcheray et al. 2006). Not all proinflammatory signals have such a consistent effect of EAAT expression and function in microglia and macrophages. Indeed, interferon-γ (IFN-γ) increases microglial glutamate uptake (Shaked et al. 2005), whereas it has no effect on human MDM (Porcheray et al. 2006).

Anti-inflammatory mediators may also modulate microglia and macrophage ability to express EAATs. Jacobsson et al. (2006) found that corticosterone inhibits EAAT2 expression and function in murine microglia, probably by inhibiting the positive autocrine action of TNF-α. Likewise, corticosterone decreased glutamate clearance capacity in hippocampal glial cultures (Brooke and Sapolsky 2003), which suggests deleterious activities of anti-inflammation from this point of view. Alternatively, in our model of human MDM, dexamethasone is the most potent inducer of EAAT1 gene expression and increases glutamate uptake with an effect close to the one of TNF-α (Porcheray et al. 2006). This difference between human MDM and murine microglia may be due to differences in TNF-α sensitivity as our MDM do not secrete detectable TNF-α when differentiated.

Another interesting point is that dexamethasone and TNF-α, that similarly increase glutamate uptake capacity of human MDM, have opposite effects on xCT gene expression. TNF-α indeed increases xCT gene expression level by more than one log while dexamethasone decreases it by about 80% (Porcheray et al. 2006). These two factors may thus differentially influence the interplay between EAATs and the cystine/glutamate antiporter, which is critical to antioxidant defence and GSH synthesis regulation in the presence of extracellular glutamate. This suggests that TNF-α and the other factors that increase both EAATs and xCT expression levels may induce an antioxidant activation programme, whereas dexamethasone would rather lead to glutamate clearance and maybe the production of glutamine. As a consequence, TNF-α and dexamethasone could accordingly constitute two kinds of stimuli modeling the two main functions that microglia and macrophages may acquire when they express EAATs.

Conclusion

A number of studies have now shown, in a variety of in vitro and in vivo paradigms, that both microglia and macrophages express the molecular effectors of the glutamate–glutamine cycle upon induction. Nevertheless, our understanding of the physiological significance of this feature remains partial. It might correspond to antioxidant defence mechanisms as well as to trophic and neuroprotective abilities to counterbalance altered astrocyte functions (see Fig. 1). From this point of view, EAAT expression and function should not be considered alone but rather with a larger view integrating the other actors that interplay with them such as, the cystine/glutamate antiporter and the glutamine synthetase. The intricate regulation of these mechanisms, which can only be glimpsed based on the available data, is supported by the very complex nature of microglial cell and macrophage activation, two closely related cell types that not only share common fate but also exhibit true specificities.

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