Calcium dyshomeostasis in multiple sclerosis

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ABSTRACT. Calcium (Ca^{2+}) dyshomeostasis is a major event in the pathophysiology of multiple sclerosis. All cellular components involved in that disease, including macroglial cells and axons, are endowed with membrane Ca^{2+}-permeable receptors and channels lodged in the cell membrane. Intracellular Ca^{2+} overload resulting from deregulated activity of channels, such as those opened by glutamate and ATP, is deleterious to glia and axons. In this review, I summarize recent advances in understanding of Ca^{2+} dyshomeostasis in experimental paradigms which are relevant to multiple sclerosis, and discuss some of the clinical implications of these findings.
Key words: demyelination, excitotoxicity, mitochondria, reactive oxygen species, cell death.

RESUMEN. La dishomeostasis del calcio es un evento importante en la patofisiología de la esclerosis múltiple. Todos los componentes celulares relacionados con esta enfermedad, incluyendo las células macrogliales y los axones, están dotados con receptores y canales de membrana permeables al calcio. La sobrecarga intracelular de calcio como consecuencia de la desregulación de la actividad de canales, tales como los activados por el ácido glutámico y el ATP, daña los oligodendrocitos y los axones. En esta revisión resumiré algunos avances recientes sobre la comprensión de la dishomeostasis del calcio en paradigmas experimentales relevantes a la esclerosis múltiple, y discutiré las implicaciones clínicas de esos hallazgos.
Palabras clave: desmielinizacion, excitotoxicidad, mitocondria, especies reactivas de oxígeno, muerte celular.

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The main vulnerable cellular elements in multiple sclerosis (MS) are oligodendrocytes, myelin and axons. Oligodendrocytes are the major cell type of CNS white matter which in humans comprises about 50% of brain volume. In addition, oligodendroglia is also present in gray matter, in particular throughout the cerebral cortex.

White matter resembles gray matter more than previously expected, in several aspects which are relevant to disease. Thus, functional synapses between axons and glial progenitors have been reported in the corpus callosum, indicating that axons within white matter are engaged in rapid neuron-glia communication in a manner that may also resemble classical synaptic transmission (Ziskin *et al*., 2007; Kuckley *et al*., 2007). In turn, white matter is endowed with the molecular machinery necessary to employ most neurotransmitters in glia- and axon-glia interactions (Stys, 2005; Matute *et al*., 2006, 2007; Butt, 2006; Constantinou and Fern, 2009; Matute, 2010).

As in neurons, prolonged and excessive elevation of the cytosolic concentration of Ca^{2+} can be lethal to white matter glia and directly disrupt axon function and structure. In this review, I briefly summarize some of the major specific processes regulating Ca^{2+} homeostasis in glial cells as well as in axons, and how aberrant Ca^{2+} signaling can lead to white matter damage in MS.

❑ Characteristics of white matter

White matter is composed of myelinated and unmyelinated axons, astrocytes, oligodendrocytes, microglia and blood vessels. Myelinated axons in the Central Nervous System (CNS) are designed to support rapid and efficient saltatory conduction by means of an insulating myelin sheath which covers 99% of the axon surface and a high density of nodal Na channels and internodal K channels which preserve electrical polarization and stability (Poliak and Peles, 2003).

Astrocytes participate in numerous functions including structural support (the word "glia" is derived from a Greek word meaning "glue"), uptake and release of neurotransmitters, regulation of extracellular cation levels, and metabolic support. Some astrocyte processes contact the blood-brain barrier, which protects the CNS from being exposed to endogenous neurotoxins present in blood. A major mediator of astrocyte signalling is Ca^{2+}, which can propagate through the astrocytic syncitium over long distances and thus, modulate activity at a distant location (Jessen and Richardson, 2001). In turn, oligodendrocytes enwrap axons and form the myelin sheaths, which provide electrical insulation around the axon. Oligodendrocyte death results in demyelination, impaired axonal conduction and ultimately axon death.

Microglial cells are the immune cells of the

CNS and are rapidly activated and recruited to sites of infection, neurodegeneration and inflammation. Microglia are stimulated by a variety of cytokines, neurotransmitters, modulators and neurotoxins, as well as by extracellular matrix molecules and proteases present in the areas of the CNS undergoing inflammation (Raivich and Banati, 2004). They are competent antigen presenters of antigen and interact with T cells entering the CNS. Microglial cells also synthesize cytokines, chemokines, complement, cell adhesion proteins, reactive oxygen radicals and neurotrophins which can exert damaging or protective effects on adjacent neurons, axons, oligodendrocytes and myelin (Raivich and Banati, 2004).

❑ Regulation of Ca^{2+} signalling in white matter

White matter axons and glial cells control intracellular Ca^{2+} levels through a complex interplay between Ca^{2+} flux across the plasma membrane, and Ca^{2+} release from and sequestration into internal stores (for reviews, see Verkhratsky *et al*., 1998; Stys, 2005, Alberdi *et al*., 2005). Two of the major players in Ca^{2+} signaling are glutamate and ATP which are released from axons and glial cells. In this section, I will outline recent knowledge about how these two excitatory neurotransmitters exert their effects in white matter.

Glial cells

White matter glial cells express ligand-gated channels which are permeable to Ca^{2+}, including glutamate and ATP receptors. Thus, metabotropic (mGluR) and ionotropic glutamate receptors of the AMPA, kainate and NMDA type are commonly present in astrocytes and oligodendrocytes, as well as in resting and activated microglia (for review, see Verkhratsky and Steinhäuser, 2000; Matute *et al*., 2006; Butt, 2006; Matute, 2006; Verkhratsky and Kirchhoff, 2007; Bakiri *et al*., 2009).

Overall, ionotropic glutamate receptors expressed in glial cells have similar properties to their neuronal counterparts. However, the fact that these receptors are edited to a lesser extent in the white matter, and that AMPA receptors in oligodendrocytes do not have GluR2 subunit, suggests that white matter glia are more Ca^{2+} permeable (Matute *et al*., 2006). In turn, NMDA receptors are expressed in white matter oligodendrocytes at all developmental stages and their activation generates a membrane depolarization and a rise in cytosolic Ca^{2+} (reviewed in Bakiri *et al*., 2009). Interestingly, NMDA receptors are expressed in clusters on oligodendrocyte processes and myelin, whereas AMPA and kainate receptors are diffusely located on oligodendrocyte somata (Káradóttir *et al*., 2005; Salter and Fern, 2005, Micu *et al*., 2006). In addition, oligodendrocytes also express all three subtypes of mGluRs, but their levels are developmentally regulated and are very low in mature cells of this lineage (Deng *et al*., 2004). However, little is still known about the specific properties of ionotropic and metabotropic glutamate receptors expressed on white matter astrocytes and microglia (see Matute *et al*., 2006).

Prolonged activation of AMPA, kainate and NMDA receptors causes oligodendrocyte death and primary and/or secondary myelin destruction. A central event to this process is Ca^{2+} influx upon receptor activation and the ensuing accumulation of this cation within mitochondria, which leads to depolarization of this organelle, increased production of radical oxygen species, and release of pro-apoptotic factors which activate caspases (Sánchez-Gómez *et al*., 2003; Matute *et al*., 2006). The types of oligodendrocyte death induced by activation of AMPA and kainate receptors depend on the intensity and duration of the excitotoxic insult. Notably, the molecular cascades initiated by AMPA and kainate receptors are not identical indicating that different intracellular domains are involved in executing the death program triggered by these receptors (Matute *et al*., 2007). In particular, insults channeled through kainate receptors activate caspases 9 and 3 leading to apoptosis. In contrast, those activating AMPA receptors induce apoptosis by recruiting caspase 8, which leads to the truncation of the Bid protein. This in turn activates caspase 3 and PARP-1 polymerase, or cause necrosis (Matute *et al*., 2007). The mechanisms triggered by NMDA receptor-mediated insults to oligodendrocytes have not been studied in detail yet.

Glial cells also express a heterogeneous repertoire of ATP receptor including an ample variety of ionotropic (P2X) and metabotropic (P2Y) purinergic receptor subtypes (Butt, 2006, Verkhratsky *et al*., 2009). ATP-gated P2X channels are formed by P2X1-P2X7 subunits and have marked Ca^{2+} permeability. Activation of P2X1 and P2X3 results in fast, rapidly desensitizing currents. In contrast, P2X7, and also P2X2 and P2X4, are capable of a conformational change which results in larger pore diameter following prolonged exposure to ATP.

Astrocytes express most of the P2X and P2Y receptor subtypes whose activation mediates signaling through the astrocyte syncytium (James and Butt, 2002; Fields and Burnstock, 2006). In particular, activation of P2X7 receptors in astrocytes increases $[Ca^{2+}]_i$ and causes the release of purines. Optic nerve astrocytes also express a variety of P2X receptors, which are highly permeable to Ca^{2+}, and of P2Y re-

ceptors which mobilize this cation from intracellular stores (James and Butt, 2002), as reported in grey matter astrocytes.

Cells of the oligodendrocyte lineage are endowed with P2X and P2Y receptors which can act as mediators of axo-oligodendroglial communication implicated in myelination control. In particular, ATP induces a rise in cytosolic Ca^{2+} in oligodendrocytes by activating ionotropic P2X7 receptors (James and Butt, 2002; Matute *et al*., 2007) and metabotropic P2Y receptors (Kirischuk *et al*., 1995; James and Butt, 2002). Moreover, mature oligodendrocytes of the optic nerve express most of the P2X receptor subtypes, with the P2X7 subtype being the most predominant; this is located in the oligodendrocyte soma and in the myelin sheath; James and Butt, 2002; Matute *et al*., 2007). P2X receptors with higher affinity may be activated by ATP released during axonal electrical activity and from astrocytes (Butt, 2006). In contrast, the functional significance of lower affinity P2X7 receptors in oligodendrocytes is not known, since unusual high concentrations of ATP in the extracellular space are needed to activate them. However, ATP levels may rise sufficiently upon tissue damage to stimulate P2X7 receptors and therefore they may be relevant to pathologies involving acute and chronic injury to white matter (Matute *et al*., 2007). Indeed, sustained activation of P2X7 receptors in oligodendrocytes in vitro and in vivo results in overload of the cytosol with Ca^{2+}, caspase-3 activation and chromatin condensation and cell death (Matute *et al*., 2007).

Microglia expresses several P2X and P2Y receptors which act as sensors of astrocyte activity and trigger cytokine release (Fields and Burnstock, 2006; Färber and Kettenmann, 2006). In particular, microglial P2X7 receptors drive microglial activation and proliferation (Monif *et al*., 2009) and are functionally linked to the release of several substances including pro-inflammatory cytokines such as interleukin-1β, which influence pathological processes and promote neurodegeneration (Färber and Kettenman, 2006). Moreover, ATP is a potent immunomodulator controlling microglial recruitment and activation (Davalos *et al*., 2005; Nimmerjahn *et al*., 2005) by acting at P2Y12 receptors to induce microglial chemotaxis at early stages of the response to local CNS injury (Haines *et al*., 2006).

Altogether, the findings discussed above indicate that activation of glutamate receptors and purinoceptors by extracellular glutamate and ATP can cause primary and/or secondary damage to white matter and that signalling by both neurotransmitters is an important component of the glial response to injury in the CNS.

Axons

Axons and astrocytes release glutamate and ATP during electrical activity, which in turn evoke Ca^{2+} signals in nearby glia (Bakiri *et al*., 2009). In addition, axons are competent sources of neurotransmission within white matter since they form functional synapses with glia (Ziskin *et al*., 2007; Kuckley *et al*., 2007; Bakiri *et al*., 2009). Conversely, astrocytes can release glutamate (Volterra and Meldolesi, 2005) and NG2-expressing glia also are endowed with the synaptic protein synaptophysin which suggests that they may also be capable of vesicular release and bidirectional communication with axons via their cellular contacts (Bakiri *et al*., 2009). Nevertheless, the details of glutamate and ATP signalling to axons remain scarce.

Electrophysiological recordings of the axon resting potential revealed that axons in the dorsal column of the spinal cord are depolarized via activation of AMPA receptors (Ouardouz *et al*., 2006). Consistent with these observations, central axons are damaged by activation of AMPA/kainate receptors (Matute, 1998; Fowler *et al*., 2003), and protected by blockers of these receptors in models of white matter injury (Tekkök and Goldberg, 2001; Pitt *et al*., 2000). Indeed, recent findings indicate that myelinated spinal cord axons have AMPA receptors formed by the GluR4 subunit and kainate receptors composed of at least GluR5 and GluR6 subunits, which are located in the internodes in all instances (Ouardouz *et al*., 2009a,b). However, axon demise could also be secondary to oligodendrocyte loss by excitotoxicity and the ensuing demyelination (see above), rather than by activation per se of glutamate receptors in axons.

Axonal AMPA receptors are weakly permeable to Ca^{2+}, the entry of which in turn releases further Ca^{2+} from the axoplasmic reticulum by opening intracellular calcium channels known as ryanodine receptors (Ouardouz *et al*., 2009a). In contrast, axonal kainate receptors with the GluR5 subunit are coupled to phospholipase C activation (Ouardouz *et al*., 2009a). In addition, activation of kainate receptors with the GluR6 subunit induces a small amount of Ca^{2+} entry that stimulates nitric oxide synthase, as well as a local depolarization which activates L-type Ca^{2+} channels and subsequently ryanodine receptors in the axoplasmic reticulum (Ouardouz *et al*., 2009b). The functional significance of these signalling mechanisms by glutamate receptors in axons is unknown but they may serve to amplify axonal Ca^{2+} signals which seem to be weak because of the limited quantity of cation available in the narrow space (Ouardouz *et al*., 2009b). In turn, high local concentrations of Ca^{2+} generated by these receptors may result in focal swellings and irreversible axonal transactions (Ouardouz *et al*., 2009b).

Table I	Mechanisms of Ca^{2+} dyshomeostasis in multiple sclerosis	
MOG-mediated demyelination	Ca^{2+}-dependent MAPK/Akt activation	[58]
Acute and chronic EAE	AMPA receptors	[40, 61, 64, 65]
Microglia activation	Oligodendrocyte excitotoxicity	[67]
Oligodendrocytes in vitro and in situ	Sensitization to excitotoxicity by kainate receptor	[70]
Chronic EAE	P2X7 receptor	[27]

Abbreviations: EAE: experimental autoimmune encephalomyelitis; MOG: myelin oligodendrocyte glycoprotein.

❑ Perturbation of Ca^{2+} homeostasis in glial cells and axons in white matter pathology

Glial cells and axons, like neurons, are vulnerable to Ca^{2+} overload resulting from deregulation of channels and/or pumps. I summarize below current evidence regarding the specific mechanisms linking alterations in Ca^{2+} homeostasis to glial cell death and axonal damage, and its relevance to human diseases involving white matter (Table I).

The major demyelinating disease of the CNS is multiple sclerosis (MS) which is the foremost disabling pathology among young adults. MS is a chronic, degenerative disease of the CNS, which is characterized by focal lesions with inflammation, demyelination, infiltration of immune cells, oligodendroglial death and axonal degeneration (Prineas *et al*, 2002). These cellular alterations are accompanied by neurological deficits such as sensory disturbances, lack of motor coordination and visual impairment. It is widely accepted that the etiology of this illness has autoimmune and inflammatory grounds and that a derailment of the immune system leads to cell- and antibody-mediated attacks on myelin. Notably, treatment of oligodendrocytes with antibodies to myelin-oligodendrocyte glycoprotein (MOG) leads to an increase in Ca^{2+} influx and activation of the MAPK/Akt pathways, a signalling cascade relevant to the initial steps of MOG-mediated demyelination (Marta *et al*., 2005). In turn, breakdown of the blood-brain barrier caused by inflammation allows the entry into the brain parenchyma of blood constituents, which may be deleterious to neurons and glia. Thus, elevated levels of albumin induced a rise in intracellular Ca^{2+} in microglia, but not in astrocytes or macrophages, which is mediated via Src tyrosine kinase and phospholipase C. This Ca^{2+} response is coupled to microglial proliferation suggesting that this signalling mechanism may play a role in microglial activation in pathological situations involving blood-brain barrier impairment as occurs in multiple sclerosis and in other neurodegenerative diseases (Hooper *et al*., 2005).

Both genetic and environmental factors contribute to MS susceptibility (Zamvil and Steinman, 2003). Among them, primary and/or secondary alterations in glutamate signalling cause excitotoxicity that contribute to MS pathology. Thus, numerous studies carried out in cellular and animal models of MS as well as in post-mortem brain and in patients indicate that excitotoxicity mediated by Ca^{2+}-permeable glutamate receptors contributes to oligodendrocyte death, demyelination and tissue damage in MS (Matute *et al*., 2001; Srinivasan *et al*., 2005; Vallejo-Illarramendi *et al*., 2006). In particular, experimental autoimmune encephalomyelitis (EAE), an animal model which exhibits the clinical and pathological features of MS, is alleviated by AMPA and kainate receptor antagonists (Pitt *et al*. 2000; Smith *et al*. 2000). Remarkably, blockade of these receptors in combination with anti-inflammatory agents is effective even at an advanced stage of unremitting EAE, as assessed by increased oligodendrocyte survival and remyelination, and corresponding decreased paralysis, inflammation, CNS apoptosis and axonal damage (Kanwar *et al*. 2004). In contrast, blockade of NMDA receptors with MK-801 does not attenuate EAE symptoms (Figure 1), an event that calls into question the proposed relevance of NMDA receptors in demyelinating diseases (Bakiri *et al*., 2009). EAE experiments carried out in genetically modified mice lacking NMDA receptors specifically in oligodendrocytes may help clarifying this issue.

Glutamate levels are increased in the human brain (Srinivasan *et al*., 2005) as a consequence of altered glutamate homeostasis (Vallejo-Illarramendi *et al*., 2006) and thus, trigger excitotoxic destruction of oligodendrocytes and myelin as well as of axons (Domercq *et al*., 2005). Glutamate dyshomeostasis results from primary and/or secondary inflammation as a consequence of the autoimmune attack to the CNS and/or resulting from ongoing cell damage within the brain and spinal cord. Thus, activated microglia release cytokines and free radicals that diminish glutamate uptake. This in turn elevates the extracellular levels of this transmitter, resulting in over-activation of Ca^{2+}-permeable glutamate receptors, which leads to oligodendrocyte excitotoxicity (Domercq *et al*., 2007). Moreover, activated microg-

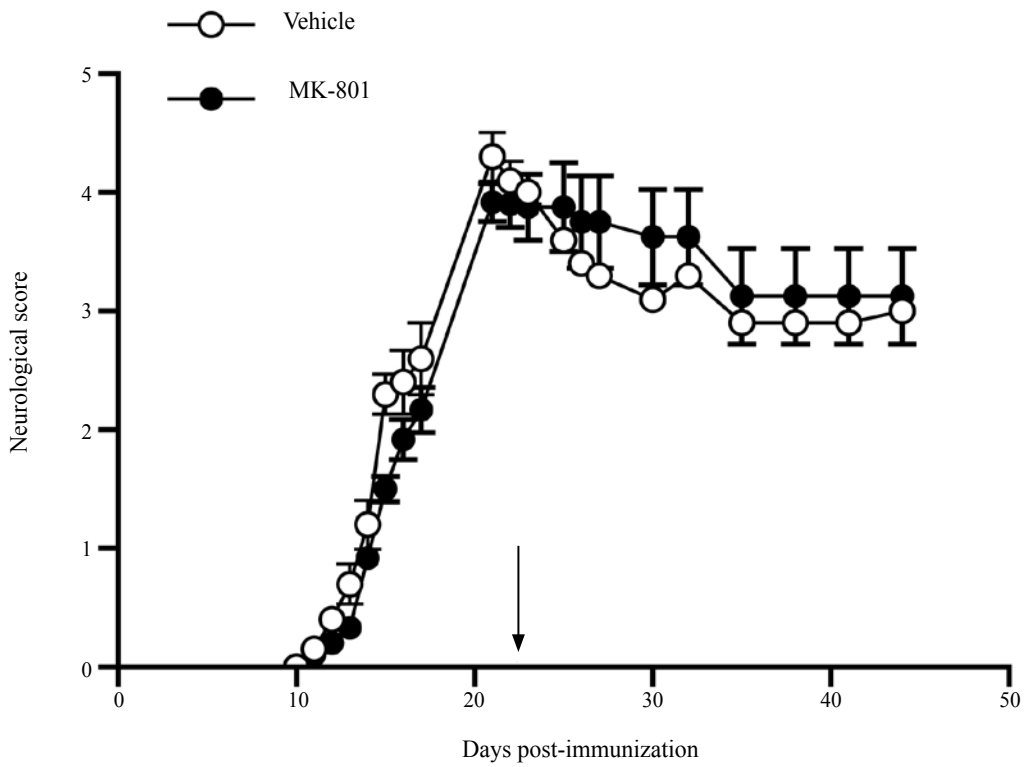

Figure 1 *The NMDA receptor antagonist MK-801 does not ameliorate chronic EAE-associated neurological symptoms. Chronic EAE was induced in C57/BL6 mice by immunization with myelin-oligodendrocyte glycoprotein, and MK-801 (0.5 mg/kg per day and body weight) was administered after 21 days (arrow) of immunization. The clinical score indicates level of motor symptoms as follows from 0-8: 0, no detectable changes in muscle tone and motor behavior; 1, flaccid tail; 2, paralyzed tail; 3, impairment or loss of muscle tone in hindlimbs; 4, hindlimb hemi-paralysis; 5, complete hindlimb paralysis; 6, complete hindlimb paralysis and loss of muscle tone in forelimbs; 7, tetraplegia and 8, moribund.*

lia increase their expression of the glutamate-cystine exchanger which contributes further to raising the levels of glutamate and its toxicity (Domercq *et al*., 2007). Other mechanisms accounting for glutamate dyshomeostasis include genetic variability in the promoter of the major glutamate transporter, EAAT2, which results in lower transporter expression (Pampliega *et al*., 2008). Finally, an additional component of the genetic background linking MS and deregulation of glutamate signalling and Ca^{2+}-dyshomeostasis may lie in a polymorphism in the Ca^{2+}-permeable AMPA receptor subunit GluR3, an abundantly expressed subunit in oligodendrocytes, which is associated with a subgroup of patients responding to interferon beta therapy in MS (Comabella *et al*., 2009).

Glutamate at non-toxic concentrations (within the micromolar range) can also contribute to demyelinating pathology by inducing Ca^{2+}-dyshomeostasis and oligodendrocyte death via sensitization of these cells to complement attack (Alberdi *et al*., 2006). Intriguingly, complement toxicity is induced by activation of kainate, but not of AMPA, NMDA or metabotropic glutamate receptors. Oligodendrocyte death by complement requires the formation of the membrane attack complex, which in turn increased membrane conductance, induced Ca^{2+} overload and mitochondrial depolarization as well as a rise in the level of reactive oxygen species (Alberdi *et al*., 2006). Sensitization to complement attack may initiate MS lesions and thus be a pathophysiological feature of this disease or any of its neuropathological subtypes.

In addition to glutamate, ATP signalling can trigger oligodendrocyte excitotoxicity via activation of Ca^{2+} permeable P2X7 purinergic receptors expressed by these cells (Matute *et al*., 2007). Thus, sustained activation of P2X7 receptors in vivo causes lesions which are reminiscent of the major features of MS lesions, i.e. demyelination, oligodendrocyte death and axon damage. In addition, treatment of acute and chronic EAE with P2X7 antagonists reduces demye-

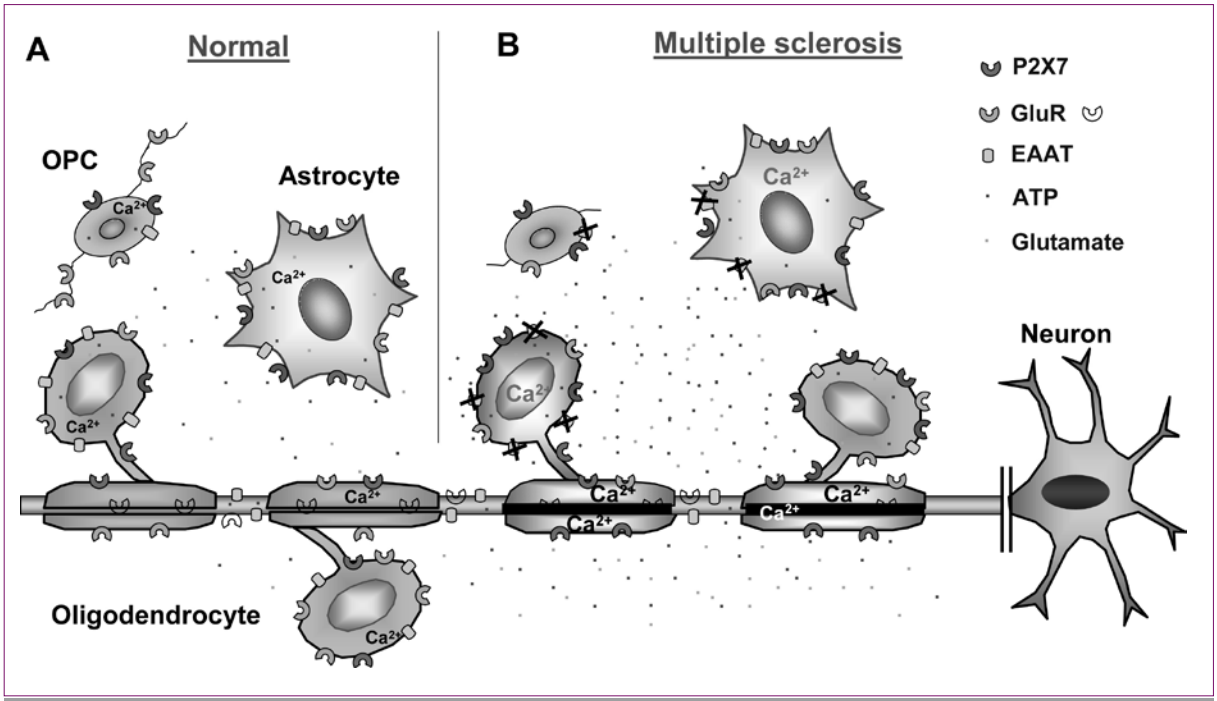

Figure 2 *Glutamate- and ATP-mediated Ca^{2+} dyshomeostasis in multiple sclerosis. (A) Astrocytes and oligodendrocytes and their myelin sheaths express highly Ca^{2+} permeable glutamate and P2X7 receptors which are critical to Ca^{2+} homeostasis in white matter. In addition, axons express glutamate receptors which permeate Ca^{2+} and mobilize Ca^{2+} from the intracellular stores. Under normal conditions, glutamate is taken up by glutamate transporters which prevent overactivation of glutamate receptors in oligodendrocyte progenitors (OPC), oligodendrocytes, myelin and axons, and the ensuing cell damage by excitotoxicity. (B) In multiple sclerosis, increased extracellular levels of glutamate as a result of oxidative stress, which impairs glutamate uptake (illustrated with an X on the glutamate transporter), can lead to excitotoxic injury to oligodendrocytes, myelin (illustrated in pink) and axons (represented in black), and the release of ATP from destroyed cells. This in turn activates P2X7 receptors on glial cells, including oligodendrocytes which may die by ATP excitotoxicity and thus amplify tissue destruction. Compounding the problem further, enhanced signaling by both glutamate and ATP, particularly in oligodendrocytes, alters Ca^{2+} homeostasis and may initiate and/or contribute to secondary white matter damage. EAAT, glutamate transporter; GluR, glutamate receptor; OPC, oligodendrocyte progenitor cell. Elevated levels of cytosolic Ca^{2+} are depicted in red. See text for further details.*

lination and ameliorates the associated clinical symptoms. Importantly, P2X7 expression is elevated in oligodendrocytes of normal-appearing axon tracts in MS patients, suggesting that signalling through this receptor is enhanced in this disease (Matute *et al*., 2007). The increased expression of P2X7 receptors in axon tracts before lesions are formed indicates that this feature may constitute a risk factor associated with newly forming lesions in MS; this receptor subunit may thus prove to be a diagnostic and/orprognostic clinical biomarker for MS-type leukoencephalopathy. Finally, blockade of ATP P2X7 receptors protects oligodendrocyte from dying; this property has enormous therapeutic potential for halting the progression of tissue damage in MS.

❑ Conclusions

Ca^{2+} dyshomeostasis in astrocytes, oligodendrocytes and axons leads to white matter demise in multiple sclerosis, and contributes to the severity of symptoms. Glutamate and ATP signalling in white matter are critical to Ca^{2+} homeostasis in health and pathology (Figure 2), and drugs attenuating their excitotoxic potential offer great therapeutic promise.

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