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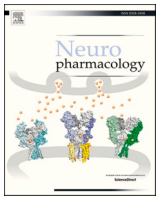

Extracellular ATP: A powerful inflammatory mediator in the central nervous system

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ABSTRACT

Nucleotides play a crucial role in extracellular signaling across species boundaries. All the three kingdoms of life (Bacteria, Archea and Eukariota) are responsive to extracellular ATP (eATP) and many release this and other nucleotides. Thus, eATP fulfills different functions, many related to danger-sensing or avoidance reactions. Basically all living organisms have evolved sensors for eATP and other nucleotides with very different affinity and selectivity, thus conferring a remarkable plasticity to this signaling system. Likewise, different intracellular transduction systems were associated during evolution to different receptors for eATP. In mammalian evolution, control of intracellular ATP (iATP) and eATP homeostasis has been closely intertwined with that of Ca^{2+}, whether in the extracellular milieu or in the cytoplasm, establishing an inverse reciprocal relationship, i.e. high extracellular Ca^{2+} levels are associated to negligible eATP, while low intracellular Ca^{2+} levels are associated to high eATP concentrations. This inverse relationship is crucial for the messenger functions of both molecules. Extracellular ATP is sensed by specific plasma membrane receptors of widely different affinity named P2 receptors (P2Rs) of which 17 subtypes are known. This confers a remarkable plasticity to P2R signaling. The central nervous system (CNS) is a privileged site for purinergic signaling as all brain cell types express P2Rs. Accruing evidence suggests that eATP, in addition to participating in synaptic transmission, also plays a crucial homeostatic role by fine tuning microglia, astroglia and oligodendroglia responses. Drugs modulating the eATP concentration in the CNS are likely to be the new frontier in the therapy of neuroinflammation.

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1. Introduction

Information exchange has always been a crucial issue in the human society as well as in the biological world, especially when information is needed to signal danger and avoid life-threatening situations. Therefore, it is likely that ever since the first forms of life appeared on earth, primordial cells developed elementary means to detect potentially harmful situations, and what is more alarming and potentially harmful than cell injury or even death of nearby cells (Di Virgilio and Giuliani, 2022)? The most obvious mean to sense danger would be to develop surface receptors for intracellular factors (damage-associated molecular patterns, DAMPs) that are normally absent or present at a very low concentration in the external world, and whose changes due to cell damage could be easily detected due to the high signal-to-noise ratio. *Mutatis mutandis*, this is the same rationale behind pathogen-sensing via PAMPs (pathogen-associated molecular patterns): it is the detection of something which is "out-of-place" that alerts nearby cells that something is wrong and potentially dangerous in their neighborhood environment (Bianchi, 2007).

PAMP- or DAMP- sensing alerts cells that molecules belonging to a different life kingdom, or which are normally segregated within the intracellular milieu, respectively, are present in the extracellular space (Janeway and Medzhitov, 2002) (Matzinger, 1994). Ever since primordial cells learnt to use inorganic phosphate and purines to synthesize nucleotides for energy metabolism and for genetic coding these phosphorylated species became very abundant intracellularly, and as a result were depleted from the extracellular milieu (Ponnamperuma et al., 1963) (Galimov, 2009) (Wimmer et al., 2021). This compartmentalization of phosphorylated compounds likely contributed to speed up evolution of living organisms. It is speculated, but not generally accepted, that massive depauperation of phosphate compounds from the extracellular environment was a consequence of the increase in Ca^{2+} content of the early aquatic habitat caused by the progressive acidification of the primordial oceans. Phosphate (PO_4^{3-}), generated by ATP hydrolysis, readily precipitates as calcium phosphate [$Ca_3(PO_4)_2$] in the

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Abbreviations

Extracellular ATP eATP
intracellular ATP iATP
P2 receptors P2Rs
P2X7 receptor P2X7R
central nervous system CNS
damage-associated molecular patterns DAMPs
pathogen-associated molecular patterns (PAMPs)
systemic inflammatory response syndrome SIRS
plasma membrane luciferase pmeLUC
multi organ failure MOF
cortical spreading depression CSD
G protein-coupled receptor GPCR
G protein-coupled receptor activation-based, GRAB
pannexin-1 Panx-1
Src family kinases SFKs
adenosine deaminase ADA

presence of elevated Ca^{2+} concentrations (Kazmierczak et al., 2013). Memory of the low Ca^{2+}, moderately alkaline and phosphate-rich environment of the early oceans was arguably preserved in the intracellular milieu that is still now characterized by low Ca^{2+} (in the 10^{-7} M range) and high ATP (in the 10^{-3} M range) concentrations. This is also reflected in the biochemical composition of the body of multicellular organisms (e.g. mammals) where Ca^{2+} and ATP concentrations in the extracellular fluid are in the 10^{-3} and 10^{-9} range, respectively (Plattner and Verkhratsky, 2016). Therefore, it is likely that the extreme paucity in well separated but interacting environments laid the basis for the evolution of Ca^{2+} and ATP into ubiquitous and essential intracellular and extracellular messengers, respectively (Plattner and Verkhratsky, 2016).

Existence of a large concentration gradient across the boundary between different compartments is a prerequisite for a biological mediator to have a messenger function since on one hand it allows to generate, with an insignificant expense of energy, a sharp increase in concentration upon release of a minute amount of the putative messenger molecule, and on the other allows a rapid return to pre-stimulation levels by removal of a small amount of the released messenger. Thus, Ca^{2+} and ATP have become universal intracellular and extracellular messengers, respectively. The parallel but topologically distinct functions of Ca^{2+} and ATP show additional intriguing similarities. Generation of the intracellular Ca^{2+} signal is fundamental for cell well-being: controlled increases in the cytoplasmic Ca^{2+} concentration support cell trophism, promote proliferation, drive motility and expression of differentiated responses (Pinto et al., 2015) (Tonelli et al., 2012), but on the contrary uncontrolled increases trigger cell death, whether by necrosis, necroptosis, pyroptosis, or apoptosis (Cheng et al., 2006) (Danese et al., 2021). Likewise, moderate increases in the eATP concentration provide a healthy cell stimulation by supporting differentiation, proliferation, motility, and secretion of neurotransmitters or growth factors (Di Virgilio, 2000). On the contrary , uncontrolled eATP accumulation may ignite a detrimental tissue response either by direct cytotoxicity or by starting inflammation (Vultaggio-Poma et al., 2022). An unchecked elevation of the cytoplasmic Ca^{2+} concentration signals a severe inability of the cell to preserve the biochemical features of its inner environment, and thus to maintain its vital functions, while a large increase in the eATP concentration signals cell inability to maintain the integrity of its boundaries with the outer world, and thus warns nearby cells of this dangerous, often pre-lytic, situation. In a few words, if a bold parallel is allowed, Ca^{2+} might be considered an "intracellular" while eATP an "extracellular" danger signal.

2. ATP as an intercellular messenger

How nucleotides originated in the pre-biotic world is unknown, but it is clear that the appearance of the pyrophosphate bonds (phosphate groups linked via a P–O–P bond) was a critical step in evolution since it made available a remarkably reactive biological molecule [ATP is one of the most reactive molecules on earth (Verkhratsky and Burnstock, 2014)] capable of releasing a very large amount of energy upon hydrolysis of the phosphoanhydride bonds, often indicated with a "squiggle" (~) in the ATP molecule (Lipmann, 1941). It is speculated that in the early phases of molecular evolution, ATP became a universal energy currency due to its versatility as a phosphate donor under thermodynamic (ATP ↔ ADP + Pi) or kinetic (ATP → AMP + PPi) control (Wimmer et al., 2021). Once primordial cells started to exploit nucleotides to store genetic information and as energy currency, ATP and other phosphorylated compounds were accumulated by the cells, causing an exponential increase in their intracellular concentration and a parallel depletion from the extracellular milieu. In other words, nucleotides, chiefly ATP, became biochemical hallmarks of the intracellular milieu.

It is thought that ATP-gated plasma membrane ion channels (the P2X receptor, P2XR, ancestors) appeared very early in evolution (Verkhratsky and Burnstock, 2014) (Verkhratsky, 2021). Since cell surface receptors initially evolved to sense the chemical composition of the extracellular world, it might be speculated that P2XRs were initially used to detect extracellular PPi, which was plenty in the prebiotic world (Wimmer et al., 2021), and became then essential for the development of intracellular energy metabolism. At later times, P2XRs acquired the ability to detect eATP. This might have been a primeval shift of P2XRs from "environmental sensors" to sensor of distress and avoidance signals released from neighboring cells. Arguably, the primordial "distress and avoidance signals" (the primeval DAMPs) were small molecules released by dying organisms, such as amino acids or nucleotides (Verkhratsky and Burnstock, 2014). Two very ancient sensors of distress are the tetrameric glutamate and the trimeric ATP ionotropic receptors that have conserved their ligand selectivity throughout evolution up to the present times (Verkhratsky and Burnstock, 2014). Incidentally, this established an intriguing functional parallel between these two receptor families in the central nervous system (CNS), where both excessive glutamatergic (excitotoxicity) and purinegic (eATP, neuroinflammation) stimulations are injurious (Olney, 1971).

Although not obvious for students focused on energy metabolism and intracellular ATP generation and turnover, this nucleotide is an ideal extracellular messenger. In fact, its extracellular levels can be increased several fold upon release of minute amounts thanks to the very low concentration in the extracellular space under physiological conditions (estimated to be in the 10^{-9}–10^{-8} M range, thus virtually close to zero), while its intracellular concentration is in the 5×10^{-3}-10^{-2} M range, i.e. at least five-six orders of magnitude higher. This concentration gradient, together with the outward directed electric gradient (ATP bears two to four negative charges, therefore being repulsed by the negative inside plasma membrane potential), allows a large and rapid ATP release following even minute breaks in plasma membrane integrity. But ATP efflux also easily occurs upon activation of non-lytic release pathways. Once in the extracellular space, ATP swiftly diffuses through the aqueous interstitial milieu owing to its high hydrophilicity, thus allowing the interaction with multiple specific receptors expressed by virtually all cell types (Vultaggio-Poma et al., 2020). Ubiquitous extracellular nucleotidases allow rapid termination of the signal and prevent desensitization (Giuliani et al., 2020). These properties epitomize the essential features of the ideal extracellular messenger molecule that should be characterized by (a) very high signal-to-noise ratio, i.e. low if not zero extracellular concentration; (b) high water solubility to allow unrestricted diffusion through the aqueous tissue interstitium; (c) presence of specific cell receptors to allow faithful signal decoding; (d) rapid degradation to prevent overstimulation or receptor desensitization.

A controlled release system increases the efficiency of an extracellular messenger generation system, but probably sophisticated mechanisms to control release were a late development in the evolution of eATP signaling. Currently, many different controlled release pathways are known ranging from plasma membrane channels and transporters to stimulated or constitutive exocytosis (Giuliani et al., 2020) (Taruno, 2018). These pathways are variably involved in eATP homeostasis in different tissues and in different pathophysiological conditions. Once in the extracellular environment, eATP is hydrolysed by at least four enzyme systems: the ecto-nucleoside triphosphate diphosphohydrolyses (E-NTPDases), the ecto-nucleotide pyrophosphatases phosphodiesterases (E-NPPs), the ecto 5′-nucleotidase (5′-NT), and ecto-alkaline phosphatases (Zimmermann et al., 2012). As recently illustrated by Nicholas Dale in a simulation of diffusion of ATP released from a point source, ecto-nucleotidases, that quickly degrade this nucleotide to adenosine, establish rigid constrains to its diffusion away from the release site (Dale, 2021). Thus, eATP increases in the pericellular space are anticipated to be highly localized and restricted to the release site. These simulations suggest that in order to build up and maintain the pericellular "eATP halo" highlighted by the plasma membrane-expressed probe (plasma membrane luciferase, pmeLUC) (Pellegatti et al., 2005) cells must constantly release this nucleotide. On the other hand, to fulfil its role as a signaling molecule eATP must necessarily diffuse away from the release point, which may require either strong stimulation of release or a transient inhibition of ecto-nucleotidase activity.

3. Role of eATP in systemic inflammation and as an universal inter-kingdom signaling molecule

All organisms, from prokaryotes to mammals, respond to the application of ATP, and most (if not all) living organisms release this nucleotide (Verkhratsky and Burnstock, 2014) (Spari and Beldi, 2020). Heterotrophic bacteria are reported to secrete elevated amounts of eATP in a process dependent on the properties of different attachment surfaces (Ivanova et al., 2006). Relevant to human diseases, ability to secrete eATP was investigated in various commensal bacteria, among which *Enterococcus gallinarum*, that was found to cause an accumulation of up to 1–3 μM eATP in the culture medium (Iwase et al., 2010). Since intestinal bacteria are suggested to cause colitis by enhancing Th17 cell differentiation via secretion of eATP (Atarashi et al., 2008), this finding might be of relevant pathophysiological significance given the pathogenic role played by Th17 cells in different tissues, CNS included (Jiang et al., 2022). Many bacterial species (e.g. *Escherichia coli* and *Staphylococcus aureus*), both Gram-positive and negative, are now known to release eATP by non-lytic mechanisms especially during the exponential growth phase and depending on glucose supplementation (Hironaka et al., 2013) (Mempin et al., 2013). There is no doubt that eATP accumulates to varying levels into the supernatant of bacterial cultures, but more importantly, eATP is present in the small intestine and in the portal vein of specific pathogen free mice to a concentration of 60–80 μM and 0.3–0.4 μM, respectively, and to a considerably lower level in germ-free mice (Proietti et al., 2019), showing that enteric bacteria produce a large amount of eATP that permeates across the intestinal wall and reaches systemic circulation.

Changes in the systemic blood eATP concentration may affect several distal organ systems, among which possibly the CNS. It is well documented that byproducts of gut bacterial metabolism and its alterations (for example during dysbiosis) may reach the brain via the circulation or even the vagus nerve, negatively affecting CNS homeostasis and paving the way to the ignition of neuroinflammation and neurodegeneration. Several molecules with potential adverse effects on CNS are secreted or released by the intestinal flora (e.g. tryptophan metabolites, trimethylamine-N-oxide, LPS, the amyloid-like curli protein) have been identified as pathogenic factors in experimental autoimmune encephalomyelitis, stroke or Parkinson's disease (Bostick et al., 2022). More complex is the interpretation of the role that short chain fatty acids have on neuroinflammation since butyrate, which induces Treg in the gut (Furusawa et al., 2013), is beneficial, while acetate may be beneficial but also an aggravating factor of amyloid-beta-dependent pathology (Erny et al., 2021). Whether eATP might be one of those "toxins" of bacterial origin released by a dysbiotic gut is not known, but is a possibility worthy of close scrutiny in view of its now well established pro-inflammatory role. Grassi and co-workers postulated that the near universal ability of bacteria to release ATP and the ubiquitous expression of eATP-sensing receptors in mammals make eATP a universal inter-kingdom signalling molecule (Proietti et al., 2019). In this more general sense, eATP goes above its function as a DAMP and may be considered a PAMP, i.e. a general signal of danger and distress. In fact, by sensing eATP immune cells are not only alerted of the presence of cell damage but in principle also of eATP-releasing foreign organisms. As it is always the case in the endless host/pathogen relationship, bacteria-derived signaling molecules are processed by host cells in multiple fashions, generating a multiplicity of different pathophysiological responses, protective but in some cases unwillingly detrimental. Immune cells may be alerted by eATP sensed as a bacterial metabolite and by consequence start a defensive response, but at the same time their differentiation is modulated by eATP. Effects of eATP maybe larger on those cells that express P2 receptors to a higher level (or ecto-nucleotidases to a lower level), an example being follicular T helper (Tfh) cells in the intestinal mucosa (Proietti et al., 2014) (Perruzza et al., 2017) (Salles et al., 2017). Thus, eATP released by enteric bacteria might also indirectly affect the inflammatory homeostasis of the CNS by shaping the maturation of the immune system. The intestinal tract is indeed a perfect system to investigate the role of bacteria-derived eATP as an interkingdom messenger due to the large bacterial flora and the tight interaction with host tissues. Tfh cells express the P2X7R to a very high level, thus making them sensitive to large increases in the eATP concentration, and therefore also to the well-known pro-apoptotic effects of this nucleotide (Proietti et al., 2014). Depletion of the mucosal Tfh cell population hinders the ability to support Tfh-mediated differentiation of B lymphocyte into SIgA-secreting plasma cells, thus decreasing SIgA levels and immunity against intestinal pathogens. Extracellular ATP derived from commensal bacteria may also promote differentiation of Th17 lymphocytes from naïve Th cells. Takeda and co-workers showed that administration of the ATP analogue ATPγS stimulated secretion of IL-6, IL-23 and TGF-β from a subset of $CD11^{+}$ lamina propria cells identified as $CD70^{high}$ $CD11c^{low}$, positive for myeloid markers such as CD11b and F4/80 (Atarashi et al., 2008), and accordingly increased Th17 differentiation of naïve $CD4^{+}$ lymphocytes. Stimulation by commensal bacteria-derived eATP might have a relevant role in the differentiation of "pathogenic" Th17 cells implicated in several inflammatory disorders of the bowel as well as of the CNS (Jiang et al., 2022). Pathogens do not only release eATP but also trigger ATP release from host cells. This may be conducive to pathogen survival and dissemination, or alternatively might support a host defense response (Spari and Beldi, 2020). Certain bacterial toxins such as hemolysin or LPS can trigger ATP release from cells as a different as erythrocytes or microglia (Skals et al., 2009) (Ferrari et al., 1997); released eATP can then feedback on the injured or nearby cells to activate cytolytic P2 receptors such as P2X1R and P2X7R, and cause amplification of the lytic process (Skals et al., 2009) (Skals et al., 2014).

Accumulation of eATP in the blood raises the issue of the contribution of eATP to the pathogenesis of sepsis, and more in general to systemic inflammatory response syndrome (SIRS). Sepsis is a life-threatening systemic response unleashed by the massive release of inflammatory cytokines caused by bacterial infections (Singer et al., 2016), but a similar pathophysiological picture can also be triggered by sterile inflammation driven by a massive release of DAMPs (Cauwels et al., 2014) (Cheng et al., 2020). Thus, both septic (PAMP-driven) and sterile (DAMP-driven) SIRS can trigger a life-threatening shock reaction that may culminate in the dreadful clinical condition of multi organ failure (MOF), and severe CNS involvement. Current mortality rates for

patients with SIRS are estimated to be below 10%, while septic shock mortality rates are about 40%, and in those patients that develop MOF mortality rates range from 45% to 75% and even higher (Rangel-Frausto et al., 1995) (Baue et al., 1998). Thus, generalized inflammation and its possible evolution to shock and MOF are still in need of an effective treatment. Extracellular ATP, whether released from host or pathogen cells, is likely to be a triggering as well as an amplifying factor in this cascade of events suggesting that use of agents aimed at reducing its blood concentration might be a useful therapy (Cauwels et al., 2014). So far very few reliable measurements of blood ATP levels in disease states are available, but all show an increased concentration versus healthy subjects, and a correlation with disease severity (Forrester, 1966), (Hlapcic et al., 2019) (Kauffenstein et al., 2018) (Moritz et al., 2017) (Ledderose et al., 2022).

4. ATP as an inflammatory mediator in the central nervous system

The eATP role as a neurotransmitter has been a focus of investigation for several decades now. It has long been known that eATP is an excitatory transmitter or co-transmitter in the CNS (Edwards et al., 1992) (Nieber et al., 1997), and it is also well established that cognate eATP receptors, P2XRs and P2YRs, are widely expressed in the CNS (Khakh and North, 2012). However, over the years, it has also become clear that eATP has additional important roles outside synapses. Neurons, microglia and astroglia release substantial amounts of ATP via several pathways, e.g. pannexin-1, connexins, secretory granules and the P2X7 receptor (P2X7R) (Illes et al., 2019) (Seo et al., 2021) (Montero and Orellana, 2015), thus generating eATP waves that diffuse across broad areas of the brain in various pathophysiological conditions, as for example during cortical spreading depression (CSD) (Schock et al., 2007). Cortical spreading depression, an interesting example of the pathogenic role of eATP in the brain, consists of a slowly-propagating wave of suppressed electrical activity that is considered the neurological substrate of migraine aura (Costa et al., 2013). However, CSD may also protect the brain from subsequent insults such as for example ischemia (Costa et al., 2013) (Shen et al., 2016). It has been shown that induction of CSD causes a fast and transient eATP increase in the CNS interstitium up to 100 μM (Schock et al., 2007), sufficient to activate all P2R subtypes, and possibly even the low affinity P2X7R.

Many agents and conditions cause increases in the brain eATP concentration: traumas, neuronal hyperexcitation, ischemia, infection and inflammation (Pedata et al., 2016). It was initially thought that ATP release was a passive phenomenon due to cell injury, but now several well-characterized non-lytic ATP release pathways have been identified, all active in the CNS. A seminal early study by Nedergaard and co-workers showed that mechanical insult of the spinal cord caused a local, delayed, ATP release lasting up to 6 h (Wang et al., 2004), that was only marginally due to the direct effect of the trauma, but on the contrary mainly due to the ensuing late stimulation of active release mechanisms. However, as a partial correction of these early data, a more refined technique of bioluminescence imaging now available demonstrates that the trauma itself (i.e. controlled cortical injury) causes both a fast (within 10 min) and a delayed (lasting for several minutes) eATP increase in brain tissue (Faroqi et al., 2020), confirming the late increase reported by Nedergaard and co-workers (Wang et al., 2004). Besides trauma, other challenges were reported to trigger a wave of eATP diffusion across brain tissue. Electrical stimulation generates a self-replicating eATP wave diffusing at about 2 mm/min (Kitajima et al., 2020), a speed compatible with that of neuronal depolarization during CSD (Kitajima et al., 2020). Direct and indirect experimental evidence suggest that an eATP wave is responsible for the diffusion of ischemic injury in the brain (Braun et al., 1998) (Melani et al., 2005) (Pedata et al., 2016) via both a direct P2X7R-mediated cytotoxic action and an indirect, but still P2X7R-mediated, pro-inflammatory activity. *In vivo* experiments with a transgenic mouse developed in our laboratory, constitutively expressing the bioluminescent eATP probe pmeLUC, the pmelUC-TG-mouse, neatly show that several minutes after temporal middle cerebral artery occlusion an eATP increase occurs in the ipsi- but not contra-lateral hemisphere (Wilmes et al., 2022). The eATP increase persists for up to 24 h, well after the initial necrotic death of brain cells, suggesting an ongoing release in the post-necrotic inflamed tissue.

Systemic inflammation has also been shown to cause an eATP increase in the brain, whether measured with fluorescent (Wu et al., 2022) or luminescent probe (Fig. 1). Recording LPS-promoted eATP release in the brain performed with the pmeLUC-TG-mouse is of special relevance because it does not require any previous potentially harmful procedure such as surgery or viral vector inoculation, thus minimizing possible manipulation artifacts. The increase in CNS eATP during systemic inflammation might suggest that eATP increases are associated with fever, however the few data available are controversial. By a amperometric microelectrode biosensor system in rabbits injected intravenously with LPS, Gourine and co-workers showed that in parallel with the initial increment of the febrile response there was a burst of ATP release in anterior hypothalamus, which suggests that ATP might be involved in the pathogenesis of fever (Gourine et al., 2007). However, and rather paradoxically, these authors also reported that intra-hypothalamic administration of purinergic blockers prolonged rather than inhibit the febrile response. They interpreted this finding as indicating that the eATP increase observed at the inception of the febrile response is more likely a feed-back response aimed at limiting the temperature increase than a feed-forward amplification mechanism. However, microelectrode-based eATP measurement is prone to serious mechanical artifacts that may cause cell injury and artifactual ATP release. This is not trivial as it is well known that ATP release pathways can be activated by even minor cell perturbations. In this regard, it is important to stress that the report by McLean and co-workers is one of the few documenting real time eATP changes in the CNS *in vivo* (Faroqi et al., 2020).

After we introduced pmeLUC, other laboratories generated and validated novel probes for the measurement of eATP. These are mostly fluorescent probes that have some advantages over luminescence, but also drawbacks, since while on the one hand fluorescent probes allow a better spatial resolution, on the other lower tissue permeability of emitted light precludes investigation of tissue more than 1–4 mm thick. Furthermore, auto fluorescence is an always present source of artifacts. A few fluorescent eATP indicators generally based on two components have been recently proposed and some used to measure eATP in the brain: a) a binding protein specific for ATP that triggers a conformational change, and b) a fluorescent moiety that converts the structural change into fluorescence signal (Wu and Li, 2020). Along these lines, several approaches have been proposed. A widely adopted strategy is to use genetically-encoded G protein-coupled receptor (GPCR) activation-based (GRAB) sensors, i.e. a GFP-based sensor coupled to a P2Y receptor, as a specific ATP-binding moiety, such as the one engineered by Wu et al. (2022). Alternatively, Tantama and co-workers have engineered an ATP/ADP-binding bacterial protein named Parm with FRET-inducing cyan and yellow fluorescent proteins fused either at the apical, surface-exposed loops of the protein, or at the N- and C- termini (Trull et al., 2019). The epsilon subunit of F0F1-ATPase from *Bacillus PS3* coupled to a circularly permuted superfolder GFP has also been used as a binding structure for ATP (Lobas et al., 2019). Finally, the F0F1-ATPase was used a FRET-based Team ATP-sensor modified to be expressed on the plasma membrane (ecATeam3.10) (Conley et al., 2017).

Thus, multiple pieces of evidence support the view that brain injury/stimulation triggers a self-amplifying eATP wave spreading across brain tissue. In principle, this wave of ATP release might be a mere epiphenomenon of brain cell damage/stimulation: given an intracellular ATP concentration in the millimolar range (10^{-3} M), and an eATP concentration in the nanomolar range (10^{-9}), the chemical gradient, further enhanced by the electrical gradient negative inside, driving ATP efflux

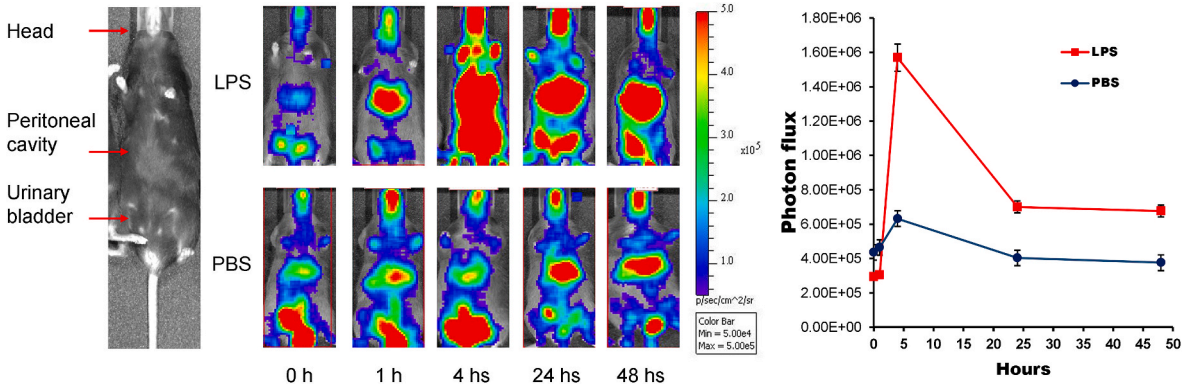

Fig. 1. Time course of systemic increase in the eATP concentration caused by intra peritoneal injection of LPS (100 μg) into the pmeLUC-Tg-mouse. This mouse expresses constitutively the pmeLUC probe, thus it signals increases in the eATP concentration generated in virtually any body compartment. Administration of LPS triggers a widespread luminescence increase (i.e. eATP increase), including the brain. Please note the increased luminescence in the bladder, also present in the control mouse (PBS) due to elimination of luciferin via the urinary route. The right hand panel shows the time course of luminescence increase highlighting the peak increase at 4 h after LPS injection. Data are shown as average ± SE of triplicate determinations.

outside the cell is very large (10^5–10^6 fold). However, ATP release dependent on membrane damage should be self-limiting while on the contrary all available data show that, even at site of injury or in the perilesion areas, the eATP increase persists for tens of minutes or even hours. In addition, the wave-like diffusion of the eATP increase is clear evidence for an active process (Wang et al., 2004) (Faroqi et al., 2020). Many years ago, we proposed that eATP could be the trigger of additional ATP release, generating a regenerative, self-amplifying process, or in other words an "ATP-induced ATP release" (Di Virgilio, 2006), very much akin the "Ca^{2+}-induced Ca^{2+} release" well known to muscle students (Rios, 2018). Of course, postulating the "ATP-induced ATP release mechanism" calls for the identification of eATP-activated, ATP conductive, plasma membrane pathways. An obvious candidate is pannexin-1 (Panx-1), the ubiquitous, CNS expressed, plasma membrane oligomeric channel permeable to aqueous molecules of MW up to 1 kD (Chiu et al., 2018). Interestingly, the Panx-1 channel can be opened not only by increases in intracellular Ca^{2+}, as for example those triggered by P2YR stimulation, but also by an increase in the extracellular K^+ concentration, an ionic dysregulation known to occur in CNS disturbances such as CSD (Pietrobon and Moskowitz, 2014). A role in the activation of Panx-1 has also been suggested for Src family kinases (SFKs) (Lohman et al., 2015), c-Jun NH_2-terminal kinases (Xiao et al., 2012) and protein kinase G (Poornima et al., 2015), intracellular pathways known to be activated during brain injury. Furthermore, Panx-1 can be irreversibly activated by cleavage of its C-terminal tail mediated by apoptotic caspases 3, 7 or 11 (Chiu et al., 2018). Extracellular alkalinization has also been shown to trigger Panx-1 permeability increase, a finding relevant in CNS pathophysiology since a rise in pH is recorded at the inception of CSD (Pietrobon and Moskowitz, 2014). In addition, a major role in sustaining ATP release and eATP propagation in the CNS might be played by the P2X7R. This receptor is thought to be associated with Panx-1 channels (Pelegrin and Surprenant, 2006), thus supposedly mediating Panx-1 opening in response to an eATP increase, but more cogently has been shown to be a conduit for ATP release (Pellegatti et al., 2005) (Suadicani et al., 2006). Direct activation of the P2X7R by eATP would be a very efficient way to trigger a regenerative wave of eATP propagation. Incidentally, the interstitial biochemical milieu of CSD would be strongly supportive of an eATP-mediated P2X7R activation since in this condition the extracellular Ca^{2+} concentration is known to plummet from the physiological 1.0/1.5 mM level to 0.2/0.8 mM (Pietrobon and Moskowitz, 2014), thus substantially lowering the ATP threshold for P2X7R activation. It is in fact well known, although often overlooked, that affinity of the P2X7R is much higher for ATP in its tetra-anionic form (ATP^{4-}) than in the divalent (Ca^{2+} or Mg^{2+})-complexed form (ATP^{2-}) (Cockcroft and Gomperts, 1980) (Di Virgilio, 1995). Lowering divalent cation concentration of the interstitial CNS fluid may cause a 10–20 fold increase of eATP affinity for the P2X7R (Steinberg and Silverstein, 1987) (Greenberg et al., 1988).

Although measurements of the eATP increases in the CNS interstitial fluid are currently only semiquantitative, based on the *in vivo* recordings made in mouse brain expressing the pmeLUC sensor (Faroqi et al., 2020) it can be estimated that post-traumatic levels up to 100 μM and higher can be achieved, thus sufficient to open the P2X7R macropore, especially in the presence of low Ca^{2+}/Mg^{2+} concentrations and at a slightly alkaline pH, thus promoting a large ATP efflux (Di Virgilio et al., 2018). On this bases, it can be hypothesized that at the initiation of CSD a coordinated series of events precipitating P2X7R opening occur in the CNS interstitium: 1) a sudden eATP increase; b) an extracellular Ca^{2+} drop; c) a pH increase (Fig. 2). Gating of the P2X7R by eATP on one hand allows further ATP release, thus powering the regenerative eATP wave, and on the other aggravates the ionic unbalance allowing massive K^+ efflux and Na^+ and Ca^{2+} influx. Such cation fluxes are of special relevance as CSD is specifically characterized by a large increase in the extracellular K^+ and a decrease in the extracellular Ca^{2+} and Na^+ concentrations. Ca^{2+} and Na^+ influx will of course cause an increase in the intracellular levels of these two cations, which is another feature of CSD, as well as a trigger

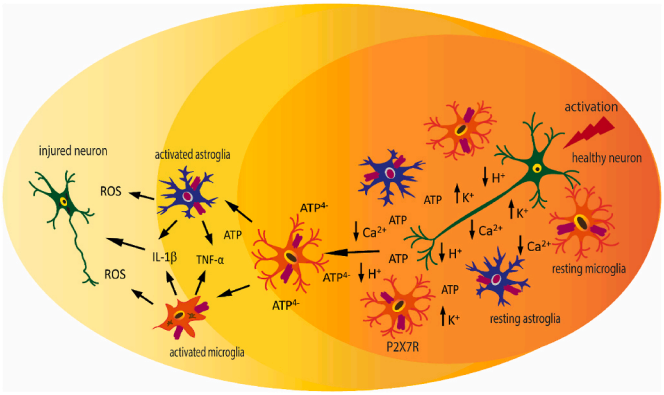

Fig. 2. The ATP wave during cortical spreading depression. It is hypothesized that ionic conditions in brain interstitial milieu (alkaline pH, high K^+, low Ca^{2+}) during cortical spreading depression (CSD) increase eATP affinity for the P2X7R (Gudipaty et al., 2001), at least in part due to accumulation of eATP in its tetra-anionic form ($eATP^{4-}$) (Cockcroft and Gomperts, 1980) (Steinberg and Silverstein, 1987). In fact, affinity of the P2X7R for $eATP^{4-}$ is 10–20 fold higher than for the divalent-complexed form ($eATP^{2-}$), thus enhancing P2X7R-dependent microglia and astroglia activation, and the associated release of inflammatory cytokines and other neurotoxic factors.

(namely the cytosolic Ca^{2+} increase) of Panx-1 activation. The intracellular K^+ depletion, consequent to the extracellular K^+ increase, is a triggering factor for caspase activation (Hughes et al., 1997), and thus a stimulus for Panx-1 cleavage and activation (Taylor et al., 2015). Quite obviously, turning on a powerful pro-inflammatory receptor such as the P2X7R has additional far reaching implications on CNS pathophysiology because this receptor is one of the most potent stimulants of the NLRP3 inflammasome, thus promoting the release of IL-1β and of many other pro-inflammatory cytokines (Di Virgilio et al., 2017). These inflammatory mediators will act on glial cells, blood vessels, and nerve terminals to generate the train of changes associated to migraine aura and pain (Pietrobon and Moskowitz, 2014).

Long-standing evidence supports the view that ischemia causes massive ATP release *in vivo* into brain interstitium (Juranyi et al., 1999) (Melani et al., 2005), and *in vitro* during anoxic depolarization in hippocampal slices (Frenguelli et al., 2007). Released ATP then either acts as an extracellular messenger in its own right or is degraded to adenosine by plasma membrane ecto-nucleotidases (Pedata et al., 2016). Ischemic stroke causes sterile inflammation, a leading cause of post-ischemic brain injury, ignites release of a wealth of alarm factors, among which eATP, as shown by *in vivo* recording with the pmeLUC probe (Wilmes et al., 2022). The post-ischemic eATP increase contributes to the generation of a strong pro-inflammatory milieu that critically aggravates brain damage.

While on the one hand eATP acts as a stimulant and an amplifier of inflammation, on the other hand it is also the main precursor of adenosine which, in the brain as in the periphery, is a powerful immunosuppressant, and therefore a neuroprotectant (Cunha, 2001) (Pedata et al., 2016). Thus, ATP release sets also the conditions for the activation of an anti-inflammatory feed-back mechanism. Whether the pro-inflammatory or anti-inflammatory response prevail depends on the balance of various factors: eATP release, eATP hydrolysis by ecto-nucleotidases, adenosine removal via cellular uptake, or adenosine degradation to inosine by extracellular adenosine deaminase (ADA). Extracellular ATP might also have a more subtle regulatory role in the complex dynamic interaction of glial cell with neurons, as recently shown by Schaefer and co-workers (Badimon et al., 2020), by recruiting microglia protrusions at the site of neuronal activity (i.e. at the synapses) and in the downmodulation of neuronal firing. Key events in this process are stimulation of microglia $P2Y_{12}$Rs and microglia-dependent adenosine production that acts at neuronal adenosine A1Rs to suppress firing (Lin et al., 2020) (Badimon et al., 2020). Overall, microglia has a central role in the regulation of synaptic activity owing to its ability to control neuronal activity by "pruning" exorbitant dendrites or remove damaged or excess synapses by phagocytosis.

Extracellular ATP has additional homeostatic functions, as for example in the fine tuning of cortical circuits involved in the modulation of mood. Sound experimental evidence shows that a physiological level of ATP release is necessary to sustain a trophic activity of P2X2R in the medial prefrontal cortex and thus prevent the insurgence of depression-like symptoms (Lin et al., 2022) (Cao et al., 2013). Astrocyte-derived eATP was shown to be necessary for the anti-depressant effect promoted by deletion of soluble epoxide hydrolase (Xiong et al., 2019). Calhm2 channels seem to be a main pathway for ATP release from astrocytes as their deletion caused decrease of eATP levels, reduction of hippocampal spines, neuronal dysfunction, and depression-like behavior (Jun et al., 2018). In this regard, purinergic signaling has been variably involved in the pathogenesis of depression with an hypothesized beneficial regulatory function for P2X2Rs (Cao et al., 2013) and a putatively adverse function of P2X7Rs (Bhattacharya and Jones, 2018) (Zhang et al., 2022), emphasizing the crucial hormetic function of eATP, whereby a basal physiological concentration is necessary (not to low, not to high) to allow healthy neuronal activity, while on the contrary an uncontrolled increase is a trigger of CNS damage.

5. Conclusions

ATP is also dubbed "the fuel of life". However, as we proposed several years ago (Di Virgilio et al., 1989) (Di Virgilio, 1998) (Di Virgilio, 2000), ATP may be a harbinger of death when acting from outside the cell. More generally, release at sites of inflammation makes ATP a nearly ubiquitous and very versatile inflammatory mediator (Di Virgilio et al., 2001) (Di Virgilio et al., 2020). The CNS is the most energy consuming tissue in the body: about 20% of total body oxygen, 25% of glucose, 20% of ATP consumption against only 2% of total body mass. This underlines the relevance of intracellular ATP metabolism in the brain. Now novel studies emphasize the parallel fundamental role of eATP in brain homeostasis not just as a neurotransmitter but rather as a general homeostatic factor, both directly and via the generation of its major metabolite adenosine. Maintaining an adequate control on eATP levels in the CNS interstitium is of fundamental importance for neuronal physiology, synaptic activity, dendrite arborization, astrocyte and microglia function and in one word for brain health. Targeting excessive eATP increases may open novel and up to now unfathomed avenue for the prevention and therapy of neuroinflammation and neurodegeneration.

Authors' contributions

All authors contributed to conceptualization, writing and revision of the review.

Declaration of competing interest

FDV is a member of the Scientific Advisory Board of Biosceptre Ltd, a biotech Company involved in the development of anti-P2X7 antibodies, and a Consultant with Axxam SpA. The other authors declare no conflict of interest.

Data availability

Data will be made available on request.

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