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REVIEW

Purinergic signalling at the plasma membrane: a multipurpose and multidirectional mode to deal with amyotrophic lateral sclerosis and multiple sclerosis

Susanna Amadio, Savina Apolloni, Nadia D'Ambrosi and Cinzia Volonté

CNR, Institute of Neurobiology and Molecular Medicine/Santa Lucia Foundation, Rome, Italy

Abstract

ATP is a widespread and multipurpose signalling molecule copiously released in the extracellular environment of the whole nervous system upon cell activation, stress, or damage. Extracellular ATP is also a multidirectional information molecule, given the concurrent presence at the plasma membrane of various targets for ATP. These include ectonucleotidases (metabolizing ATP down to adenosine), ATP/adenosine transporters, P2 receptors for purine/pyrimidine nucleotides (ligand-gated ion channels P2X receptors and G-protein-coupled P2Y receptors), in addition to metabotropic P1 receptors for nucleosides. All these targets rarely operate as single units, rather they associate with each other at the plasma membrane as multi-protein complexes. Altogether, they control the duration, magnitude and/or direction of the signals triggered and propagated by purine/pyrimidine ligands, and the impact that each single ligand has on a variety of short- and long-term functions. A strict control system allows assorted, even divergent, biological outcomes. Among these, we enumerate cell-to-cell communication, tropic, trophic, but also noxious actions causing the insurgence/progression of pathological conditions. Here, we show that purinergic signalling in the nervous system can be instrumental for instance to neurodegenerative and neuroinflammatory diseases such as amyotrophic lateral sclerosis and multiple sclerosis.

Keywords: experimental autoimmune encephalomyelitis, microglia, mutant SOD1, oligodendrocytes, $P2X_7$ receptor, $P2Y_{12}$ receptor.

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Purinergic signalling

In the extracellular environment, purinergic and pyrimidinergic molecules target many distinct cell types and accordingly give rise to several different, even divergent, biological outcomes. These can culminate for instance into neurotransmission, muscle contraction, immune surveillance, or even fertilization, reproduction and development. Because of this heterogeneity, the purinergic and pyrimidinergic information can be properly defined as 'multipurpose' at a cellular and functional level.

The purinergic and pyrimidinergic signalling can continue for up to 30 s to 30 min, corresponding to the average half life of ATP outside the cells, and diffuse for about 300 $\mu m^2/s$, indicative of the approximate diffusion radius of ATP in the extracellular surroundings (Zimmermann *et al.* 1998). Within these space-time coordinates, the ATP concentration drops from 1–10 mM intracellularly, down to about 1–10 μM in the pericellular space, back down to 1 nM–1 μM extracellularly. This concentration range is wide enough to comprise all the K_M/V_{max} and K_B/B_{max} of the multiple purinergic and pyrimidinergic targets present at the plasma membrane. On the other hand, it is too wide to predict which specific targets will be functional at any time in each cell type. These comprise nucleotide and nucleoside transporters (Damaraju

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Address correspondence and reprint requests to Cinzia Volonté, CNR, Institute of Neurobiology and Molecular Medicine/Santa Lucia Foundation, Via Del Fosso di Fiorano 65, 00143 Rome, Italy.
E-mail: cinzia.volonte@inmm.cnr.it

Abbreviations used: ALS, amyotrophic lateral sclerosis; CD39, nucleoside triphosphate diphosphohydrolase-1; CD73, ecto-5′-nucleotidase; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; mSOD1, mutant SOD1; OPCs, oligodendrocyte progenitor cells; SOD1, superoxide dismutase 1.

et al. 2009), in addition to ectonucleotidases (Zimmermann 2006), P2 and P1 receptors for purinergic/pyrimidinergic nucleotides and nucleosides, respectively (Burnstock 2008a; Sebastião and Ribeiro 2009). The receptors are in turn classified into ionotropic P2X for ATP (seven distinct subtypes termed $P2X_{1–7}$ with different pharmacological and molecular properties have been identified so far in mammalian species), metabotropic P2Y for ATP, ADP, UTP, UDP, UDP-glucose (eight independent subtypes defined $P2Y_{1,2,4,6,11–14}$) and metabotropic P1 proteins for adenosine (A_1, A_{2A}, A_{2B}, A_3 subtypes).

ATP cannot be transported across lipid bilayers by simple diffusion, but it can pass the intact cellular membrane by either electrodiffusional movement through ATP release channels (Sabirov and Okada 2005) or facilitated diffusion by nucleotide-specific transporters (carriers or pumps) and vesicular exocytosis (Pankratov *et al.* 2006). In the nervous system, there is compelling evidence for exocytotic release of ATP from astrocytes and neurons under physiological conditions, whereas diffusion through ion channels is more likely to be involved in pathological conditions. For instance in response to ischemia, Domercq and co-workers recently described the pannexin-mediated mechanism by which oligodendrocytes release ATP (Domercq *et al.* 2010). Finally, ATP can be released through the damaged plasma membrane of basically all cell types via lytic mechanisms, during trauma, injury, apoptosis and necrosis. Also adenosine can move through the plasma membrane under different physiopathological conditions (Podgorska *et al.* 2005) and the in/out transporters (equilibrative and concentrative nucleoside transporters) are instrumental to the nucleoside salvage pathway.

In the extracellular space, the ectonucleotidases (namely ecto-nucleotide triphosphate diphosphohydrolases; ecto-nucleotide pyrophosphatase/phosphodiesterases; alkaline phosphatase non-specific phosphomonoesterases; and ecto-5′-nucleotidases, CD73) at last metabolize nucleotides consecutively down to nucleosides. As a result, all these transporters and enzymes work synergistically or consecutively to modulate ligand availability at the different P2/P1 purinergic receptors. A near-equilibrium phosphotransfer network thus exists among release-signalling-termination of purinergic and pyrimidinergic information, and the final duration, magnitude and direction of such information is coordinated by dynamic shifts between nucleotides/nucleosides consuming and regenerating pathways.

Purinergic cooperation and membrane domains

The purinergic/pyrimidinergic targets are not physically separated units, rather they associate with each other within multiprotein complexes and intermolecular interaction networks (Volonté *et al.* 2008a,b). For instance, ectonucleotidases form oligomeric complexes with P1 and P2 receptors (Schicker *et al.* 2009); also nucleoside and nucleotide transporters combine with P1 (Escudero *et al.* 2008) and P2 receptors (Jiang *et al.* 2005), respectively; A_1 and A_{2A} receptors associate with each other and form dimers, trimers and higher order complexes also with P2Y receptors (Schicker *et al.* 2009); finally, homo- and hetero-oligomeric complexes have been demonstrated within the P2X and P2Y subfamilies of receptors (D'Ambrosi *et al.* 2006; Köles *et al.* 2008). As a result, other than being multipurpose at the cellular and functional level, the biological information delivered by purinergic and pyrimidinergic ligands can be also defined as 'multidirectional' at the molecular level. This means that a single ligand can concomitantly or sequentially merge with various subtypes of receptors (although within different molecular affinities), with different classes of ectonucleotidases and transporters, even with assorted oligomeric complexes forming among these same targets. Moreover, a single ligand can give rise to several diverse metabolic ligands, with the final aim of amplifying, or attenuating, the original signal. A network of overlapping, mutually not exclusive, biological reactions and a dynamic cross-regulation of signalling is thus generated. A precise space-time coincidence is then the only possible framework where purinergic and pyrimidinergic signals can be operative, that is, perceived, discriminated, maintained and terminated.

This is accomplished in specialized submembrane compartments (lipid rafts, rafts-like structures, caveolae) that permit complex control systems involving molecular associations, cooperation, conformational or electronic state changes in receptors or channels. Indeed, compartments either exclude or include certain proteins, separate unrelated reactions, favour proper cooperative behaviour by decreasing the search time for an enzyme to find a substrate, or for a ligand to find a receptor. This leads to highly sophisticated cellular diversities in response to common epigenetic factors and/or modifications in the extracellular environment, and to a modelling of the cell architecture and biochemistry. Purinergic receptors, particularly A_1 (Escriche *et al.* 2003), A_{2A} (Mojsilovic-Petrovic *et al.* 2006), $P2X_{1,3,4,7}$ (Vacca *et al.* 2003; Garcia-Marcos *et al.* 2009), $P2Y_{1,2,4,6,12}$ subtypes (Bhatnagar *et al.* 2004; Kittel *et al.* 2004; Quinton *et al.* 2005; D'Ambrosi *et al.* 2007), as well as ectonucleotidases (Kenworthy and Edidin 1998; Delaunay *et al.* 2007) and nucleotide transporters (Schubert *et al.* 2002; Kowalski and Pier 2004) localize to lipid rafts/caveolae. Often, these proteins translocate out of the membrane micro-domains upon stimulation, and this could be interpreted as a different mechanism for regulation, coupling to effectors, or desensitization and inhibition. Disruption of lipid rafts by cholesterol sequestering agents can even shift the purinergic nucleotidases, transporters and receptors from raft to non-raft fractions, thus abolishing their ability to activate lipid signalling pathways and to integrate with additional signal-

ling events. This indicates that the topology of the purinergic components at the cell surface organizes the signal transduction machinery and contributes to its fine-tuning, by controlling the local kinetics of extracellular agonist metabolism and the integration with different purinergic signal inputs to generate the final cellular response.

In the present work, we describe in the nervous system that purinergic and pyrimidinergic signalling at the plasma membrane depends on finely integrated and simultaneous molecular reactions, and that the occurrence, modulation of expression and interactions of purinergic/pyrimidinergic targets are tightly specialized features in neuronal versus glial cells in different cerebral areas. In particular, we show that purinergic signalling is instrumental to neurodegeneration as well as neuroinflammation, and to diseases such as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a relentlessly progressive, fatal disorder with a pronounced delay between onset of symptoms and diagnosis, perhaps beyond the therapeutic window. It is the most common and aggressive form of adult-onset degeneration of both upper and lower motor neurons and their connections, leading to muscle weakness, atrophy, spasticity, dysarthria, dysphagia and eventually paralysis because of denervation. The patient loses the ability to initiate and control almost all voluntary movements, while cognitive functions are generally spared. Death generally occurs by respiratory failure within 1–5 years from onset (Cozzolino *et al.* 2008). Most of ALS cases are classified as sporadic, while 10–15% are inherited and defined as familial, but all affect the same neuronal population with comparable etiopathology (Rothstein 2009). The most frequent (20–25%) cause of familial ALS is linked to mutations in the ubiquitous free radical scavenger enzyme Cu, Zn superoxide dismutase 1 (SOD1), by far the strongest risk-conferring factor for ALS, with an almost complete penetrance (Chattopadhyay and Valentine 2009). Although a wealth of evidence has been collected especially from preclinical studies of transgenic animals expressing human mutant SOD1 (mSOD1) protein, scientists have neither found an unique key mechanism, nor an effective treatment for ALS, simply because this is a multifactorial and multisystemic disease (Chiò *et al.* 2005; Dion *et al.* 2009). Experiments in mSOD1 cell and animal models have established that the neurons do not die alone, but rather the process depends on the active participation of non-neuronal cells such as microglia, astrocytes, muscle and T cells, which differently contribute to the different phases of the disease (Appel *et al.* 2010). Expression of mSOD1 within the most susceptible motor neurons is a primary determinant for disease onset; synthesis of the mutant protein by interneurons also positively contributes to disease initiation. Neighbouring glial cells, especially astrocytes and microglia, then undergo mSOD1-mediated damage and cause acceleration of disease progression (Ilieva *et al.* 2009). A current hypothesis is that also Schwann (Chen *et al.* 2009) and muscle cells (Dupuis and Loeffler 2009), which are partnered to the injured motor axons, may be respectively recipients or initiators of the primary damage.

Purinergic signalling in ALS

Purinergic and pyrimidinergic transmission, one of the most conserved and wide-ranging extracellular signalling system, plays an unique role in integrating cellular circuits, because virtually every type of cell possesses the entire purinergic machinery (Volonté and D'Ambrosi 2009). As a general consequence, dysfunction and modulation of purinergic receptors, ectonucleotidases or transporters, in addition to altered levels of nucleotides/nucleosides in the extracellular environment, are instrumental to several forms of neurodegeneration and neuroinflammation characterized by a failure in the cellular communication network (Burnstock 2008b). Given that ALS is definitely a non-cell autonomous disease and that extracellular purine and pyrimidine molecules, through transporters, P2 receptors, ectonucleotidases and P1 receptors, constitute a well known neuron-to-glia alarm signal, purinergic mechanisms might definitely play a central role in the pathogenesis of this disease, also becoming an attractive novel strategy for fighting ALS. On this matter, Yiangou *et al.* (2006) have recently established that human postmortem ALS spinal cords having greater density of microglial/macrophages-like cells with increased cyclooxygenase-2 production in dorsolateral white matter (but not in dorsal columns spared in ALS), concomitantly display a greater density of $P2X_7$ receptor-positive microglial cells/macrophages. Negligible $P2X_7$-immunoreactivity is conversely found in grey matter from either control or ALS spinal cord. In mSOD1 rat at advanced disease stages, Casanovas and colleagues (Casanovas *et al.* 2008) confirmed that a conspicuous $P2X_7$ receptor immunolabeling in spinal cord clearly delineates microglial cells, in accordance with the abundance of activated microglia found in ALS patients (Yiangou *et al.* 2006). Further substantiating the relevance of purinergic mechanisms in ALS, Andries *et al.* (2007) obtained a 10% life span extension in the mSOD1 mice treated with ivermectin, an allosteric modulator of $P2X_4$ receptor. Casanovas and co-workers (Casanovas *et al.* 2008) then demonstrated in mSOD1 rat that a strong $P2X_4$-like protein immunoreactivity is present in degenerating motor neurons, but not in glial cells, in the ventral horns of spinal cord, that is, in tissue susceptible to ALS degeneration. Recruitment of microglial cells with neuronophagic activity is, moreover, observed in cerebral cortex and brainstem around neurons with positive $P2X_4$-like immunostaining. Neuronal populations other than motor neurons, such as

Purkinje cells in cerebellum, serotonin-containing neurons in raphe nucleus and noradrenergic neurons in locus coeruleus, elicit a similar pattern of modulation of $P2X_4$ receptor and degeneration. Finally, the decline in the number of motor neurons strictly correlated with the increase in $P2X_4$-like immunoreactive structures, and with the loss of motor performance (Casanovas *et al.* 2008). Further work also reported that parallel to neuronal degeneration and over-expression of the 42.7 kDa $P2X_4$ protein, a misfolded form of mSOD1 with newly exposed antigenic sites is also detected by the antibody against $P2X_4$ receptor in neurons, but not in glial cells. Intracerebral injections of this misfolded mSOD1 protein in control mice apparently activates microglia and astrocytes, suggesting its pathogenetic relevance (Hernández *et al.* 2010). Further confirming the potential involvement of purinergic signalling in ALS, an extended analysis of P2 receptors in primary and immortalized microglial cells from mSOD1 mice has shown strong up-regulation of $P2X_4$, $P2X_7$ and $P2Y_6$ receptors concomitantly to down-regulation of ATP-hydrolyzing activities (D'Ambrosi *et al.* 2009). Expression of $P2X_7$ protein on activated microglia is also proved in spinal cord tissue from mSOD1 mice (Fig. 1). The functional consequence of the described dysregulation of ATP receptors and degrading enzymes is the amplification of mSOD1 microglia inflammatory properties upon purinergic stimulation. In particular, activation of $P2X_7$ receptor by the preferential agonist 2′-3′-*O*-(benzoyl-benzoyl) ATP enhances the morphological transition of mSOD1 microglia into activated state, together with the content and release of the proinflammatory mediator tumour necrosis factor alpha and the induction of cyclooxygenase-2. These effects are prevented by the $P2X_7$ receptor preferential antagonist Brilliant Blue G. Remarkably, only microglia expressing mSOD1 and pre-activated by the $P2X_7$ receptor agonists exerts toxic effects toward neuronal cells (D'Ambrosi *et al.* 2009). In a similar way, endogenous ATP or extracellularly added 2′-3′-*O*-(benzoyl-benzoyl) ATP also causes astrocyte neurotoxicity in culture inducing motor neuron death. Mutant SOD1 astrocytes also display increased ATP-dependent proliferation and basal increase in extracellular ATP degradation, all prevented again by Brilliant Blue G (Gandelman *et al.* 2010). Taken together, these results clearly suggest that microglia, astrocytes and motor neurons might cross-talk via ATP release and $P2X_7$ receptor activation during the progression of the pathology, generating a feedback loop that drives a sustained pro-inflammatory and detrimental response. The inhibition of $P2X_7$ receptor might thus reduce neuroinflammation and motor neuron relapse in ALS by the decrease of microglia (D'Ambrosi *et al.* 2009) and astrocyte activation (Gandelman *et al.* 2010). Moreover, a systemic inhibition of $P2X_7$ receptor can also directly rescue the motoneuronal population, as exposure to ATP leads to spinal cord neuron death and administration of $P2X_7$ receptor antagonists protects motor neurons and promotes functional recovery after injury (Wang *et al.* 2004; Peng *et al.* 2009).

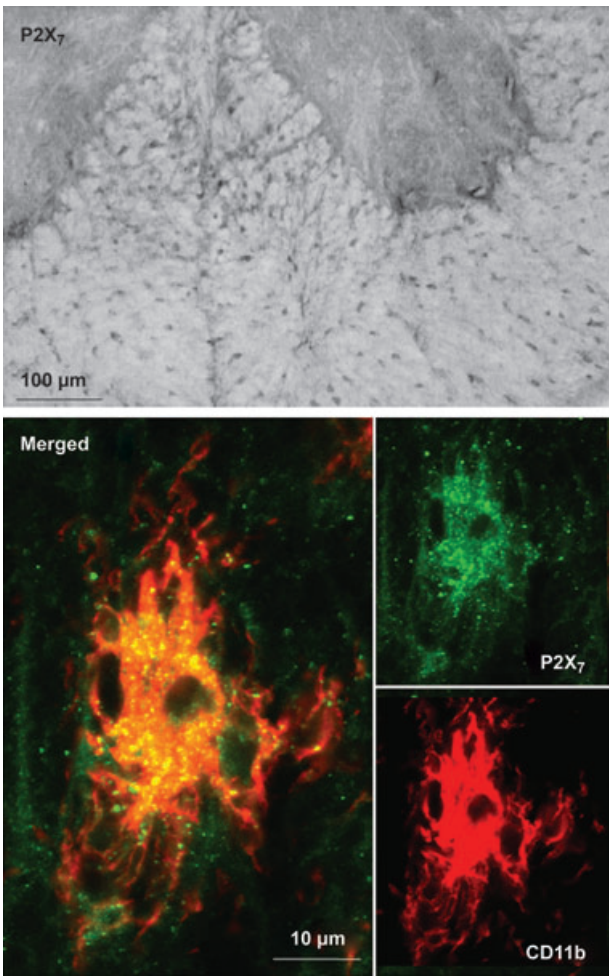

Fig. 1 $P2X_7$ receptor is present in mSOD1 mouse and co-localizes with CD11b in activated microglia. Lumbar sections from mSOD1 spinal cord were subjected to immunohistochemical staining using antiserum for $P2X_7$ receptor and 3,3′-diaminobenzidine as a chromogen. Scale bar: 100 µm. Double immunofluorescence and confocal microscopy analysis was performed using antibodies for $P2X_7$ receptor (green, Cy2 immunofluorescence) and for mouse complement type 3 receptor (CD11b, microglia marker) (red, Cy3 immunofluorescence). Co-localization is visualized in yellow. Scale bar: 20 µm (left panels).

Also adenosine receptors and particularly the A_{2A} subtype has a renowned role in ALS. In spinal cord cultures, antagonists of A_{2A} receptor significantly protect motor neurons from toxicity directly following the expression of mSOD1. This occurs through inhibition of TrkB receptor trans-activation by A_{2A} receptors (Mojsilovic-Petrovic *et al.* 2006).

A strict balance and mutual interaction exists between extracellular ATP and adenosine, and between P2 and P1 receptor occupancy (Volonté *et al.* 2008a). This is tightly

Table 1 Purine and pyrimidine agonists/antagonists, receptors, enzymes and transporters in ALS

	Effects	References
$P2X_7$ receptor	Inflammation	Yiangou *et al.* (2006); Casanovas *et al.* (2008)
BzATP ($P2X_7$ agonist)	Microglia/astrocyte inflammation, TNF-α release, COX-2 activation, neurodegeneration	D'Ambrosi *et al.* (2009); Gandelman *et al.* (2010)
BBG ($P2X_7$ antagonist)	Neuroprotection	D'Ambrosi *et al.* (2009); Gandelman *et al.* (2010)
$P2X_4$ receptor	Neurodegeneration, loss of motor performance	Andries *et al.* (2007) ; Casanovas *et al.* (2008)
ATP (acting at $P2X_4$ receptor)	Motor neuron survival or death, in the absence or presence of ivermectin, TNF-α, COX-2 activation	Andries *et al.* (2007); D'Ambrosi *et al.* (2009)
Ivermectin ($P2X_4$ allosteric modulator)	Motor neuron survival or death in the absence or presence of ATP	Andries *et al.* (2007)
$P2Y_6$ receptor	Neuroinflammation	D'Ambrosi *et al.* (2009)
UDP ($P2Y_6$ agonist)	Morphological effect on microglia	
A2 receptor	Neurotoxicity	Mojsilovic-Petrovic *et al.* (2006)
KW6002 (A_{2A} antagonist)	Neuroprotection	
MRS1754 (A_{2B} antagonist)	No effect	
Apyrase (ectonucleotidase)	Inhibition of astrocyte proliferation	Gandelman *et al.* (2010)
P-glycoprotein (ATP-transporter)	Up-regulation during neurodegeneration	Boston-Howes *et al.* (2008)

BzATP, 2′(3′)-*O*-(4-Benzoylbenzoyl)ATP; BBG, Brilliant Blue G; COX-2, cyclooxygenase-2; TNF-α, tumour necrosis factor alpha.

regulated by the activity of both ectonucleotide metabolizing enzymes and nucleoside/nucleotide transporters. While no information is still available about the role of ectonucleotidases in ALS patients, the efflux transporter P-glycoprotein responsible for releasing endogenous ATP into the extracellular environment is up-regulated in patients with impaired neurological conditions characterized by inflammatory processes. This is demonstrated also in spinal cord of ALS mice, where the content of P-glycoprotein that is barely detectable at disease onset remarkably increases during disease progression (Boston-Howes *et al.* 2008).

In general, these results suggest that induction of the ALS phenotype especially through mSOD1 expression indeed modifies the overall purinergic signalling in motor neurons, microglia and astrocytes (Table 1). A shift in the dynamic equilibrium regulating the purinergic/pyrimidinergic biomolecular network present at the plasma membrane and involving P2/P1 receptors, ectonucleotidases and purine/pyrimidine transporters might in our opinion constitute a previously overlooked pathogenetic feature in ALS.

Multiple sclerosis

Multiple sclerosis is an inflammatory demyelinating disease of the CNS in which autoreactive myelin-specific T cells cause extensive tissue damage resulting in neurological deficits. In the early disease process, T cells are primed in the periphery by antigen presenting dendritic cells, crucial regulators of specific immune responses. In the chronic phase, particularly oligodendrocytes, myelin and axons degenerate in the CNS, causing numerous symptoms often progressing into physical and cognitive disabilities. MS patients can be affected by a relapsing/remitting early form of the disease, but a large proportion of the patients soon evolves into primary and secondary progressive phases (Rovaris *et al.* 2006). Although MS is in general regarded as a white matter disease, the incidence of demyelination and oligodendrocyte or neuron/axon injury are prominent and widespread in grey matter too (Stadelmann *et al.* 2008). MS lesions are abundant in cerebral cortex (Lassmann 2007), where they constitute a significant proportion of the overall pathology of the brain, with a particularly high prevalence of plaques being observed in progressive stages of the disease. In addition to changes to oligodendrocytes and neurons, current knowledge also emphasizes an important dual role for astrocytes and microglia in MS (He and Sun 2007). Astrocytes, for instance, can promote inflammation, damage to oligodendrocytes and axons, formation of the glial scar but, at the same time, can support migration, proliferation and differentiation of oligodendrocyte progenitors (Williams *et al.* 2007). Likewise, microglia may play an essential causative function in MS pathogenesis, but also restore the damaged tissue (Muzio *et al.* 2007; Sanders and De Keyser 2007). As a result, all glial cells are likely to play significant parts in both the destructive and restorative phases of MS. Hence, a major challenge in MS research is to discern the conditions and factors that might contribute to the outcome of this unsteady equilibrium.

Purinergic signalling in MS

Extracellular purine/pyrimidine nucleotides and nucleosides are among the most widespread exogenous signals playing important either detrimental or protective roles in neuron-to-glia and glia-to-glia communication, in the normal and injured brain (Inoue *et al.* 2007; Apolloni *et al.* 2009). However, not much is known regarding purinergic signalling and MS. Not only ATP, but also adenosine can directly modulate migration, proliferation, and differentiation of oligodendrocyte progenitor cells (OPCs). Adenosine inhibits OPCs proliferation while promotes OPCs differentiation and myelination (Stevens *et al.* 2002), and stimulates OPCs migration via A_{1A} receptors (Othman *et al.* 2003). ATP would instead trigger proliferation, migration, and differentiation of OPCs primarily via several different P2Y receptors (Morán-Jiménez and Matute 2000; James and Butt 2001; Agresti *et al.* 2005), and activation of P2 receptors evokes Ca^{2+} signals in OPCs and oligodendrocytes *in situ* and in culture (James and Butt 2001; Alberdi *et al.* 2002; Agresti *et al.* 2005; Butt *et al.* 2005). Thus, the general opinion is that axons release adenosine and ATP during propagation of action potential, in order to control oligodendrocyte development, with an overriding role for adenosine in stimulating terminal differentiation, and for ATP in promoting myelination also via astroglial components (Ishibashi *et al.* 2006). A recent work has hypothesized that extracellular ATP might directly contribute to MS lesion-associated release of interleukin-1β via $P2X_7$ receptor-dependent induction of cyclooxygenase-2 protein and downstream pathogenic mediators (Yiangou *et al.* 2006), and the $P2X_7$ receptor is described as one among the subtypes predominantly expressed in differentiated oligodendrocytes (Yu *et al.* 2008). Consistently, Matute and co-workers (Matute *et al.* 2007) have shown that oligodendrocytes and myelin indeed express functional $P2X_7$ receptor mediating cell death *in vitro* and *in vivo*. Activation of $P2X_7$ receptor, moreover, contributes to tissue damage in experimental autoimmune encephalomyelitis (EAE) pathology (an animal model for studying MS). Finally, $P2X_7$ receptor blockade prevents oligodendrocyte excitotoxicity and ameliorates EAE and receptor expression is even increased in MS human tissue before lesion formation (Matute *et al.* 2007). It was also demonstrated that mice deficient in $P2X_7$ receptor function are less susceptible to EAE than wild-type mice, also showing reduced CNS inflammation (Sharp *et al.* 2008). However, authors have also reported that $P2X_7$ receptor knockdown displays a reduction in interleukin-1 and -6, with concomitant decrease in lymphocytic apoptosis and exacerbation of the EAE phenotype (Chen and Brosnan 2006). Also the $P2X_4$ subtype is probably involved in EAE pathology, being expressed by macrophages infiltrating in the brain and spinal cord from early and asymptomatic phase, to recovery phase of EAE. Moreover, the kinetics of accumulation of $P2X_4$ receptor in macrophages are parallel to those of infiltration and disease severity, therefore suggesting a likely role for this receptor in immunoregulation during CNS inflammation (Guo and Schluesener 2005). In addition, by analyzing the distribution pattern of all P2 receptors in sections of cerebral cortex from postmortem MS brains, a clear immunoreactive signal for $P2X_1$ protein is found in blood vessels on cells of the haematopoietic origin; $P2X_{2,4}$ receptors appear localized in grey matter neuronal nuclei; a strong signal for $P2X_3$ protein is present only in degenerating cortical pyramidal neurons in grey matter, and for $P2Y_{2,11}$ in the entire frontal cortex. $P2Y_{6,14}$ immunoreactivities are instead very weak and localized to small areas. Finally, $P2X_6$ and $P2Y_1$ receptors seem absent from white and grey matter MS frontal cortex, whereas $P2X_5$, $P2Y_{4,13}$ proteins could not be detected (Amadio *et al.* 2010). The metabotropic $P2Y_{12}$ receptor is instead abundantly expressed in myelin and interlaminar

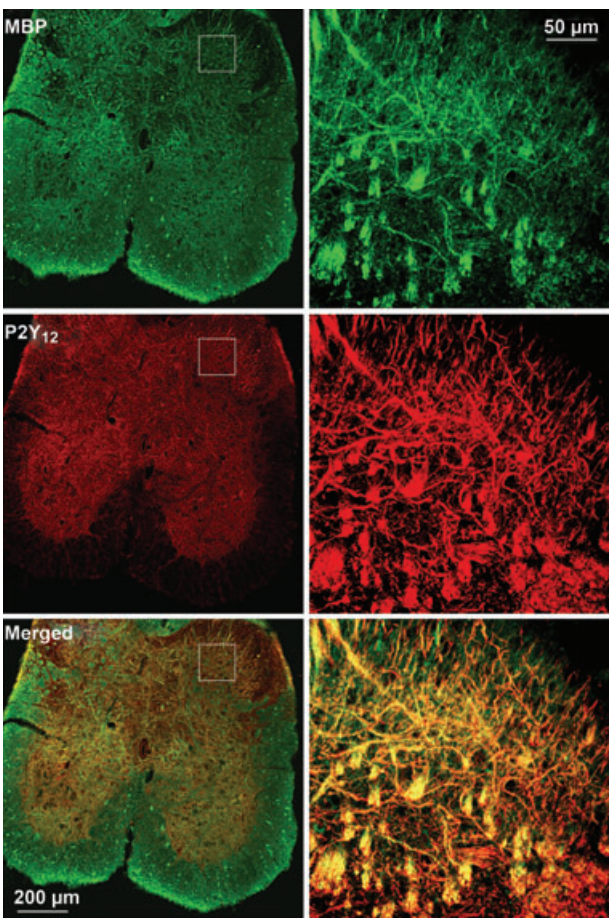

Fig. 2 $P2Y_{12}$ receptor is present in EAE mouse and co-localizes with myelin basic protein in oligodendrocytes. Double immunofluorescence and confocal microscopy analysis was performed on sacral sections from EAE spinal cord using antibodies for myelin basic protein (green, Cy2 immunofluorescence) and $P2Y_{12}$ receptor (red, Cy3 immunofluorescence). Co-localization is visualized in yellow. Scale bars: 200 μm (left panels), 50 μm (right panels).

astrocytes, but absent from protoplasmic astrocytes of the deeper cortical layers, absent from microglia/macrophages and from intact demyelinated axons in MS brain. Moreover, a decreased $P2Y_{12}$ protein in proximity to the lesions is directly correlated with the extent of demyelination found in all types of grey matter cortical plaques and subcortical white matter. It was hence suggested that loss of purinergic $P2Y_{12}$ receptor might be detrimental to tissue integrity in MS (Amadio *et al.* 2010). This is still to be confirmed in the EAE mouse model, where the $P2Y_{12}$ receptor is shown to co-localize with myelin basic protein in spinal cord sacral sections (Fig. 2).

Because of the strong immunosuppressive and anti-inflammatory properties of adenosine, dysfunction particularly of the A_1 adenosinergic system in the CNS has been implicated in the development of MS in humans and EAE in animals. In particular, the A_1 receptor expressed principally on cells of monocyte/macrophage lineage in both brain and blood, is selectively diminished in MS patients, potentially leading to increased macrophage activation and CNS inflammation. This suggests that modulation of neuroinflammation by A_{1A} receptors may represent a novel mechanism providing new therapeutic opportunities for MS and other demyelinating diseases (Johnston *et al.* 2001). Caffeine, a non-selective adenosine receptor antagonist indeed provides protection against myelin oligodendroglia glycoprotein-induced EAE in mice, not by inhibition of adenosine receptors, but by up-regulation of A_1 receptors and transforming growth factor-beta mRNAs and suppression of interferon-gamma mRNA (Chen *et al.* 2010). Caffeine decreases the incidence of EAE and attenuates EAE pathology at behavioural, histological (inflammatory cell infiltra-

Table 2 Purine and pyrimidine agonists/antagonists, receptors, enzymes and transporters in MS

	Effects	References
ATP, α,β-metATP (P2X agonist), 2MeSATP (P2Y agonist)	Calcium mobilization in glial cells	James and Butt (2001)
ATP, 2MeSATP	Myelination	Ishibashi *et al.* (2006)
$P2X_7$ receptor	Induction of COX-2	Yiangou *et al.* (2006)
	Neuroinflammation	Chen and Brosnan (2006)
	Development of disease	Sharp *et al.* (2008)
ATP, BzATP ($P2X_7$ agonist)	Oligodendrocyte, tissue damage	Matute *et al.* (2007)
PPADS (P2X antagonist), OxATP, BBG ($P2X_7$ antagonist)	Oligodendrocyte survival, Reduced demyelination	
$P2X_4$ receptor	Neuroinflammation	Guo and Schluesener (2005)
ATP (acting at $P2X_7$, $P2Y_1$), ADP/ADPβS ($P2Y_1$ agonist)	OPCs migration, inhibition of proliferation	Agresti *et al.* (2005)
MRS2179 ($P2Y_1$ antagonist)	Reverse of effects	
$P2Y_1$ receptor	To be defined	Morán-Jiménez and Matute (2000)
$P2X_{1\text{-}4}$, $P2Y_{2,6,11,14}$ receptor	To be defined	Amadio *et al.* (2010)
$P2Y_{12}$ receptor	Correlation to myelination	Amadio *et al.* (2010)
Adenosine NECA (A_{1-3} agonist)	Inhibition of OPCs proliferation, induction of myelination	Stevens *et al.* (2002)
MRS1191, DPCPX (A_{1-3} antagonists)	Reverse of effects	
Caffeine (A_{1-3} antagonist)	Neuroprotection by A_1 receptor up-regulation	Chen *et al.* (2010)
A_{1A} receptor	Neuroinflammation	Johnston *et al.* (2001); Tsutsui *et al.* (2004)
CPA (A_{1A} receptor agonist)	OPCs migration	Othman *et al.* (2003)
SCH58261 (A_{2A} receptor agonist)	Neuroprotection	Mills *et al.* (2008)
CD_{73} (ectonucleotidase)	Contribution to INF-β effects	Airas *et al.* (2007)
	Vulnerability to EAE development	Mills *et al.* (2008)
CD_{39} (ectonucleotidase)	Neuroprotection	Borsellino *et al.* (2007); Fletcher *et al.* (2009)
Pyrophosphatase/phosphodiesterase (ectonucleotidase)	Neuroprotection	Spanevello *et al.* (2010a,b)

α,βmetATP, α,β-methyleneATP; BBG, Brilliant Blue G; BzATP, 2′(3′)-*O*-(4-Benzoylbenzoyl)ATP; COX-2, cyclooxygenase-2;CPA, N6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; EAE, experimental autoimmune encephalomyelitis; INF-β, interferon-beta; NECA, *N*-ethylcarboxamido-adenosine; 2Me-SATP, 2-methylthio ATP; OPCs, oligodendrocyte progenitor cells; OxATP, oxidized ATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid.

tion and demyelination) and neurochemical (expression of inflammatory cytokines) levels (Chen *et al.* 2010). Consistently, A_{1A} receptor null ($A_{1A}R-/-$) mice develop a more severe progressive/relapsing form of EAE compared with their wild-type ($A_{1A}R+/+$) littermates, together with worsened demyelination, axonal injury, and enhanced activation of microglia/macrophages (Tsutsui *et al.* 2004). Finally, blockade of adenosine receptor signalling by the A_{2A} receptor-specific antagonist SCH58261 also protects wild-type mice from EAE induction (Mills *et al.* 2008), but no information is yet available on the contribution of the additional A_{2B} and A_3 adenosine receptor subtypes during MS in humans and EAE in animals.

Adenosine is generated from breakdown of AMP by CD73 (ecto-5′-nucleotidase), a cell surface enzyme of the purine catabolic pathway. Mills *et al.* (2008) demonstrated the presence of CD73 on brain endothelial cells choroid plexus epithelium, which regulates lymphocyte immunosurveillance between blood and cerebrospinal fluid. Moreover, they reported that CD73 is required for the efficient entry of lymphocytes into the CNS during EAE development, as CD73(−/−) mice with preserved T cell responsiveness are instead resistant to EAE. As interferon-beta is known to increase the expression of CD73 on endothelial cells, both *in vitro* and after systemic treatment of MS patients *in vivo*, as detected in brain samples taken at autopsy, it was, however, postulated that CD73-derived adenosine might in part contribute to the therapeutic effects of interferon-beta (Airas *et al.* 2007). Modulation of ATP, ADP and AMP hydrolysis was further confirmed to operate during EAE and suggested to represent the basis of novel therapeutic strategies in immune-mediated diseases such as MS. As a matter fact, after induction of EAE, the hydrolysis of ATP, ADP and AMP shows a significant decrease in blood serum, but a prominent increase in spinal cord membrane preparation, compared to control groups (Lavrnja *et al.* 2009). Moreover, it was demonstrated that CD39 (nucleoside triphosphate diphosphohydrolase-1), an ectonucleotidase which hydrolyzes ATP, is expressed only on a subset of human natural T regulatory cells, primarily by immune-suppressive Foxp3(+) regulatory effector/memory-like T cells, which play a central role in maintaining self-tolerance. Notably, patients with the relapsing/remitting form of MS have strikingly reduced numbers of CD39(+)T regulatory cells in the blood (Borsellino *et al.* 2007). Recent findings also suggested that CD4(+) CD25(+)Foxp3(+)CD39(+) T regulatory cells play an important role in constraining autoimmune pathogenesis, and their reduction in MS patients might thus lead to inability to control interleukin-17 mediated autoimmune inflammation (Fletcher *et al.* 2009). In addition to CD73 and CD39, also ectonucleotide pyrophosphatase/phosphodiesterase and adenosine deaminase (enzymes responsible for extracellular ATP/adenosine metabolism and for altering the levels of nucleotides and nucleosides in the circulation) are modulated in lymphocytes and decreased in platelets of relapsing/remitting MS patients, thus contributing to alterations in lymphocytes and platelets function in MS (Spanevello *et al.* 2010a,b).

All these results are the clear evidence that a general shift in the dynamic equilibrium regulating the purinergic biomolecular network present at the plasma membrane likely constitutes a previously overlooked pathogenetic feature not only in ALS, but also in MS (Table 2).

Conclusion

What we have learned so far about the involvement of purinergic mechanisms in the insurgence/progression of neurodegenerative and neuroinflammatory conditions such as ALS and MS, is that a large-scale interplay occurring at the plasma membrane among receptors, enzymes and transporters drives the overall response of neurons and glial cells to purine and pyrimidine ligands. Not a single interaction becomes privileged on another, but a choral event showing space and time coincidence explains the multipurpose and multidirectional nature of the purinergic/pyrimidinergic information. In other words, we can say that purinergic/pyrimidinergic signalling at the plasma membrane is built up of innumerable layers and each layer is worth exploring, as long as we do not forget that it is simply one of many.

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