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REVIEW ARTICLE

Toward a new role for plasma membrane sodium-dependent glutamate transporters of astrocytes: maintenance of antioxidant defenses beyond extracellular glutamate clearance

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Abstract The primary function assigned to the sodium-dependent glutamate transporters, also known as excitatory amino acid transporters (EAATs), is to maintain the extracellular glutamate concentration in the low micromolar range, allowing glutamate to be used as a signaling molecule in the brain and preventing its cytotoxic effects. However, glutamate and cyst(e)ine, that is also a substrate of EAATs, are also important metabolites used for instance in the synthesis of the main antioxidant glutathione. This review describes the evidence suggesting that EAATs, by providing glutathione precursors, are crucial to prevent oxidative death in particular cells of the nervous system while being dispensable in others. This differential importance may depend on the way antioxidant defenses are maintained in each cell type and on the metabolic fate of transported substrates, both being probably controlled by EAAT interacting proteins. As oxidative stress invariably contributes to various forms of cell death, a better understanding of how antioxidant defenses are maintained in particular brain cells will probably help to develop protective strategies in degenerative insults specifically affecting these cells.

Keywords Astrocytes · EAATs · Glutathione · Oxidative stress · Ischemia · Parkinson's disease

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Introduction

Glutamate is an important signaling molecule in mammalian brain, being not only the main excitatory neurotransmitter but also a potential gliotransmitter. Moreover, glutamate is a crucial metabolite involved in different cycles between neurons and astrocytes important for neuronal viability and functions. Finally, at high extracellular concentrations, glutamate is a potent cytotoxin able to induce not only neuronal death but also glial cell death through either excitotoxicity or oxidative glutamate toxicity. To allow glutamate to exert its signaling and metabolic functions, as well as to prevent its toxicity, extracellular glutamate concentrations are maintained below the low micromolar range by secondary active transport mediated by the sodium-dependent plasma membrane glutamate transporters, also named excitatory amino acid transporters (EAATs), mainly those found on astrocytes.

Classically, the function of EAATs in astrocytes is seen in terms of extracellular glutamate clearance. Our work contributed to demonstrate that they are also required for maintenance of antioxidant defenses and prevention of oxidative cell death by providing intracellular precursors of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH), the main brain antioxidant. At first, the different functions of the amino acid glutamate in the brain shall be reviewed, followed by the structure, mechanism and inhibitors of EAATs. The final part of this review is dedicated to the description of the relative role played by plasma membrane glutamate transporters either sodium-dependent (EAATs) or independent (system x_c^-) in maintenance of antioxidant defenses in astrocytes. For further details on the nomenclature of glutamate transporters and an overview of their respective functions refer to the editorial by Had-Aissouni (2011) and also to Fig. 1. The

function of these transporters in maintenance of neuronal antioxidant defenses are detailed by Aoyama et al. (2011) and Lewerenz et al. (2011) while Persson and Rönnbäck (2011) and Gras et al. (2011) concentrated on their role in maintenance of microglia and macrophages antioxidant defenses, respectively. Conrad and Sato (2011) further detailed the structure, regulation and function of system x_c^- particularly in non-neural cells.

Glutamate, much more than a simple neurotransmitter

Glutamate as a neurotransmitter

Nowadays and since the 80s, glutamate is considered as the main excitatory amino acid neurotransmitter in the mammalian brain (Fonnum 1984). The first description of its depolarizing action was made by Curtis et al. (1959). Full acceptance of its transmitter role required a further 20 years, mainly because its widespread effects argued against its specificity (Watkins 2000). Other excitatory amino acids, such as aspartate, have also been suspected to be neurotransmitters (Robinson and Coyle 1987), but their role in neurotransmission was ruled out by the fact that only glutamate is a substrate for vesicular uptake (Hisano 2003; Naito and Ueda 1985). In glutamatergic neurons, glutamate is mainly synthesized from glutamine (Bak et al. 2006; Hamberger et al. 1979) as de novo synthesis from glucose is limited by the lack of pyruvate carboxylase (EC 6.4.1.1; Hassel 2000; Shank et al. 1985). Then, glutamate is stored into synaptic vesicles through transport by specific vesicular transporters of the VGLUT family (Bellocchio et al. 2000; Takamori et al. 2000; Takamori 2006). Upon depolarization of active glutamatergic neurons, glutamate is released into the synaptic cleft by calcium-dependent exocytosis from synaptic vesicles of the presynaptic terminals (Ghijsen et al. 2003; Tibbs et al. 1989). Glutamate exerts its fast excitatory effects on postsynaptic neurons through binding to ligand-gated (ionotropic) glutamatergic receptors of NMDA and non NMDA (AMPA/kainate) subtypes (named after preferential agonists; Lodge 2009) and a modulatory effect by acting on G-protein coupled (metabotropic) glutamatergic receptors (Conn 2003). Glutamate may also diffuse outside the synaptic cleft and affect extrasynaptic receptors of the postsynaptic neurons (Sem'yanov 2005) as well as glutamatergic receptors found presynaptically (Pinheiro and Mulle 2008), both regulating the efficiency of synaptic transmission. It is worth noting that specific scaffolding proteins containing PDZ or EVH domains, which interact with the intracellular C-terminal part of the receptors or their subunits, control the distribution of glutamate receptors in the different neuronal membrane compartments and may assemble different complexes determining glutamate receptor signaling properties (Bockaert et al. 2004; Feng and Zhang 2009; Newpher and Ehlers 2008). Another level of complexity is due to the fact that glutamate spillover may also contribute to synaptic crosstalk between adjacent synapses (Huang

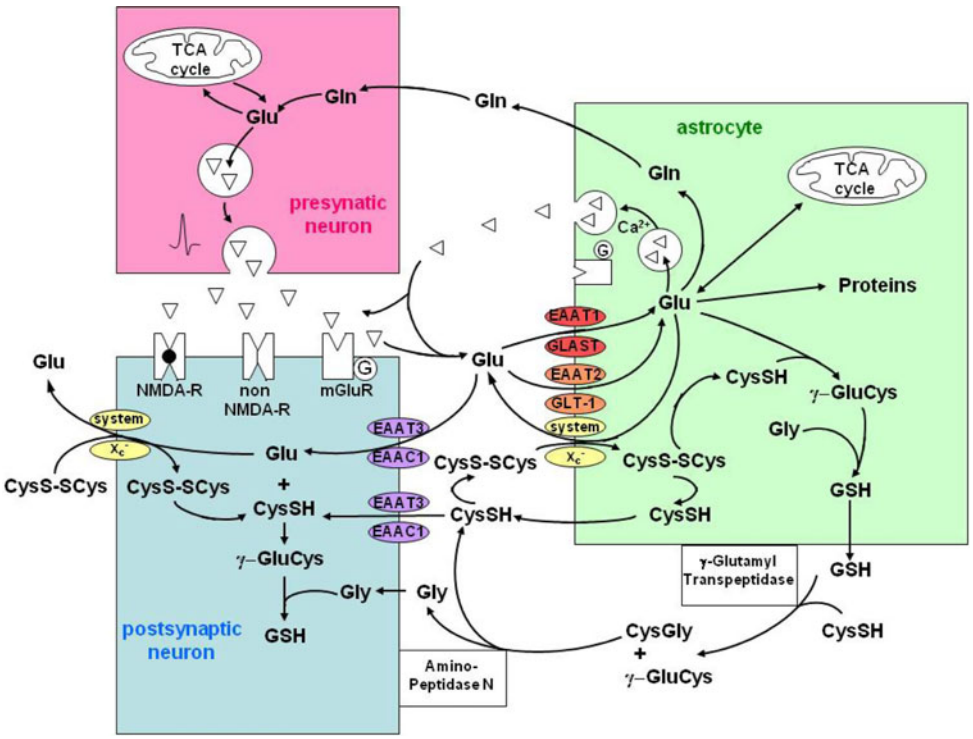

Fig. 1 EAATs and system x_c^- in glutamate and glutathione precursors cycling between neurons and astrocytes. The different cycles and the relative contribution of each transport system are described in the text

and Bergles 2004; Kullmann et al. 1996). Finally, glial cells (astrocytes, oligodendrocytes and microglia), that are the neuron-supporting cells in the brain parenchyma, also possess different types of glutamatergic receptors either ionotropic or metabotropic (D'Antoni et al. 2008; Gallo and Ghiani 2000; Verkhratsky and Kirchhoff 2007), so that they can sense the neuronal activity. Of functional importance for glutamatergic transmission are the perisynaptic astrocytes processes that are in close apposition to glutamatergic synapses. First, glutamate volume transmission depends on their synaptic coverage. In synapses completely wrapped by these glial processes only wired transmission is allowed while glutamate spillover may lead to synaptic crosstalk when the glial coverage is less complete (Oliet et al. 2004; Piet et al. 2004). Second, they are the main contributors to glutamate inactivation and recycling, through the glutamate-glutamine cycle (see below). Third, they seem to be able to mediate bi-directional communication with the neuronal synaptic elements so that glutamatergic synapses are now considered as tripartite (see below; Araque et al. 1999).

Glutamate as a gliotransmitter

Even still controversial, several recent evidences suggest that glutamate may also be a gliotransmitter. In parallel to the neuronal network, astrocytes, the main neuron supporting cells in the brain, form also a network where cells are not coupled through synapses but by gap-junctions (Brightman and Reese 1969) made primarily of connexin 43 (Dermietzel et al. 1989; Rouach et al. 2002). Under in vitro conditions, astrocyte activation, by mechanical stimulation or glutamate receptors agonists, induces calcium wave propagation in the astrocyte network (Charles et al. 1991; Cornell-Bell et al. 1990; Scemes and Giaume 2006) and stimulates the release of different sets of neuroactive substances that may impact on neuronal activity (Parpura et al. 1994; Volterra and Meldolesi 2005). This suggests that the astrocyte network is also able to process information in the brain although at a lower speed than neurons (Hansson and Ronnback 2003; Hertz et al. 2001; Verkhratsky 2009). Among the neuroactive substances that can be released by astrocytes, glutamate may have a crucial role in the crosstalk between the neuronal and the astrocytic networks (Parpura et al. 1994). In astrocytes, the high expression of pyruvate carboxylase enables de novo synthesis of glutamate from glucose (Shank et al. 1985). This glial synthesized glutamate is thought to be rapidly transformed into glutamine, a non neuroactive amino acid, which is then released to neurons as a precursor for neuronal glutamate (Hertz et al. 1999). This glutamate–glutamine cycle may also be supported by direct transport of the released neurotransmitter glutamate into astrocyte processes (see below). Finally, other precursors may also be released by astrocytes to sustain neuronal glutamate synthesis (Maciejewski and Rothman 2008). According to their high capacity to metabolize glutamate, astrocytes are thought to have very low cytoplasmic glutamate concentrations (Attwell et al. 1993). However, other authors have found intracellular glutamate concentrations in astrocytes to be comparable to the cytoplasmic neuronal ones (Longuemare et al. 1999) and astrocytes to be able to release glutamate through different channels or transport systems (volume sensitive organic anion channels, reverse operation of EAATs, gap-junction hemi-channels, system x_c^-; Agulhon et al. 2008). Calcium-dependent exocytosis of glutamate from astrocytes has also been shown (Araque et al. 2000; Montana et al. 2004). Indeed, astrocytes surprisingly do possess the whole machinery necessary for such a regulated release (VGLUT, SNARE proteins, synaptotagmin; Montana et al. 2006). In these conditions, the astrocyte processes that surround the synapse are able to listen to the synapse due to the presence of glutamatergic receptors, and it is very likely that they are also able to talk to the synapse by releasing glutamate through calcium-regulated exocytosis. Therefore, glutamate is now not only considered as a neurotransmitter but also as a gliotransmitter, even if in vivo evidence for such a function is still lacking.

Glutamate as the main brain metabolite

Years of research on glutamatergic transmission has obscured the fact that glutamate in the brain may have other functions than just being a signaling molecule. Glutamate is not only the main excitatory amino acid; it is simply the main amino acid, glutamate concentration being above all of that of other amino acids in the brain (Krebs et al. 1949). Moreover, most of the glutamate is found intracellularly, not only in glutamatergic terminals but also in neuron and glia cytoplasm (Montana et al. 2006), suggesting an important metabolic role. Indeed, the first function attributed to glutamate by Krebs (1935) was a role in ammonia detoxification through its transformation into glutamine. Transformation of glutamate into glutamine is now known to occur only in astrocytes (Martinez-Hernandez et al. 1977), while transformation of glutamate into GABA only occurs in GABAergic neurons (Buddhala et al. 2009; Martin and Rimvall 1993) and both neurons and astrocytes may use glutamate as an energetic substrate or for peptide and protein synthesis (McKenna 2007). Among all these metabolic fates of intracellular glutamate, the use of glutamate for the enzymatic synthesis of the tripeptide GSH, the main brain antioxidant molecule (Dringen 2000), may be of crucial importance for cell viability. Intracellular glutamate may have a dual role in GSH synthesis first, as a direct substrate (Machiyama et al. 1970) and second, by

being released via the transport system x_c^- in exchange of cystine (Bannai 1986), cystine being the preferred source of cysteine for GSH synthesis in astrocytes (Cho and Bannai 1990; Kranich et al. 1998).

Glutamate as a potent cytotoxin

Finally, glutamate is not only a transmitter, a signaling molecule and an important metabolite; it is also a potent toxin that may trigger different cytotoxic processes at high extracellular concentrations. Glutamate may trigger neuronal death through excitotoxicity, i.e. overstimulation of glutamatergic receptors, mainly those of the NMDA subtype that are located extrasynaptically (Hardingham et al. 2002). Indeed, opposite to synaptic NMDA receptors whose activation promotes neuronal survival, activation of extrasynaptic NMDA receptors trigger pro-death signaling (Gouix et al. 2009; Hardingham et al. 2002; Ivanov et al. 2006; Papadia and Hardingham 2007). This signaling dichotomy may be linked to interaction with different scaffolding proteins (Yi et al. 2007). Glutamate may also trigger oligodendrocyte death through overstimulation of AMPA receptors (Matute et al. 1997, 2007) while other glial cell types, particularly astrocytes, do not seem to be sensitive to excitotoxicity, unless under particular conditions of low desensitization of AMPA receptors (David et al. 1996; Kovacs et al. 2002). However, astrocytes may be sensitive to another form of glutamate toxicity, i.e. the oxidative glutamate toxicity. When extracellular glutamate concentration is high enough to inhibit cystine transport by system x_c^-, the subsequent decrease of intracellular cysteine availability for GSH synthesis will ultimately lead to oxidative cell death of astrocytes when cultured in the absence of neurons (Cho and Bannai 1990), but not of astrocytes in mixed neuron-astrocyte cultures (Murphy et al. 1989). This oxidative glutamate toxicity has also been shown to affect immature neurons and neuronal cell lines (Had-Aissouni et al. 2002; Murphy et al. 1989, 1990; Tan et al. 2001; Tobaben et al. 2011).

Regulation of glutamate functions by glutamate uptake

Extracellular glutamate concentrations are maintained below the low micromolar range by secondary active transport through EAATs. This is important to maintain the phasic aspect of neurotransmission, shape the time course of synaptic receptor activation, as well as to prevent glutamate spill-over, extrasynaptic receptor activation, synaptic cross-talk, excitotoxicity and oxidative glutamate toxicity (Danbolt 2001). EAATs use the ionic gradient maintained by the sodium/potassium pump to transport glutamate against its concentration gradient (see below). Five distinct glutamate transporters, now classified in the solute carrier 1A (SLC1A) family, EAAT1/glutamate-aspartate transporter (GLAST)/SLC1A3, EAAT2/glutamate transporter-1 (GLT-1)/SLC1A2, EAAT3/excitatory amino acid carrier 1 (EAAC1)/SLC1A1, EAAT4/SLC1A6 and EAAT5/SLC1A7, have been cloned from mammalian tissues (Arriza et al. 1994, 1997; Fairman et al. 1995; Kanai and Hediger 1992; Pines et al. 1992; Storck et al. 1992) and shown to be differently expressed in brain cells. Astrocytes express both EAAT1/GLAST and EAAT2/GLT-1 subtypes (Lehre et al. 1995). Most of the neurons express the EAAT3/EAAC1 subtype post-synaptically (Rothstein et al. 1994) while GLT-1 or a splice variant may be the presynaptic neuronal transporter at least in some neurons of the brain (Chen et al. 2002, 2004; Holmseth et al. 2009). EAAT4 and EAAT5 are only expressed by a subset of neurons, EAAT4 in the cerebellum (Yamada et al. 1996) and EAAT5 in the retina (Pow and Barnett 2000). Transport of glutamate into astrocyte processes is responsible for the bulk clearance of the neurotransmitter glutamate from the synaptic cleft (Rothstein et al. 1996). The main neuronal transporter, EAAC1, may exert a more subtle control on glutamatergic transmission by shaping the time course of postsynaptic receptor activation (Diamond 2001). It can also supply GABAergic neurons with glutamate for GABA synthesis (Sepkuty et al. 2002). Into astrocytes, transported glutamate is thought to be the more efficient substrate to feed the glutamate-glutamine cycle and to recycle the neurotransmitter pool (Hertz et al. 2007). However, the glutamate-glutamine cycle is not stoichiometric. As stated above, glutamate may serve as an energetic substrate and/or as a precursor for the synthesis of different sets of molecules and proteins in glia as well as in neurons (McKenna 2007). At odds with the classical view that EAATs are only required to buffer extracellular glutamate, we have contributed to the view that they are also required for maintenance of antioxidant defenses and prevention of oxidative cell death by providing intracellular precursors of GSH, the main brain antioxidant molecule (Re et al. 2003, 2006; Nafia et al. 2008).

EAAT structure, mechanism of transport and inhibitors

To unveil the importance of glutamate transporters for cell viability and maintenance of antioxidant defenses, a pharmacological approach was chosen, with different sets of glutamate transporter inhibitors being used. To better understand the different ways by which inhibitors may differently impair glutamate uptake, it is necessary to have a closer look at the functional properties of glutamate transporters.

EAAT structure

The structure of the mammalian EAATs may be significantly related to that of their archaea orthologue from Pyrococcus horikoshii (Glt_{ph}) as Glt_{ph} shows 37% sequence identity to human EAAT2 (GLT-1). Its crystallized structure was solved at 3.5 Å resolution (Yernool et al. 2004). In the presence of glutamate or aspartate, the transporter is a trimeric complex forming a characteristic bowl with a large aqueous basin at the bottom of which are located three substrate-binding sites (one within each protomer, each protomer functioning independently). Each substrate-binding site is bordered by two helical hairpins that may function as gates controlling substrate binding from the extracellular and intracellular environment (Gouaux 2009). Indeed, according to the alternating access mechanism that is also supported by functional evidence, glutamate transporters cycle between different conformational states, one of them allowing access of substrates to their binding sites from the extracellular side, the other one allowing access from the cytoplasm (Grewer et al. 2008). The structure is in remarkable agreement with predictions made from previous mutagenesis studies performed on the mammalian transporters, including the existence of the two hairpins previously described as two re-entrant loops (Brocke et al. 2002). The most notable difference between Glt_{ph} and its mammalian orthologues may reside in the number of sodium-binding sites as only two binding sites have been identified in Glt_{ph} while three Na^+ are known to be transported with one glutamate in mammalian transporters (Boudker et al. 2007).

EAAT mechanism of transport

This system demonstrates a high affinity not only for L-glutamate and L- and D-aspartate but also for both cysteine and cystine (Hayes et al. 2005). Under normal conditions, translocation of the substrates across the plasma membrane involves loading the empty protomer with sodium and substrate, followed by closure of the external gate, opening of the internal gate and then release of the substrate and sodium at the intracellular face. Thereafter, K^+ binds to the protomer at the intracellular face and promotes the relocation of the empty binding site for extracellular accessibility. Based on the stoichiometry described above, glutamate transport is electrogenic, i.e. associated with net positive charge transport across the membrane. However, glutamate transporters also behave like ligand-gated chloride channels as glutamate transport is associated with an anion conductance not stoichiometrically coupled to the transport of glutamate but that may help counteract the depolarization triggered by Na^+-co-transport. Using the Na^+ gradient and the negative membrane potential as driving forces, glutamate transporters can concentrate glutamate 5×10^6-fold inside cells under physiological conditions. Assuming an intracellular glutamate concentration of 10 mM, they may be able to achieve an extracellular concentration of 2 nM (Grewer et al. 2008).

For net uptake of glutamate, EAATs have to complete the aforementioned cycle. If the empty transporters do not enter the relocation step and do not complete the cycle, then, they may bind Na^+ and an intracellular substrate at the inside of the cell and translocate back in the opposite direction mediating facilitated substrate exchange, like the ASC (Alanine Serine and Cysteine preferring) transporters, the other members of the SLC1A family (Grewer et al. 2008). Under normal conditions, the exchange component is small compared with the uptake component but disruption of the K^+-coupling will result in glutamate efflux (Kavanaugh et al. 1997). Finally, glutamate transport may be totally reversed. Although the conventional transport direction is inward under physiological conditions, glutamate can also be transported in the outward direction when extracellular $[Na^+]$/intracellular $[K^+]$ decrease and/or intracellular $[Na^+]$/extracellular $[K^+]$ increase such as occurring during ischemic conditions, when impaired Na^+/K^+-ATPase function due to energetic failure leads to the collapse of ionic gradients. The driving force for uptake also decreases under membrane depolarization, so that during intense and repeated stimulations, neuronal membrane depolarization will also result in a reversal of the transport direction (Grewer et al. 2008).

EAAT inhibitors

Historically, a large number of compounds, mostly glutamate and aspartate analogues or derivatives, were tested for possible effects on glutamate transport to define the most probable functions of EAATs in the mechanisms of the excitatory synaptic transmission. Thereafter, glutamate transport inhibitors were more specifically designed to identify the structural and conformational features responsible for binding and translocation as well as to develop glutamate analogs that act selectively among the various EAAT subtypes. Most of the first-generation inhibitors were competitive substrates, inducing both a transport current and a substrate-dependent chloride flux. Competitive substrates possess also the particular property to induce the efflux of endogenous glutamate by a process termed heteroexchange probably by impairing potassium binding and favoring the relocation of the transporter through substrate exchange. Therefore, the use of such compounds usually results in an increase in the extracellular glutamate concentration that complicates the assessment of EAAT function. L-trans-pyrrolidine-2,4-dicarboxylate (PDC) is a conformationally restricted structural analog of glutamate

and the most widely used substrate inhibitor (Bridges and Esslinger 2005; Shigeri et al. 2004). For elucidating some of the intrinsic properties and physiological roles of EAATs, non-transportable inhibitors (blockers) were developed. These recently introduced analogues contain specifically placed substituents that may not impair binding but translocation. DL-threo-beta-benzyloxyaspartate (TBOA) is one of the most potent blocker inhibitors of EAATs identified to date (Bridges and Esslinger 2005; Shigeri et al. 2004). When Glt_{ph} was crystallized with TBOA, it was found that the benzyl group of TBOA prevents the closing of the extracellular gate and the subsequent translocation step (Boudker et al. 2007). Except for dihydrokainate (DHK) a long-known specific, if not very potent, blocker of GLT-1, inhibitors with selectivity toward one particular EAAT subtype were not used in our studies as they were not developed until recently (Dunlop 2006).

As they control extracellular glutamate concentration and therefore glutamate toxicity, EAAT dysregulation of activity and/or expression has been specifically implicated in the pathology of several neurodegenerative conditions such as amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease, Parkinson's disease and ischemic injury. Therefore, several groups have concentrated their efforts to understand the mechanisms that control EAAT expression and activity, including those that regulate gene expression, splicing and post-translational modification, trafficking and interacting proteins. The identification of these mechanisms has aided the development of therapeutic strategies while inhibitors as well as gene inactivation or silencing has helped to understand the role of glutamate transporters during pathology. Blocking EAAT activity or preventing their expression has been useful to delineate their function in synaptic transmission and in preventing extracellular glutamate toxicity, mimicking pathological conditions where down regulation or loss of activity is observed (Beart and O'Shea 2007). Substrate inhibitors may be more useful to probe the intracellular role of transported glutamate. Indeed, glutamate may be synthesized through different metabolic pathways both in neurons and astrocytes (transamination, reverse operation of glutamate dehydrogenase EC 1.4.1.3; Frigerio et al. 2008) so that just blocking glutamate uptake may be compensated for by glutamate synthesis intracellularly. By both inhibiting glutamate uptake and inducing glutamate release by heteroexchange, substrate inhibitors may potentially deplete the intracellular glutamate pool more rapidly, compared with blockers or techniques preventing gene expression. Moreover, by inducing glutamate release through EAATs, substrate inhibitors partly mimic the reverse operation of these transporters that may occur during ischemic insults. Therefore, to study the intracellular consequences of EAAT dysfunction, application of PDC was our pharmacological treatment of choice (Re et al. 2003, 2006; Nafia et al. 2008).

Glutathione metabolism in brain cells

Differential preferences in glutathione precursors between brain cells

GSH is the main brain antioxidant molecule. It can non-enzymatically scavenge radicals and it is also the electron donor for the reduction of peroxides by glutathione peroxidase (EC 1.11.1.9). The product of the oxidation of GSH is glutathione disulfide (GSSG). Glutathione reductase (EC 1.6.4.2) regenerates GSH by transferring reducing equivalents from NADPH to GSSG. GSH is also used in detoxification processes by glutathione-*S*-transferases (EC 2.5.1.18) to form conjugates that are ultimately released from cells. GSH itself may also be released (Dringen 2000). Consumed GSH is replaced by resynthesis. It is a tripeptide composed of glutamate, cysteine and glycine. It is synthesized through two steps by ATP-driven enzymatic reactions. The first reaction is catalyzed by glutamate cysteine ligase (EC 6.3.2.2), also named γ-glutamyl cysteine synthetase, which makes an unconventional peptide bond between the γ-carboxyl group of glutamate and the amine group of cysteine. The second reaction is catalyzed by glutathione synthase (EC 6.3.2.3) which links the carboxyl group of cysteine with the amine group of glycine. Synthesis of GSH is regulated by feedback inhibition of the first reaction by GSH (Richman and Meister 1975).

Among brain cells, astrocytes do posses higher GSH levels than neurons both in vivo and in culture (Dringen 2000). Dringen et al. (2000), using amino acid deprivation and subsequent refeeding with potential GSH precursors revealed striking differences between cultured neurons and astrocytes in their precursor preferences. For instance, mature neurons depend on extracellular cysteine for their GSH synthesis (Kranich et al. 1996) while extracellular cystine is the better donor of GSH cysteine moiety in astrocytes (Dringen and Hamprecht 1996; Kranich et al. 1998). As cysteine is oxidized into cystine in the extracellular milieu, GSH synthesis in neurons is therefore limited by cysteine availability (Kranich et al. 1996). On the contrary, the glutathione content of cultured astrocytes seems to be limited by the availability of glutamate (Dringen and Hamprecht 1996). Aspartate, which may be rapidly converted intracellularly into glutamate by transamination in astrocytes, is the amino acid that is the more efficiently used by these cells to sustain GSH synthesis in absence of glutamate (Dringen and Hamprecht 1996; Kranich et al. 1998) while neurons prefer glutamine (Kranich et al. 1996).

Supply by astrocytes of glutathione precursors to neurons

Interestingly, most neurons, when cultured in the presence of astrocytes, strongly increase their GSH content indicating that astrocytes provide neurons with a cysteine precursor (Sagara et al. 1993). Inhibition of the ectoenzyme γ-glutamyltransferase (γ-GT, also known as γ-glutamyl transpeptidase; EC 2.3.2.2) totally prevents the astrocyte-induced effect on GSH content in neurons (Dringen et al. 1999). In the brain parenchyma, this ectoenzyme is mainly expressed by glial cells (Shine and Haber 1981) where it hydrolyses extracellular GSH and derivatives to form the dipetide CysGly and different γ-glutamyl containing peptides. For instance, the dipeptides CysGly and γ-GluCys are generated from extracellular hydrolysis of GSH in the presence of cystine (Dringen 2000; Fig. 1). Both neurons and astrocytes are able to use these dipeptides as precursors for GSH synthesis although through different mechanisms. Both peptides are taken up intact into astrocytes probably by the peptide transporter PepT2 expressed in these cells (Dringen et al. 1998). While CysGly is hydrolyzed intracellularly to generate cysteine and glycine, γ-GluCys may be used directly as a substrate for glutathione synthetase, bypassing the first step of GSH synthesis in pure astrocyte culture (Dringen et al. 1997, 1998) but apparently not in astrocytes cultured in the presence of neurons (Keelan et al. 2001). Peptide transporters do not seem to be expressed in neurons (Dringen et al. 2001) and hydrolysis of the dipeptides is required before their constituent amino acids may serve as glutathione precursors (Dringen et al. 1999). This hydrolysis is probably performed extracellularly by the neuronal ectoenzyme aminopeptidase N (EC 3.4.11.2; Dringen et al. 2001). Despite this complicated way of using dipetides as GSH precursors, neurons are more efficient in utilizing these compounds than astrocytes (Dringen 2000). Therefore, a metabolic coupling between neurons and astrocytes for GSH metabolism may exist where the GSH synthesized by astrocytes is released through MRP1 (Hirrlinger et al. 2002) and hydrolyzed by γ-GT. The dipeptide CysGly produced in this reaction is cleaved by the neuronal ectoenzyme aminopeptidase N. The amino acids cysteine and glycine generated are subsequently taken up as precursors for neuronal GSH synthesis. The glutamate moiety for neuronal GSH synthesis is also provided by astrocytes under the form of glutamine through the glutamate-glutamine cycle (see above and Fig. 1). Therefore, neuronal glutathione synthesis seems to be strictly under the dependence of glial precursors. Thus, it is of importance to know how glutathione precursors are supplied to astrocytes as GSH synthesis in these cells may determine the antioxidant defenses of most cells in the brain parenchyma.

Supply of glutathione precursors to astrocytes: different possibilities

Only a few studies have addressed this issue. System x_c^-, which transports cystine in exchange of intracellular glutamate with an 1:1 ratio in physiological conditions, was first found to contribute to cystine uptake in pure astrocyte cultures where its inhibition by extracellular excess of glutamate leads to GSH deficiency and oxidative cell death (Cho and Bannai 1990) while astrocytes cultured in presence of neurons were poorly affected by oxidative glutamate toxicity (Murphy et al. 1989). Later on cystine was found to be taken up by different transport systems in astrocytes, including not only system x_c^-, but also glial EAATs (Allen et al. 2001; Bender et al. 2000; Hayes et al. 2005) and a transport system depending on the activity of γ-GT (Allen et al. 2001) that corresponds probably to PepT2 (Dringen et al. 1998). As neuronal factors may induce EAATs expression (Gegelashvili et al. 1997; Schlag et al. 1998), this may explain why astrocytes cultured in the presence of neurons may be less dependent on system x_c^- for their antioxidant defenses than astrocytes in pure cultures. However, in retinal Müller glial cells, the function of EAATs in maintaining GSH levels was shown to be primarily directed at transporting glutamate, which, as shown for astrocytes by Dringen and Hamprecht (1996), seems to be the limiting factor for GSH synthesis in these cells (Reichelt et al. 1997). The hypothesis was that EAAT-transported glutamate is required for the activity of system x_c^- and that when EAAT transport of glutamate is impaired, cystine uptake by system x_c^- is therefore prevented as system x_c^- is an exchange agency. However, the relative importance of x_c^- and EAATs to maintain GSH levels has now been tested in vivo in knock-out mice. There is no GSH deficiency in xCT deficient mice and a novel function has been recently attributed to this transport system (Sato et al. 2005). It may participate in a cystine/cysteine cycle important to maintain the external redox balance and limit lipid peroxidation (Banjac et al. 2008). On the contrary, there is a delayed GSH depletion in brain from mice deficient in the neuronal EAAC1/EAAT3 (Aoyama et al. 2006), and there is a GSH deficiency in retinal glial cells of mice deficient in one of the glial EAAT, EAAT1/GLAST (Harada et al. 2007). Therefore, EAATs may be assumed to be crucial for transport of GSH precursors both in vitro and in vivo. However, the consequences of EAAT dysfunction in term of glial cell viability have been poorly investigated.

Differentiation state selective requirement of EAATs for antioxidant defenses in astrocytes

Differential vulnerability of astrocytes to EAAT dysfunction in vivo

In neurodegenerative insults, either acute (ischemia) or chronic (amyotrophic lateral sclerosis), dysfunction of astrocyte EAATs may trigger neuron death by contributing to the excitotoxic rise in extracellular glutamate concentration (Szatkowski et al. 1990; Trotti et al. 1999). In these insults, it was also shown that astrocyte death may occur and even precede neuron loss and reactive astrogliosis (Bruijn et al. 1997; Liu et al. 1999; Martin et al. 1997). Moreover, Jean-Charles Lievens, in the laboratory, showed that pharmacological alteration of EAAT function, by chronic intrastriatal infusion of the substrate inhibitor of glutamate transport PDC, induced two types of astrocyte responses in the lesion area (Lievens et al. 2000a). There was first a disappearance of the astrocyte markers, glial fibrillary acidic protein (GFAP) and GLT-1, after 3 days (d) of PDC infusion. Thereafter, reactive astrocytes invaded the lesion area despite the presence of PDC, as indicated by the increase in GFAP immunostaining at 14 d. Altogether, these data suggested that the effects of EAAT dysfunction may differ according to astrocyte population, affecting quiescent astrocytes, whereas reactive ones are resistant. This raised the question about the mechanisms involved in astrocyte vulnerability when EAAT function is impaired, as astrocytes are generally resistant to excitotoxicity. To ascertain whether EAAT dysfunction may induce different astrocyte responses and to elucidate the mechanisms responsible for these possible differences, I studied with Diane Re the effects of EAAT dysfunction on pure striatal astrocyte cultures, either differentiated or not (Re et al. 2003, 2006).

In vitro astrocyte differentiation

Pure striatal astrocyte cultures from striata of new-born rats (P0) were set up. The same protocol used during my PhD thesis to obtain pure cortical astrocyte cultures (Had et al. 1993) was applied on striata dissected as described by Robertson et al. (1989). Confluent striatal astrocytes display a flat polygonal shape. If treated at that stage for 1 week with 0.25 mmol/L dibutyryl-cyclic AMP (dBcAMP), a membrane permeant analog of cyclic AMP thought to mimic beta-adrenergic receptor stimulation (Shain et al. 1987), they change their morphology to process-bearing cells. This stellate morphology more resembles that of mature astrocytes in vivo and therefore treated cells are usually referred to as differentiated astrocytes while untreated cells are referred to as undifferentiated ones. It worth noting that this treatment was long considered to induce transformation into reactive astrocytes, as dBcAMP-differentiation was found to be correlated with an increase in GFAP expression (Fedoroff et al. 1984). However, the same group showed that astrocytes expressed a marker of cell cycle arrest when treated with dBcAMP, suggesting they enter a phase of terminal differentiation and become quiescent instead of reactive (Fedoroff et al. 1990). Both types of cultures were characterized by Hayet Aït-Mamar and found to contain more than 95% of GFAP-positive astrocytes.

Differential EAAT expression and activity upon astrocyte differentiation in vitro

Hayet Aït-Mamar also analyzed the expression level of EAATs between undifferentiated and differentiated striatal astrocytes on Western blots using antibodies kindly provided by Pr. B. Danbolt (University of Oslo, Norway). In agreement with data obtained on cortical astrocytes (Gegelashvili et al. 1996; Schlag et al. 1998; Swanson et al. 1997) densitometric analysis showed that GLAST expression level was 1.5-fold higher while GLT-1 expression level was 3.3-fold higher in differentiated astrocytes after 1 week of treatment with 0.25 mmol/L dBcAMP as compared with untreated undifferentiated cells (Fig. 2). Contrary to GLAST, GLT-1 expression did not reach the level of expression found in striatum, suggesting that other factors are needed to fully induce GLT-1 expression in astrocytes and/or that other cell types may express GLT-1 in vivo (Danbolt 2001). Similar results were obtained later on by Zdenek Berger using antibodies from alpha-diagnostic (San Antonio, USA; data not shown). Finally, Hayet Aït-Mamar also performed uptake experiments using [^{3}H]-D-aspartate as substrate, a transportable but non metabolizable analog of glutamate. Saturation curves indicated that V_{max} increased 7.4-fold, from 238 to 1,762 pmol/mg protein/min, upon astrocyte differentiation (Fig. 3), in accordance with the increased expression of GLAST and GLT-1. Eadee-Hofstee plot showed that K_m also increased fourfold, from 16.7 to 74 µmol/L. These results are in agreement with those obtained by Schlag et al. (1998) for cortical astrocytes using [^{3}H]-L-glutamate as substrate. We found this culture model of two different populations of astrocytes, expressing different levels of glutamate transporters and having a different capacity of glutamate transport, with K_m values in the range of the ones obtained in different expression systems (Gegelashvili and Schousboe 1998), suitable to hopefully obtain different effects after pharmacological induction of EAAT dysfunction.

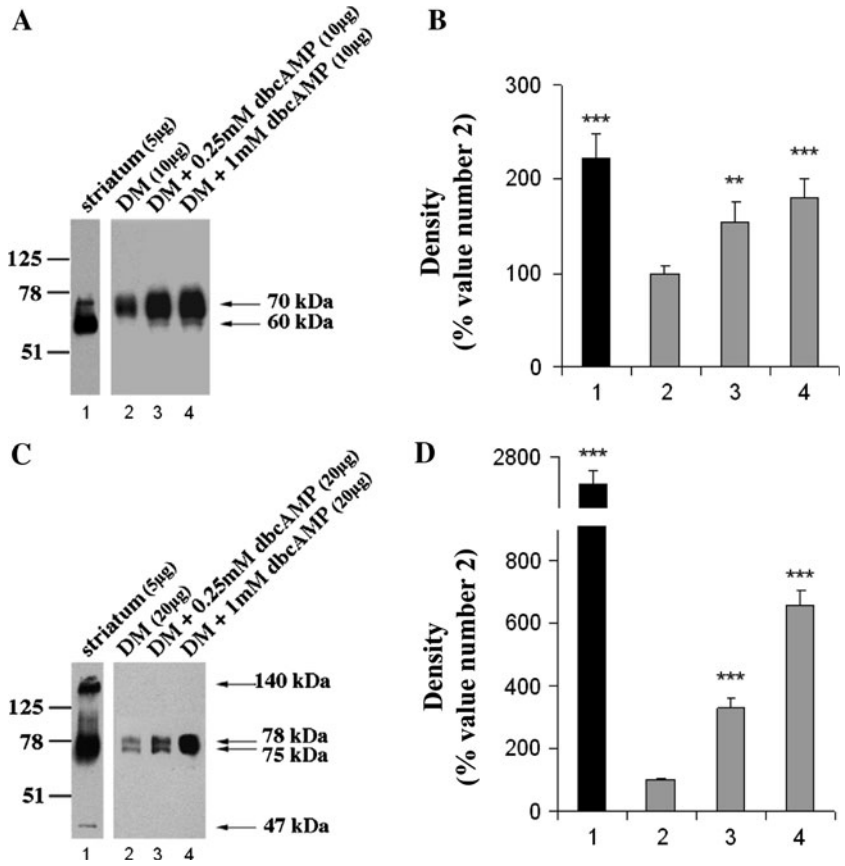

Fig. 2 Expression of GLAST and GLT1 in striatum and undifferentiated or dBcAMP-differentiated astrocyte cultures examined on Western blots. *DM* define medium. Striatal astrocyte cultures either undifferentiated (DM w/o dBcAMP) or dBcAMP-differentiated (DM with 0.25 or 1 mM dBcAMP for 1 week) were obtained as described by Re et al. (2003). Protein samples were prepared from cultured cell pellets, as well as from striatum obtained from adult rats, and Western blots were performed as described by Lievens et al. (2000b). The total amount of proteins used to load each line is indicated in brackets. Transporter expression was quantified by densitometric analysis from three different preparations. **a** The antibodies to C-terminal peptide from GLAST sequence (anti-A522; generous gift from B Danbolt, Oslo, Norway) recognized a wide band that is about 60 kDa and a minor band that is about 70 kDa in striatum, as previously described by Lehre et al. (1995). The relative proportions of these two bands are inverted in striatal astrocytes cultures probably because of differences in glycosylation processes in cultures as compared with brain tissues, as described for cortical astrocytes (Schlag et al. 1998). **b** GLAST expression is increased 1.5 fold by treatment for 1 week with 0.25 mM dBcAMP. **c** The antibodies to N-terminal peptide from GLT-1 sequence (anti-B12; generous gift from B Danbolt, Oslo, Norway) recognized several bands in striatum as described by Lehre et al. (1995). The main immunoreactive band is relatively broad, apparently heterogeneous with maximum density at about 75 kDa. The band at about 140 kDa represents probably homomultimers, which were not dissociated by treatment of the samples with β-mercaptoethanol (Haugeto et al. 1996). The band at about 47 kDa is probably a degradation product. In striatal astrocyte cultures, the anti-B12 antibody recognized a doublet (a band of about 75 kDa and a band of about 78 kDa) that could represent two different GLT-1 isoforms issued from alternative splicing (Chen et al. 2002). **d** GLT-1 expression is increased 3.3 fold by treatment for 1 week with 0.25 mM dBcAMP. $^{***}p < 0.001$ and $^{**}p < 0.01$ as compared with value number two (undifferentiated astrocytes)

Differential vulnerability to EAAT dysfunction upon astrocyte differentiation in vitro

To induce EAAT dysfunction, PDC was chosen. First, PDC has a weak affinity for glutamate receptors (Bridges et al. 1991) and we would like not to interfere with the large set of glutamatergic receptors expressed by astrocytes (Kimelberg et al. 1995). Second, PDC-induced heteroexchange can partly mimic the reverse glutamate uptake that may occur in ischemia and ischemic conditions were known to induce astrocyte death in perinatal striatum (Martin et al. 1997). Third, PDC effects obtained on cultured striatal astrocytes could be directly compared with results obtained in vivo after intrastriatal PDC infusion in adult rats (Lievens et al. 2000a). The consequences of such EAAT dysfunction were analyzed in terms of (1) cell viability, mostly by using MTT reduction assay which monitors primarily mitochondrial activity (Musser and Oseroff 1994); (2) glutamate levels in the culture media and intracellularly using HPLC and finally (3) GSH contents and ROS production in live cells using monochlorobimane and dichlorofluorescein diacetate, respectively, and flow cytometry. The mechanisms

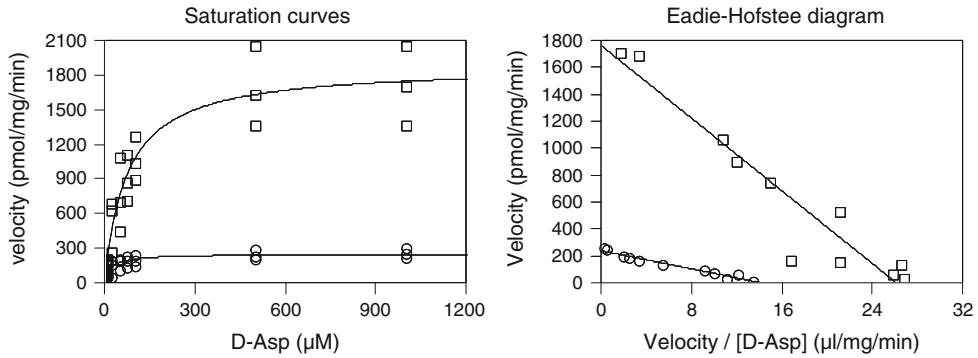

Fig. 3 Determination of uptake parameters. Undifferentiated and dBcAMP-differentiated striatal astrocyte cultures were obtained as described by Re et al. (2003). Uptake experiments were performed as described for neuronal culture by Lortet et al. (1999) except that [^{3}H]-D-aspartate was used as substrate, this non-metabolizable glutamate analog being more suitable to perform uptake experiments in cells that highly metabolize glutamate such as astrocytes. Uptake parameters were determined from three independent experiments using the curve fitting program WinCurveFit (Kevin Raner Software, Victoria, Australia). Undifferentiated astrocytes (*open circles*); differentiated astrocytes (*open squares*)

involved in the death of differentiated astrocytes were assessed using different protective strategies, putative protective drugs being usually applied in co-treatment with PDC, unless stated differently.

Differentiated astrocytes were found to be highly vulnerable to PDC treatment, compared with undifferentiated ones (Re et al. 2003). When applied for at least 12 h, PDC induced a delayed and slow progressive cell loss in differentiated astrocyte cultures (median effective at 6 days and ceiling effect at 14 days) with cells presenting some features of apoptosis such as cell body shrinkage, cell process membrane blebbing and chromatin compaction from 3 days after treatment. On the contrary, PDC did not induce any gross differences in undifferentiated astrocyte morphology or viability, compared with untreated controls at this or subsequent timepoints. Dose effects on differentiated astrocyte viability indicated an EC_{50} of about 250 μmol/L, a concentration corresponding to about ten times the K_m of PDC for glial EAATs expressed in *Xenopus* oocytes (Arriza et al. 1994) and likely to ensure near maximal occupancy of the transporter binding sites and heteroexchange (Koch et al. 1999). As the effects of PDC can be mimicked by other substrate inhibitors of glutamate uptake, such as threo-*β*-hydroxy aspartate, and partly prevented with the blocker TBOA, and as the death of differentiated astrocytes is preceded by and correlated with the increase in extracellular glutamate concentration, therefore, glutamate release by heteroexchange is probably the primary event for differentiated astrocyte death induction (Re et al. 2003, 2006).

Low intracellular glutamate-linked oxidative toxicity in differentiated astrocytes in vitro

Further investigation of the downstream pathways of PDC-induced differentiated astrocyte death showed that it was independent of glutamate receptor stimulation, since it was not protected by any of the antagonists tested (Re et al. 2003). On the other hand, PDC treatment induced a significant GSH depletion, particularly in differentiated astrocytes. Indeed, even if the percentage of depletion was similar in both type of cultures (50–55% depletion 12 h after treatment), the GSH concentration reached after PDC treatment of differentiated astrocytes was two times lower than the one reached in undifferentiated astrocytes due to difference in basal GSH content. In accordance, the death of differentiated cells was found to be oxidative as preceded by an increase in ROS content (+250% at day 1) and protected by the antioxidant molecule *N*-acetylcysteine and the antioxidant enzyme catalase (EC 1.11.1.6), while ROS production was much lower in undifferentiated astrocytes (+50%; Re et al. 2003).

The next stage of the study was to determine whether oxidative death of differentiated astrocytes shares some features with the previously described oxidative glutamate toxicity (Re et al. 2006). Inhibition of system x_c^- by 4-carboxyphenylglycine alone or in combination with PDC did not induce significant changes in differentiated astrocyte viability as compared with respective controls. These results suggested that impairment of system x_c^--mediated cystine uptake may be part of, but cannot account solely for astrocyte death mechanism upon EAAT dysfunction. Knowing that different transport systems for cystine have been shown in astrocytes in addition to system x_c^-, including EAATs and uptake processes depending on the activity of the ectoenzyme *γ*-GT (Shanker and Aschner 2001), the effects of cystine deprivation were examined alone or in combination with PDC treatment to further evaluate the contribution of failure in cystine supply (Re et al. 2006). Cystine deprivation per se induced 22% loss of differentiated astrocytes and its combination with PDC led to a

dramatic increase in cell loss reaching 94%, suggesting that under PDC treatment cystine uptake is not totally impaired. Finally, activation of arachidonic acid metabolism through the 12-lipoxygenase (12-LOX; EC 1.13.11.31) pathway has been shown to be involved in the cytotoxic processes due to GSH depletion in oxidative glutamate toxicity (Tan et al. 2001; Seiler et al. 2008) and is suggested to play a central role in neurodegenerative diseases (Khanna et al. 2003; Mytilineou et al. 1999). Therefore, the effects on PDC toxicity of inhibitors of the different pathways involved in arachidonic acid metabolism were examined. The general LOX inhibitor NDGA as well as AA861 a specific inhibitor of 5-LOX (EC 1.13.11.34) potently reduced PDC toxicity. On the contrary, indomethacin, to inhibit cyclooxygenase (EC 1.14.99.1), metyrapone, to inhibit cytochrome P450, and baicalein, a specific inhibitor of 12-LOX, had no effect. Despite the fact that baicalein is a rather weak inhibitor of 12-LOX and that AA861 may also inhibit this enzyme, these data suggest that astrocyte death through EAAT dysfunction may be different from the conventional oxidative glutamate toxicity (Re et al. 2006).

As PDC-induced death of differentiated astrocytes was correlated with glutamate release but did not seem to involve the classical death pathways triggered after an increase in extracellular glutamate concentration, intracellular mechanisms were investigated (Re et al. 2006). In PDC-treated astrocyte cultures, there was a delayed and progressive decline in glutamate intracellular levels with a threshold of 12 h and significant from 24 h. Moreover, even if glutamate alone induced significant astrocyte loss at concentrations known to inhibit system x_c^-, at lower concentrations it had significant protective effect against PDC toxicity. Glutamate is even protective when applied 6 h before or after PDC, suggesting that glutamate protection was not mediated solely by direct competition with PDC. The protective effect of glutamate was not mimicked by glutamate receptor agonists or blocked by antagonists, indicating that receptor activation was not involved. On the other hand, inhibiting transformation of transported glutamate into glutamine by methionine sulfoximine (MSO) or replacing glutamate by a transportable and metabolizable analog such as aspartate was protective against PDC. Conversely, doubling glutamine concentration or adding a transportable but not metabolizable analog D-aspartate, or membrane permeable analogs such as glutamate dimethyl ester or glutamate diethyl ester was not. Moreover, none of the treatments that were ineffective in providing protection against PDC toxicity could block the decrease in GSH levels. Conversely, all the treatments with protective effects, i.e. addition of glutamate, L-aspartate or MSO, significantly increased GSH concentration versus PDC. Altogether, these data suggested that PDC was toxic by depleting a compartmented metabolic pool of glutamate, normally fed by EAATs and essential for GSH synthesis. It is proposed that this novel form of toxicity is termed "low intracellular glutamate-linked oxidative toxicity" or LIGO toxicity to underscore that it is separate from the classically described mechanisms linked to high extracellular glutamate, i.e. excitotoxicity and oxidative glutamate toxicity (Re et al. 2006).

It is surprising that glutamate transport determines antioxidant defenses only in differentiated astrocytes (Re et al. 2003, 2006). First, cysteine is usually considered as the limiting factor for GSH synthesis. However, studies conducted on muscles in several catabolic conditions showed high correlation between decreased muscular levels of GSH and glutamate, but not cysteine (Rutten et al. 2005). Moreover, it has been shown in astrocytes that glutamate is necessary for optimal GSH synthesis in the presence of cyst(e)ine (Dringen and Hamprecht 1996). Reichelt et al. (1997) further showed that, in this condition, GSH synthesis is highly dependent on the capacity of glutamate transporters to provide glutamate intracellularly in retinal Muller cells. Accordingly, we showed that cystine uptake is not totally impaired under PDC treatment, that inhibition of cystine uptake through system x_c^- is only part of the PDC effects, that PDC effects are mediated by the same pathway as buthionine sulfoximine (BSO) and prevented by treatments which may compensate for the loss of transported glutamate. Taken together, these data suggest that transported glutamate may be a significant limiting factor for GSH synthesis when cyst(e)ine is not depleted and/or cyst(e)ine uptake not totally impaired. The mechanisms that maintain cyst(e)ine availability in case of both EAAT and system x_c^- dysfunction remain to be determined. The transsulfuration pathway may be an intracellular source of cysteine from methionine in astrocytes (McBean 2011). On the other hand, one-third of the cystine uptake in astrocytes is dependent on the activity of γ-GT (Shanker and Aschner 2001) and the dipeptides formed by this enzyme may be transported by astrocytes to sustain GSH synthesis (Dringen et al. 1997, 1998). On the contrary, glutamate metabolism is highly compartmentalised in astrocytes (McKenna et al. 1996; Schousboe et al. 1993) and the metabolic processes depending on glutamate uptake such as glutamine or GSH synthesis may not be easily compensated for by other glutamate sources in case of EAAT dysfunction.

Antioxidant defenses of undifferentiated astrocytes in vitro: independence of glutathione?

In both type of cultures, it was shown that intracellular GSH levels are highly dependent on EAAT activity but that EAAT dysfunction triggers only oxidative death of differentiated astrocytes. As undifferentiated astrocytes do

possess a twofold higher basal GSH content compared with the differentiated ones and as the GSH level reached upon PDC treatment of undifferentiated cells was similar to that of the untreated differentiated ones, we first thought that the resistance of undifferentiated astrocytes to EAAT dysfunction was a question of threshold of GSH depletion. In accordance, lowering GSH levels further by direct inhibition of GSH synthesis using BSO renders undifferentiated astrocytes vulnerable to PDC. However, treatment with BSO alone, at concentrations ranging from 50 µmol/L to 5,000 µmol/L, was ineffective at inducing loss of undifferentiated astrocytes, contrary to differentiated astrocytes, which were highly sensitive. Therefore, it could be that undifferentiated astrocytes, like Burkitt's Lymphoma cells (Banjac et al. 2008; Conrad and Sato 2011), do not rely on intracellular GSH for their survival. In the Lymphoma cells antioxidant defenses are primarily directed to prevent lipid peroxidation from the extracellular milieu through a cystine/cysteine cycle. Cystine transported inside the cells by system x_c^- is reduced into cysteine that is then released through a neutral amino acid transport system in the extracellular milieu where it can scavenge ROS by being oxidized back into cystine. In embryonic stem cell-like cells, forced expression of system x_c^- is even capable of rescuing genetic GSH deficiency (Mandal et al. 2010). To test whether undifferentiated astrocytes rely on a cycle driven by cystine uptake for their antioxidant defenses, we tested their sensitivity to cystine deprivation. Contrary to differentiated astrocytes that were not significantly affected by such a treatment, undifferentiated astrocytes cannot withstand cystine deprivation (Fig. 4). More experiments are required to ascertain the mechanism by which cystine deprivation affects undifferentiated astrocyte viability. If indeed, a cystine/cysteine cycle is responsible for antioxidant defenses in undifferentiated astrocytes, it would be of interest to determine as to what is the source of intracellular glutamate required to fuel its activity as it cannot be transported glutamate since undifferentiated astrocytes are not sensitive to EAAT dysfunction. Moreover, determining what mechanisms are responsible for the change in the way antioxidant defenses are maintained upon astrocyte differentiation will be also of interest. The shift from cystine/cysteine cycle to strict dependence on GSH could be due to differential level of expression of the different transporters and notably the one responsible for the release of cysteine whose expression could be decreased in differentiated astrocytes. It could be also due to a change in the expression of the proteins interacting with the intracellular part of EAATs that may control their distribution at the plasma membrane, their activity and interaction with other proteins. For instance, the expression level of GTRAP3-18, a glutamate transporter-associated protein for EAAC1, was recently shown

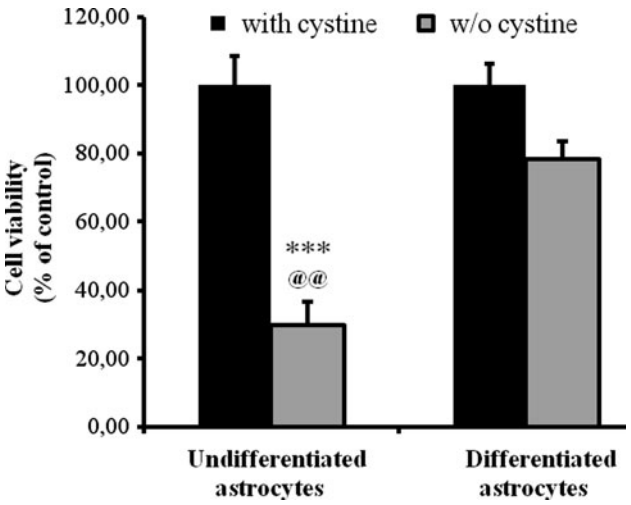

Fig. 4 Effect of cystine depletion on astrocyte viability. Striatal astrocyte cultures either undifferentiated (w/o dBcAMP) or dBcAMP-differentiated (0.25 mM dBcAMP for 1 week) were obtained as described by Re et al. (2003). Cystine depletion was performed as described for differentiated astrocytes by Re et al. (2006). Cell viability was assessed by MTT assay as described by Re et al. (Re et al. 2003, 2006). Data are mean ± SEM of values obtained in three independent experiments. $^{***}p < 0.001$ as compared with respective controls with cystine; $^{@@}p < 0.01$ when comparing values in undifferentiated versus differentiated cultures w/o cystine

to control the GSH level in neurons (Watabe et al. 2008). The association of different transport systems through interacting proteins for EAATs in the same complex may perhaps determine the way antioxidant defenses are maintained. Changes in the expression level of such proteins during differentiation may be responsible for the shift from cystine/cysteine cycle to strict dependence on GSH.

Conclusion and outlook

Acute or chronic oxidative stress is invariably involved in neurodegenerative insults. To develop neuroprotective strategies aiming at limiting the neurological deficits associated with neuronal loss, a better understanding of how antioxidant defenses are maintained in the brain is required. Astrocytes are thought to play a central role in maintenance of antioxidant defenses in the brain as they possess high content of glutathione, the main brain antioxidant molecule, and as they support neuronal antioxidant defenses by supplying neurons with glutathione precursors. However, we had shown that the way antioxidant defenses are maintained in astrocytes depended on their differentiation state. Differentiated quiescent astrocytes use EAAT transported glutamate to sustain their own GSH synthesis and resist oxidative stress, while proliferative and/or reactive astrocytes may probably use preferentially a cystine/cysteine cycle to sustain their antioxidant defenses,

independently of EAAT activity. This differential dependency of astrocytes on EAATs for maintenance of their antioxidant defenses may have possible implications in concurrent death of quiescent astrocytes and reactive gliosis in neurodegenerative insults such as ischemia where EAAT function is known to be altered. Investigating how the change in the way astrocytes maintain their antioxidant defense impact neuronal viability may be of importance as, in ischemic insults, astrocyte dysfunction/death in the penumbra may contribute to the delayed neuronal death and to the expansion of the infarct (Nedergaard and Dirnagl 2005).

Not all neuronal cells may depend on astrocytes for their antioxidant defenses. Neuronal cells with a limited glial surrounding, such as DA neurons of substantia nigra (Damier et al. 1993) which degenerate in Parkinson's disease, may have developed a particular way to maintain their antioxidant defenses. Similar to differentiated astrocytes, we found DA neurons to be highly vulnerable to pharmacologically induced EAAT dysfunction both in vitro and in vivo (Nafia et al. 2008). In vitro, we showed that PDC triggered increase in extracellular glutamate levels, GSH depletion, delayed production of ROS and death of both astrocytes and neurons, DA neurons being preferentially affected as compared with non-DA neurons. Surprisingly, different mechanisms were involved in the death of each cell type. While astrocyte death was oxidative, that of non-DA neurons mainly excitotoxic, the preferential vulnerability of DA neurons was related to PDC-induced decrease in antioxidant defenses that lowers the threshold for glutamate toxicity through NMDARs. The decrease in antioxidant defenses in DA neurons did not seem to be correlated with the loss of astrocytes. Indeed, while astrocyte death was prevented by cystine, death of DA neurons was not, being protected by cysteine instead (Nafia et al. 2008). These data are consistent with the substrate preference of EAATs of glial versus neuronal type (Hayes et al. 2005) and suggest a direct mechanism of PDC toxicity on DA neurons. In contrast, the death of non-DA neurons could be linked to astrocyte dysfunction as being protected by cystine, while being not oxidative. In vivo, we found that acute PDC injection suffices to produce SNc DA neuron degeneration, whereas it was reported as inefficient when administered in other brain structures (Massieu et al. 1995, 2001; Montiel et al. 2005). However, EAAT function may be required to protect some non-DA neurons against oxidative stress due to H_2O_2 exposure (Aoyama et al. 2006; Himi et al. 2003) or such as occurring with advancing age (Aoyama et al. 2006) while contributing to other metabolic pathways in basal conditions.

In conclusion, EAAT function seems to be crucial for the maintenance of antioxidant defenses in particular cells of the nervous system (differentiated astrocytes, DA neurons) while being dispensable in other cell types, unless challenged with oxidative stressors. This differential importance may depend on the way antioxidant defenses are maintained in each cell type and on the metabolic fate of the transported substrate, both being probably controlled by EAAT interacting proteins.

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