Adenosine as a multi-signalling guardian angel in human diseases: when, where and how does it exert its protective effects?

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Abstract

The importance of adenosine for human health cannot be overstated. Indeed, this ubiquitous nucleoside is an integral component of ATP, and regulates the function of every tissue and organ in the body. Acting via receptor-dependent and -independent mechanisms – the former mediated via four G-protein-coupled receptors, A_1, A_{2A}, A_{2B}, and A_3, – it plays a significant role in protecting against cell damage in areas of increased tissue metabolism, and combating organ dysfunction in numerous pathological states. Accordingly, raised levels of adenosine have been demonstrated in epilepsy, ischemia, pain, inflammation and cancer, in which its behaviour can be likened to that of a guardian angel, even though there are instances in which an overproduction of adenosine is pathological. This review condenses the current body of knowledge on the issue, highlighting when, where and how adenosine exerts its protective effects in both the brain and the periphery.

Keywords: adenosine, adenosine receptors, ischemia, pain, inflammation, cancer.

Adenosine as a protective agent

The purine nucleoside adenosine is a ubiquitous molecule whose importance for human health cannot be overstated. Indeed, it is the backbone of adenosine triphosphate (ATP), and regulates the functions of every tissue and organ [1], mainly, but not solely, through the activation of a family of four G-protein-coupled receptors (GPCRs), A_1, A_{2A}, A_{2B}, and A_3. Interestingly, A_1 and A_3 subtypes have an inhibitory effect on adenylyl cyclase (AC) activity, while A_{2A} and A_{2B} stimulate it, with a consequent modulation of cyclic AMP levels [1]. Although the affinity of adenosine for these receptors may vary, depending on the type of test used to evaluate it [2], adenosine seems to present a higher affinity for A_1, A_{2A} and A_3 than for the A_{2B} subtype [3].

Adenosine can be produced intracellularly, through the hydrolysis of AMP or S-adenosyl-homocysteine (SAH) by an intracellular 5'-nucleotidase or SAH hydrolase, respectively. However, ATP dephosphorylation, orchestrated by the ectonucleoside triphosphate diphosphohydrolase CD39 and the 5'-nucleotidase CD73 [1], is the main mechanism behind high extracellular adenosine levels. The bioavailability of adenosine depends upon its transformation to inosine through adenosine deaminase (ADA), of which there are intracellular and extracellular forms, and/or intracellular transport via nucleoside transporters. Once inside the cell, adenosine is phosphorylated to AMP or degraded to inosine by adenosine kinase (ADK) and ADA, respectively (Figure 1).

The formation of adenosine is strictly dependent on the metabolic state of a cell. Normally, the extracellular concentration of adenosine spans the low nanomolar range, but its levels rise during conditions involving increased metabolic demand and/or lack of oxygen, for example pathological states such as epilepsy, ischemia, pain, inflammation and cancer. The physiological actions of adenosine all tend to redress an imbalance between energy demand and availability, earning it the reputation of "retaliatory metabolite" [3]. Adenosine-mediated tissue protection, and, as a consequence, preservation of organ function, involves four main mechanisms, namely increasing/rebalancing the oxygen supply/demand ratio, preconditioning, anti-inflammatory effects, and stimulation of angiogenesis [4].

In this review we highlight this guardian angel role of adenosine in various disease states, describing when, where and how adenosine exerts its protective effects against cell damage in both the brain and the periphery.

Adenosine and epilepsy

There is a huge body of evidence that adenosine is an inhibitory modulator of brain activity, and its anticonvulsant effects, mediated by both receptor-dependent and independent pathways, have been demonstrated in several experimental models of epilepsy [5]. The ability of adenosine to prevent or ameliorate seizures induced by pentylenetetrazole, pilocarpine, NMDA, bicuculline, organophosphate treatment, and electrical stimulation has been attributed essentially to A_1 receptor activation, which inhibits pre-synaptic excitatory neurotransmitter release and hyperpolarizes the post-synaptic cell membrane [6]. Indeed, an upregulation of the A_1 receptor has been reported as a consequence of spontaneous seizures triggered by electrical stimulation [7]. Furthermore, both upregulation of the protective A_1 receptor and downregulation of the proconvulsant A_{2A} subtype have been shown in the cerebral cortex after hyperthermia-induced seizures, suggesting the existence of a specific neuroprotective mechanism [8]. The same study also showed a concomitant reduction of the adenosine-generating 5'-nucleotidase, suggesting that this may be the means by which the A_{2A} receptor effects of adenosine are attenuated, enabling adenosine to fulfil its protective role in epilepsy.

Interestingly, activation of the A_1 receptor subtype has been linked to the antiepileptic effects of a ketogenic diet (KD), a low-carbohydrate, high-fat diet protocol prescribed to treat epilepsy [9,10], and KD treatment has been shown to increase adenosine levels and reduce DNA methylation [11]. This follows, because adenosine does exert receptor-independent effects in DNA methylation homeostasis [12]. This process, triggered by DNA methyltransferases and mediated by S-adenosylmethionine (SAM)-dependent transmethylation of DNA, results in the production of

adenosine, whose removal via ADK increases the transmethylation pathway potentially implicated in epileptogenesis [13] (Figure 2). Indeed ADK is overexpressed in epileptogenic brain areas, where it induces seizures. This would seem to suggest the use of ADK inhibitors in epilepsy therapy, but, unfortunately, the chronic systemic use of these agents leads to liver toxicity, as well as cognitive and sedative side effects [14]. It is therefore vital to find alternative strategies for increasing adenosine levels in epilepsy. To this end, gene therapy directed to ADK through an antisense oligonucleotide is being explored as a means of conserving adenosine by reducing ADK expression [15,16]. Promisingly, adenosine has also been delivered directly to the brain ventricles of epileptic rats, thereby reducing DNA methylation and slowing disease progression [12].

Although there is still much work to be done, there is every indication that agents able to increase adenosine availability may have a place in the future treatment of epilepsy.

Adenosine and ischemia

Adenosine appears to play a role as an endogenous mediator of neuroprotection in the homeostatic response to changes occurring during ischemia and stroke. Indeed, by activating A_1 receptors, this nucleoside hinders Ca^{2+} influx, thereby inducing pre-synaptic inhibition and a reduction in the release of excitatory neurotransmitters. In addition it increases the conductance of K^+ and Cl^- ions, mediating a fall in neuronal excitability and playing a key role in ischemic preconditioning (IP) [17,18]. By these means, adenosine is able to reduce cellular metabolism and energy consumption in ischemia within a few hours. Moreover, during a later phase of ischemia, (i.e., in the hours and days after the insult), adenosine also exerts beneficial peripheral effects via activation of A_{2A} receptor.

The ability of A_{2A} receptor activation to inhibit platelet aggregation, mediate vasodilation, restrict leukocyte infiltration and curb the inflammatory response is crucial for attenuating neuroinflammation after ischemia [17]. By suppressing neuroinflammation, A_{2A} receptors may also

produce positive effects on neurogenesis, and those located in the CNS are also known to provide a degree of neuroprotection against brain ischemia through the increase of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which act to restore brain activities [19]. Furthermore, adenosine may limit hypoxia-triggered inflammation and vascular damage in ischemia via stimulation of A_{2B} and A_3 receptors. These slow neutrophil infiltration, promote angiogenesis, and inhibit migration of the microglia and monocytes in ischemic areas, respectively [20] (Figure 3).

Brain IP, a process by which repeated short, sublethal insults protect the tissue against subsequent ischemic damage, is an appealing therapeutic approach against stroke. However, the molecular mechanisms underlying IP, which include NMDA glutamate receptors, nitric oxide synthase, cytokines, oxidative stress and inhibition of immune cells, have only been partially defined. Nevertheless, adenosine is known to be an important mediator of this phenomenon, acting by triggering A_1 receptors, which are able to reduce glutamatergic excitotoxicity induced by overstimulation of NMDA receptors [21].

The protective effect of IP has also been observed in other tissues, particularly the heart, in which it has been most widely investigated, and where it has been found to act by stimulating A_1, A_{2B} and A_3 receptors. The molecular mechanism of cardioprotection induced by A_1 receptors has been attributed to regulation of mitochondrial ATP-dependent K^+ channels through activation of protein kinase C (PKC). This prevents the destruction of the mitochondrion and consequent cell death in myocytes [22].

The protective effects of A_{2B} have been attributed to its ability to stabilize the circadian transcription factor period (Per)2, thereby flicking a hypoxia-inducible factor (HIF)1-dependent metabolic switch essential for the adaptation of myocytes to ischemia by promoting more oxygen-efficient utilization of carbohydrates [23,24]. Moreover, A_{2B} receptor signalling has recently been ascribed different cardioprotective functions through its actions in several different tissues. Indeed, its effects in IP are exerted through the vascular endothelial cells and cardiac myocytes, while its

actions on inflammatory cells are critical for attenuating reperfusion injury after ischemia (IR) [25]. However another critical cardioprotective effect of adenosine during IP has been observed in cardiac mast cells, in which both A_{2B} and A_3 subtypes trigger a signalling cascade of PKC-ε and aldehyde dehydrogenase type-2. This prevents renin release from mast cells and therefore the dysfunctional consequences of the local renin-angiotensin system activation responsible for reperfusion arrhythmias [26]. However it is difficult to exploit IP as a therapy because it is not usually possible to anticipate an ischemic event before it occurs. It is also unfeasible to maintain IP indefinitely as a prophylactic treatment.

Nevertheless, there is significant evidence that A_3 receptor activation exerts a cardioprotective effect, both prior to ischemia and during reperfusion [27-31]. Post-ischemic protection may occur through the inhibition of either neutrophil-induced IR or apoptotic cell death in myocytes [1,32,33]. The cardioprotective effect of one A_3 agonist, IB-MECA, has recently been attributed to A_3-receptor desensitization, and the indirect activation of A_{2A} receptors on bone marrow-derived cells. This suggests that blockade of reperfusion injury by A_{2A} agonists added at reperfusion may be a promising strategy [34]. Indeed the beneficial role of A_{2A} receptors has been very well described. Treatment with A_{2A} agonists has been reported to reduce tissue injury during reperfusion in different organs, like the liver, kidney, lung, heart, skin and spinal cord, by reducing the neutrophil accumulation, pro-inflammatory cytokine/oxygen free radical release, endothelial cell activation, microvascular occlusion, and platelet aggregation that can exacerbate tissue injury during reperfusion of previously ischemic tissues [35-37].

Adenosine or compounds modulating adenosine signalling therefore represent a promising therapeutic means of both protecting the myocardium and enhancing its recovery after reperfusion [38]. In fact, encouraging results, in the form of a lower rate of microvascular obstruction, have been already obtained in a randomized, placebo-controlled trial entitled Intracoronary Nitroprusside Versus Adenosine in Acute Myocardial Infarction (REOPEN-AMI) [39-41]. In light of this, a

clinical trial to assess the cardioprotective effects of adenosine administration, in terms of reducing infarct size in patients with acute myocardial infarction (MI), is currently underway.

An attenuation of ischemia-IR has previously been reported in the lung, in which treatment with A_1, A_{2A}, or A_3 agonists significantly improved organ function, reducing neutrophil infiltration, oedema, and the production of tumour necrosis factor-alpha (TNF-α) [42]. These findings have been corroborated by various recent studies showing that activation of A_1, A_{2A}, A_{2B} or A_3 receptors improves lung function and decreases inflammation, oedema, and neutrophil chemotaxis after ischemia and reperfusion [43-46].

Likewise, beneficial effects have been attributed to the activation of A_{2A} and A_{2B} receptors in mouse models of kidney and hepatic IR, through mechanisms involving the inhibition of natural killer T cell activation and nuclear factor-kB (NF-kB), respectively [47-49]. Interestingly, A_{2A} receptor activation has also been found to trigger an increase in ATP production in hepatocytes and sinusoidal endothelial cells, thereby redressing the metabolic alteration induced by IR and increasing the availability of enzymes necessary for energy production [50]. Furthermore, inhibitors of nucleoside transporters have been ascribed a protective function in a model of renal ischemia, enrolling A_{2B} receptors in vascular endothelia and thereby increasing adenosine signalling [51]. More recently, an increase in vascular endothelial growth factor (VEGF) induced by A_{2B} receptor activation has also been associated with the reversal of renal dysfunction [52].

As a whole, these results present a very compelling case for adenosine agonists as potential therapeutic agents in strategies for the prevention of IR after organ transplant, a planned insult to the body which allows for an anticipatory care/immediate after-care strategy that is not feasible with injuries of sudden onset, like stroke, MI, etc.

Adenosine and pain

Adenosine has been recognized as a potent antinociceptive agent in several different preclinical models of chronic pain, and is therefore undergoing clinical trials for chronic regional pain syndrome, as well as perioperative and neuropathic pain [53,54]. Indeed, in the spinal cord and periphery, adenosine has been shown to reduce neuronal activity, and therefore pain, through its activation of the A_1 receptor. These results are consistent across several experimental pain models, including formalin-induced inflammation, carrageenan-triggered arthritis, hyperalgesia following surgical incision, neuropathic pain caused by spinal nerve ligation and chronic constriction injury, pain after spinal cord injury, and diabetic neuropathy provoked by streptozotocin [53,55-57].

Antinociceptive effects have been demonstrated for several different A_1 receptor activator types, specifically agonists in microglial cells, partial agonists and allosteric enhancers in acute and neuropathic pain models, and ADK inhibitors in chronic pain [58-63]. Moreover, a very recent study on A_1 receptor knockout mice (KO), previously reported as mice with an increased nociceptive response, indicates that inosine behaves as an agonist for A_1 receptors, furnishing antinociceptive effects at a potency similar to adenosine itself [64], even though inosine may also act by competing with adenosine for nucleoside transport, thereby increasing its extracellular levels. The molecular mechanisms triggered by A_1 receptors that lead to antinociception include inhibition of cyclic AMP, protein kinase A (PKA) and Ca^{2+} channels, activation of K^+ currents, and interactions with phospholipase C, inositol triphosphate, diacylglycerol and β-arrestin pathways [65]. Interestingly, recent studies demonstrate a role for A_1 receptors in the mediation of the antinociceptive effect of acupuncture, whose analgesic effect has been replicated by direct injection of an A_1 receptor agonist [66,67]. In particular, A_1 activation has been shown to mimic the effect of acupuncture on microRNA profiling and protein levels [68].

Adenosine may also provide beneficial effects against inflammatory pain by acting through A_{2A} and A_{2B} receptors located in inflammatory immune cells [69], and the latest evidence suggests that A_3 receptor activation may be useful in the treatment of chronic neuropathic pain [1,70]. Indeed, A_3 receptor activation has been shown to inhibit neuropathic pain, induced mechanically or by

chemotherapy, by enhancing the effects of available analgesics [71]. In particular A_3 agonists were able to attenuate neuropathic pain induced by paclitaxel, by modulating glial-restricted spinal signalling pathways [72]. This mechanism has also been confirmed in an oxaliplatin-induced peripheral neuropathy model, in which its beneficial effects have been attributed to the inhibition of an astrocyte-associated neuro-inflammatory response [73,74]. A_3 agonists have also been shown to reverse neuropathic pain via an increase in GABA inhibitory neurotransmission [75] (Figure 4). Furthermore, A_3 receptor stimulation has been found to reduce pain in an *in vivo* model of bone cancer [76]. This latter finding is particularly interesting in light of the fact that A_3 agonists also display anticancer activity [1]. It is also important to note that, unlike A_1 and A_{2A} receptor stimulation, the administration of potent, selective, and orally bioavailable A_3 agonists in humans induces no cardiac or hemodynamic side effects, making the A_3 receptor a particularly appealing therapeutic target in chronic pain of various aetiologies [77-79].

Adenosine and inflammation

By activating A_{2A}, A_{2B} and A_3 receptor subtypes, adenosine plays a crucial role in the regulation of tissue homeostasis, affecting the immune system. It typically inhibits endothelial cell adhesion and superoxide anion production by neutrophils, and reduces proinflammatory cytokine release from macrophages, dendritic cells and lymphocytes [80-85]. In 2001, a seminal paper by Ohta *et al.* reported increased inflammation, tissue damage, TNF-α/interferon (IFN)-γ levels, and mortality in A_{2A} KO mice treated with sub-threshold doses of inflammatory stimuli, thereby suggesting a role for A_{2A} receptors in inflammation [86]. Furthermore, several studies indicate that the A_{2B} receptor subtype is selectively induced in inflamed vascular and intestinal epithelia, as well as the kidneys, heart and lung, making it a direct target in the treatment of inflammation, which is typically characterized by a hypoxic environment [47,87-89]. The A_3 receptor subtype may also prove useful

in hypoxia, as its upregulation has been demonstrated in a variety of inflammatory conditions and immune pathologies [1] (Figure 5).

Autoimmune diseases

An enormous body of literature points towards A_{2A} and A_3 agonists potentially playing a relevant role in the treatment of rheumatoid arthritis (RA). In particular, the gold standard therapy for RA is methotrexate (MTX), which is related to adenosine production and recent evidence indicates that the capacity to generate adenosine by Treg cells is an excellent predictor of MTX response [90-93]. Furthermore, in an *in vivo* model of collagen-induced arthritis, A_{2A} receptor stimulation slowed its progression by preventing nitrosative and oxidative injury and reducing TNF-α, interleukin (IL)1-β and IL-6 levels [94]. Interestingly, an increase in A_{2A} receptors in neutrophils and monocytes in the arthritic knee joint has been shown to mirror the upregulation of CD73 in the neutrophils, monocytes and macrophages of the synovial fluid of mice affected by RA, leading to its pioneering exploitation as an A_{2A} agonist prodrug [95]. Indeed, a CD73-dependent prodrug transformation has been shown to inhibit joint inflammation by provoking the selective activation of A_{2A} receptors on immune cells, an approach that avoids the cardiovascular side effects previously encountered upon administration of A_{2A} agonists.

Multiple lines of evidence also point to the upregulation of A_3 receptors in RA, as well as psoriasis and Crohn's disease [1]. Interestingly, administration of anti-TNF-α drugs normalizes the overexpression of both A_{2A} and A_3 receptors in RA. Moreover, the endogenous activation of these receptors may play a direct role in the control of RA inflammation, as suggested by the inverse correlation between their levels and the Disease Activity Score [96]. Indeed, the upregulation of A_{2A} receptors by TNF-α and IL-1 is well known, and TNF-α has also been reported to increase A_{2A} receptor activity [97-99].

Overexpression of A_3 receptors in RA has been directly linked to an increase in NF-kB, a transcription factor regulating A_3 gene expression and a key player in the pathogenesis of arthritic diseases and osteoarthritis (OA) [77]. Activation of A_3 receptors downregulated NF-kB and Wnt

pathways, resulting in a marked improvement in disease parameters. In this regard, the A_3 agonist IB-MECA (CF101) has already completed Phase I clinical trials, in which it proved to be safe and well tolerated, and Phase II studies in RA and OA patients, which confirmed that it ameliorated their signs and symptoms [77]. This indicates that CF101 may represent a powerful weapon against rheumatic diseases, as do the results of another Phase II trial in patients affected by moderate-to-severe chronic plaque-type psoriasis. This multicenter, randomized, double-blind, dose-ranging and placebo-controlled trial confirmed the safety and good tolerance of the A_3 agonist, which brought about a progressive linear improvement in symptoms [100]. It is no surprise therefore, that CF101 has already embarked on a Phase II/III randomized, double-blind, placebo-controlled, dose-finding study to evaluate the efficacy and safety of daily oral administration in patients with moderate-to-severe plaque psoriasis (NCT01265667 Clinicaltrials.gov).

Inflammatory Bone Loss

Adenosine is known to play a role in the suppression of inflammatory bone resorption. Indeed, the drug MTX, used to inhibit bone erosion in patients with RA, exerts its anti-inflammatory effects via A_{2A} receptors [91,101,102], activation of which inhibits osteoclast differentiation and regulates bone turnover via PKA-dependent inhibition of NF-κB nuclear translocation. This suggests a mechanism by which adenosine could target bone destruction in inflammatory diseases like RA [103,104].

A_{2A} receptors also promote the proliferation of mouse bone marrow-derived mesenchymal stem cells, thereby playing a critical role in osteoblast differentiation. Osteoblast formation, on the other hand, is heavily regulated by A_{2B} receptors [102,105-107]. Accordingly, osteoclast development in bone marrow cells from healthy humans and patients with multiple myeloma is inhibited by activation of A_{2A} and A_{2B}, but with only A_{2B} receptors being implicated in human osteoblast formation [108]. The upregulation of A_{2A} and A_3 receptors in joint diseases and following pulsed electromagnetic fields, have also been shown to modulate chondrocyte biology and cartilage matrix with beneficial effects on the pathology [1,109].

Wound healing and remodelling

Adenosine plays a key role in the inflammatory response involved in wound healing and remodelling [110]. In particular, the A_{2A} receptor subtype promotes several early events in these processes, including vasodilatation, angiogenesis, matrix production, and inflammation [90]. As a consequence, topical application of selective A_{2A} agonists accelerates the healing of dermal wounds in both normal and impaired-wound-healing animals, by a mechanism involving tissue plasminogen activator (tPA) [90,111-113]. Even though a clinical trial of an A_{2A} agonist for wound healing failed, a more recent clinical study reported that intramuscular and perilesional administration of an A_{2A} agonist improves the healing of foot ulcers in diabetic patients [114,115].

A_{2B} receptors are also involved in wound healing and remodelling. Indeed, their expression in cardiac mesenchymal stromal cells after myocardial injury has been found to promote these processes in the myocardium, by inducing the transition of these cells into myofibroblasts [116]. Furthermore, the A_{2B} receptor is behind the increase in the production of proangiogenic factors like IL-6, IL-8 and VEGF by cardiac stromal cells, revealing its role in the stimulation of angiogenesis in an injured heart [117,118]. In fact, A_{2B} receptors have long been known to stimulate VEGF production and angiogenesis in various cell types, including cardiac mesenchymal stem-like cells [117,119], as well as retinal and skin endothelial cells, mast cells, tumour-infiltrating hematopoietic cells, and certain types of cancer cells. Although the main transcription factor involved in this response has been identified as HIF-1, JUN-B transcription factor has also recently been implicated [120-124]. Moreover, A_3 receptor activation also appears to be involved in tissue remodelling activity in human lung mast cells, increasing genes like IL-8, IL-6 and VEGF, all mediators of tissue remodelling and angiogenesis [125].

Lung injury

An important function of the A_{2A} receptor subtype has been observed in models of lung inflammation, and, based on encouraging *in vitro* studies, several A_{2A} agonists have been developed for treating the asthmatic response. Unfortunately, however, these presented limited efficacy in

clinical settings [84], although scientific interest in the role of A_{2A} in this context persists. Indeed, the A_{2A} receptor has been associated with lung-protective properties of propentofylline in a murine model of LPS-induced acute pulmonary inflammation [126].

Intestinal inflammation

Likewise, A_{2B} receptor signalling has been shown to protect against inflammation in the intestine; A_{2B} KO mice showed significantly more severe colitis of more acute onset, associated with a loss of intestinal epithelial barrier function [127]. Accordingly, A_{2B} signalling in epithelial cells has been shown to attenuate colonic inflammation through a specific barrier-repair response, namely phosphorylation of vasodilator-stimulated phosphoprotein [128,129]. However, it has been previously reported that A_{2B}KO mice had an attenuated colitis and that A_{2B} receptors on non-immune cells played an important role for the induction of colitis [130,131]. Overall these conflicting data suggest that A_{2B} receptor signalling has opposing effects on different elements of gut inflammation.

Eye diseases

A_3 receptors have been widely implicated in many ocular diseases, including dry eyes, glaucoma and uveitis. A_3 agonists have proven efficacy in glaucoma therapy, ascribed to their preventing the activation of the $P2X_7$/NMDA receptors responsible for the rise in Ca^{2+} and apoptosis in retinal ganglion cells [1]. A_3 receptor activation is also demonstrably useful in patients with moderate-to-severe dry-eye syndrome, in which CF101 has shown good tolerance and a statistically significant improvement in a phase II clinical trial [132].

Interestingly, in the same clinical trial, CF101 also demonstrated its efficacy as agent able to lower intraocular pressure [133]. This followed findings that oral treatment with CF101, initiated upon disease onset, improves clinical fundoscopy scores, and ameliorates the pathological manifestations of uveitis [134]. Hence daily oral CF101 is currently the subject of a Phase II, randomized, double-blind, placebo-controlled safety and efficacy trial in subjects with active, sight-threatening, non-infectious intermediate or posterior uveitis (NCT01905124 clinicaltrials.gov).

Adenosine and Cancer

Adenosine does play a protective role in cancer, but this risks partial disturbance by its concomitant effects on the immune system. Indeed, high levels of CD39 and CD73 lead to increased adenosine concentration, which, through A_{2A} and A_{2B} receptor-mediated effects on immune cells, creates an immune-tolerant tumour microenvironment [135,136]. This effect of adenosine may be considered a natural consequence of its attempting to avoid excessive inflammation during tissue injury, but suggests both inhibitors of enzyme-generating adenosine and A_{2A} and A_{2B} receptor antagonists as potential anticancer targets [137]. In addition there are in vitro and in vivo studies reporting the prosurvival and prometastatic effects of A_{2B} receptor activation [138]. However, as for the specific effects of adenosine in neoplastic cells, a large amount of data points to adenosine playing the role of protective guardian in cancer. Firstly, A_3 receptor expression is high in various tumour cells, including HL60 and K562 human leukaemia, Jurkat and U937 human lymphoma, Nb2 rat lymphoma, A375 human melanoma, PGT-beta mouse pineal gland tumour, human glioblastoma, and human prostatic and mesothelioma cells. What is more, A_3 receptor overexpression has also been reported in surgical colon, breast, hepatocellular and mesothelioma cancer tissues, as compared to their healthy counterparts. As peripheral blood cells mirror upregulation of A_3 receptors in tissues, this adenosine subtype could be a novel marker for cancer [1,139].

Indeed several *in vitro* and *in vivo* studies have demonstrated that A_3 receptor activation is responsible for inhibiting tumour cell proliferation, increasing apoptosis, and reducing tumour development and metastasis. These studies, including syngeneic, xenograft, orthotopic and metastatic experimental animal models utilizing CF-101 and Cl-IB-MECA (CF-102) in melanoma, colon, prostate and hepatocellular carcinomas, thereby suggest A_3 receptor upregulation as a potential mechanism by which adenosine may reduce tumour development [140]. The molecular pathway activated by A_3 receptors involves de-regulation of the Wnt signal, which generally actively stimulates cell cycle progression and cell proliferation during embryogenesis and

tumorigenesis. In particular, downregulation of PKA and PKB/Akt leads to an increase in glycogen synthase kinase 3β (GSK-3β) activity, in turn resulting in phosphorylation and ubiquitination of β-catenin and suppression of cyclin D1 and c-myc expression. Reduced NF-kB, by inducing apoptosis, has also been implicated in the antitumor effects of A_3 agonists, in particular IB-MECA, which provokes this effect in melanoma and hepatocellular carcinoma [77] (Figure 6).

As a consequence of the above, which opens new therapeutic perspectives against cancer, the safety and efficacy profile of A_3 agonist CF102 has been clinically tested, further to the treatment of hepatocellular carcinoma [141]. In light of the favourable results of this trial, more extensive Phase II liver cancer studies are ongoing (NCT02128958 clinicaltrials.gov).

Concluding Remarks

Adenosine has long attracted considerable attention due to its stress-induced release and homeostatic regulation capabilities. Basic research in several pathologies has generated a huge amount of data suggesting that adenosine has an important function in protecting cells and tissues against injury. As studies have shown, adenosine is implicated in stressful conditions such as hypoxia and ischemia, in which levels of adenosine dramatically increase. Accordingly, adenosine signalling plays a relevant role in epilepsy, pain, ischemic organ injury, inflammation and cancer.

The effects of adenosine are often obtained by activating specific adenosine receptors, which are widely distributed through the body. Hence, to avoid central and peripheral side effects, including sedation, headache, vasodilation, atrioventricular block, and bronchoconstriction, strategies are being targeted at the stimulation of receptors only when and where adenosine is increased.

Literature data suggest that the most promising candidates for successful clinical application may be allosteric enhancers of A_1, A_{2A} and A_3 receptors for pain, inflammation and cancer, and prodrugs of A_{2A} subtypes for inflammation (see “Outstanding Questions”). Indeed one class of selective agonists of A_3 subtypes is emerging for the treatment of cancer, and clinical trials of its efficacy in

inflammatory/autoimmune diseases are also underway. Alternatively, inhibitors of adenosine uptake and degradation seem promising means of increasing adenosine levels by potentiating the endogenous salvage pathway of this nucleoside with less risk of toxicity.

Although it is the field of adenosine agonists that is generating the molecules under clinical development today, and foreseeably the new drugs of tomorrow, we should not forget that there are instances in which an overproduction of adenosine is pathological, particularly in Parkinson's disease, SCID, fibrosis, hepatic steatosis, colitis, asthma, cancer and possibly diabetes [84,137,139,142]. As a consequence, the next 3 to 5 years should see preclinical and clinical research aimed at better understanding which adenosine subtype/ligand is the best target in different pathologies, and consequently developing novel compounds characterized by greater receptor and tissue selectivity. In particular, gene therapy with antisense oligonucleotides specific for adenosine-related enzymes may be useful in diseases that would benefit from increased adenosine concentrations e.g. epilepsy.

Another important goal is clarification of an appropriate time window for useful modulation of adenosine receptors in the management of ischemic damage, and to understand whether the protective effects obtained with adenosine following IR may be exploited during organ transplant. Finally, a challenge for the future will be to determine whether or not circulating levels of adenosine receptor subtypes could be used as biomarkers for the detection of disease development, which may ultimately prove useful in the provision of individualized treatment.

In conclusion, the current status of knowledge on the protective effects of adenosine is rather exciting. Although much work still lies ahead, scientists working in the field of adenosine receptors can be proud of the goals achieved so far.

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Figure legends

Figure 1- Schematic view of adenosine metabolism and adenosine receptors. Overview of adenosine biosynthesis and degradation and second messenger pathways that are coupled to adenosine receptor subtypes. A_1 and A_3 subtypes inhibit AC activity and induce stimulation of PLC; A_1 receptors also modulate K^+ and Ca^{2+} channels. A_{2A} and A_{2B} receptors stimulate AC, with a consequent increase in cAMP levels and A_{2B} receptors activate also PLC. Regulation of PKA and PKC trigger downstream signalling able to modulate transcription of genes involved in inflammation and cell regulation e.g. NF-kB, CREB, HIF-1.

AC (adenylyl cyclase); ADA (adenosine deaminase); ADK (adenosine kinase); cAMP (cyclic AMP); CREB (cAMP response element-binding protein); HIF-1 (hypoxia-inducible factor); NF-kB (nuclear factor-kB); NT (nucleoside transporter); PKA (protein kinase A); PKC (protein kinase C); PLC (phospholipase C).

Figure 2- Schematic view of the main adenosine-mediated effects in epilepsy. A_1 receptor activation inhibits Ca^{2+} influx whilst increasing K^+ conductance, thereby reducing excitability. Adenosine derived from SAH, increased by KD, inhibits the DNA transmethylation pathway, potentially implicated in epileptogenesis. SAH (S-adenosyl-homocysteine); KD (ketogenic diet); DNMT (DNA-methyltransferase); SAM (S-adenosylmethionine); ADK (adenosine kinase); carb (carbohydrate).

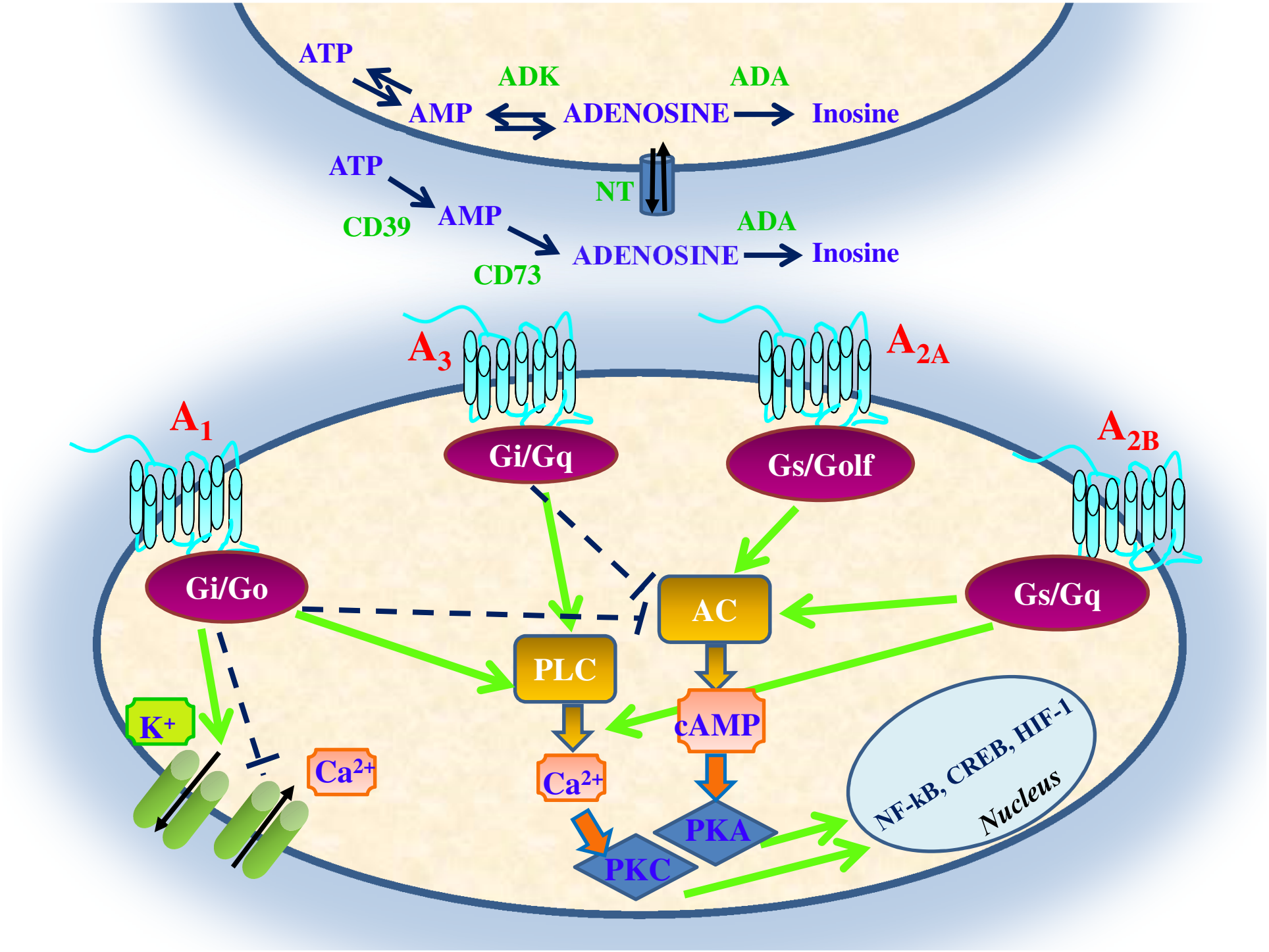
Figure 3- Schematic view of the main adenosine-mediated effects in ischemia. A) A_1 receptor activation inhibits Ca^{2+} influx whilst increasing K^+ conductance, thereby reducing excitability. By these means, adenosine is able to reduce cellular metabolism and energy consumption in ischemia within a few hours (early phase). Recruitment of A_{2A} receptor increases NGF and BDNF. **B)** Hours and days after the insult (Late phase), A_{2A} receptor activation inhibits platelet aggregation, mediates vasodilation, reduces leukocyte infiltration, and suppresses the inflammatory response, thereby

attenuating neuroinflammation after ischemia. NGF (nerve growth factor); BDNF (brain-derived neurotrophic factor).

Figure 4- Schematic view of the main adenosine-mediated effects in pain. A_1 and A_3 receptors mediate antinociceptive effects in neurons through the modulation of Ca^{2+}/K^+ ions and GABA neurotransmission, respectively. A_3 receptors mechanically inhibit allodynia in astrocytes by modulating inflammatory cytokines. Peripheral anti-inflammatory effects mediated by A_{2A}/A_{2B} receptors also contribute to the reduction of inflammatory pain. IL (interleukin); TNF (tumour necrosis factor).

Figure 5- Schematic view of the main adenosine-mediated effects in inflammation. Top) Adenosine exerts anti-inflammatory effects by orchestrating the response of immune cells through the activation of A_{2A}, A_{2B} and A_3 receptors. Bottom) An overview of the inflammatory diseases affected by adenosine. RA (rheumatoid arthritis); OA (osteoarthritis); VEGF (vascular endothelial growth factor).

Figure 6- Schematic view of the main adenosine-mediated effects in cancer. A_3 receptors induce downregulation of PKA and PKB/Akt, and increase GSK-3β activity, resulting in phosphorylation and ubiquitination of β-catenin, and suppression of cyclin D1 and c-myc expression. A_3 agonists may also exert antitumor effects by inhibiting NF-kB, and thereby inducing apoptosis. GSK-3β (glycogen synthase kinase 3β); NF-kB (nuclear factor-kB).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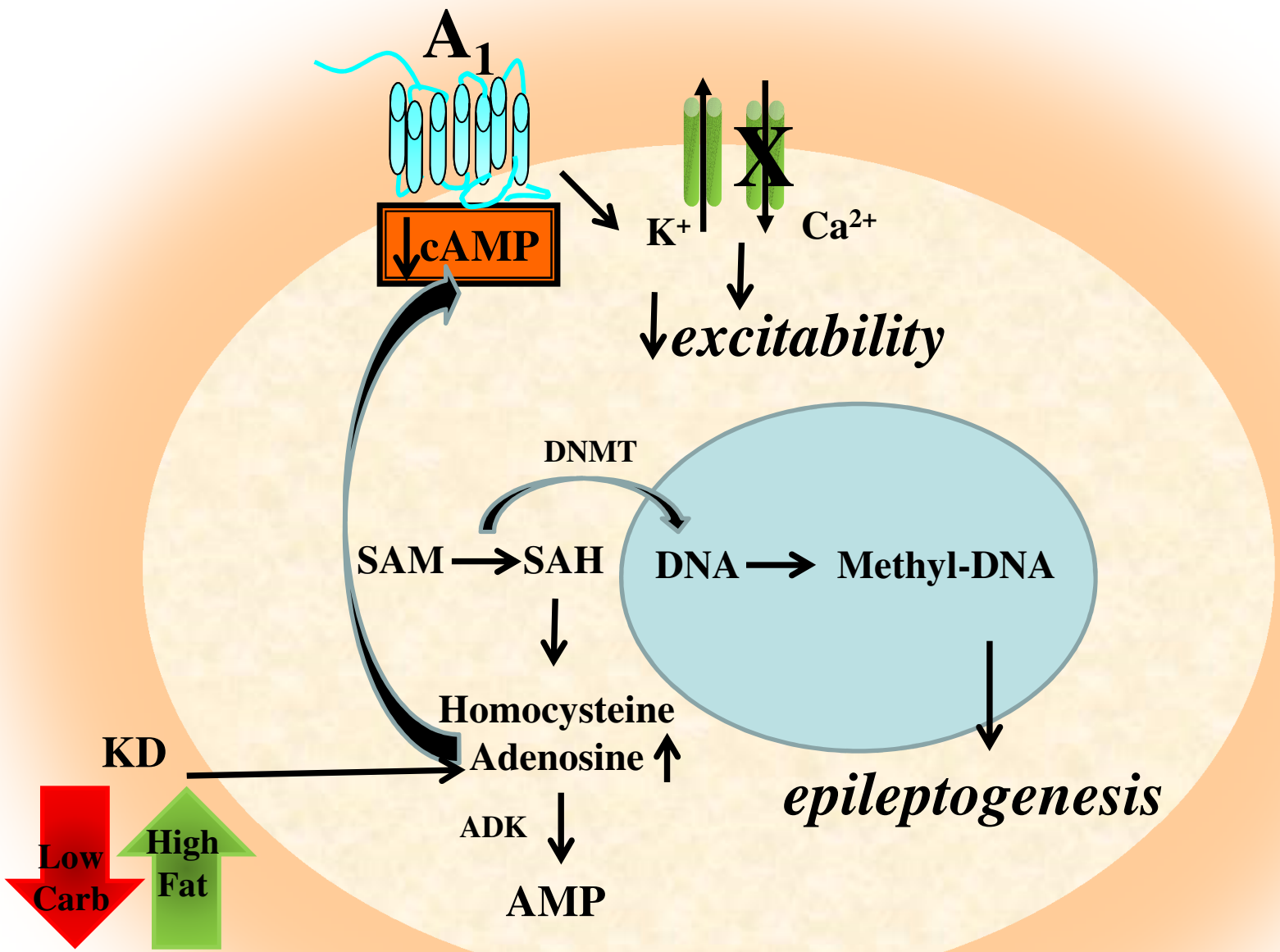
A1
A2B
Gi/Gq
Gs/Golf
Gi/Go
Gs/Gq
AC
PLC
K+
cAMP
Ca2+
Ca2+
PKA
PKC
NF-kB, CREB, HIF-1
Nucleus


A1
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DNMT
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DNA → Methyl-DNA
Homocysteine
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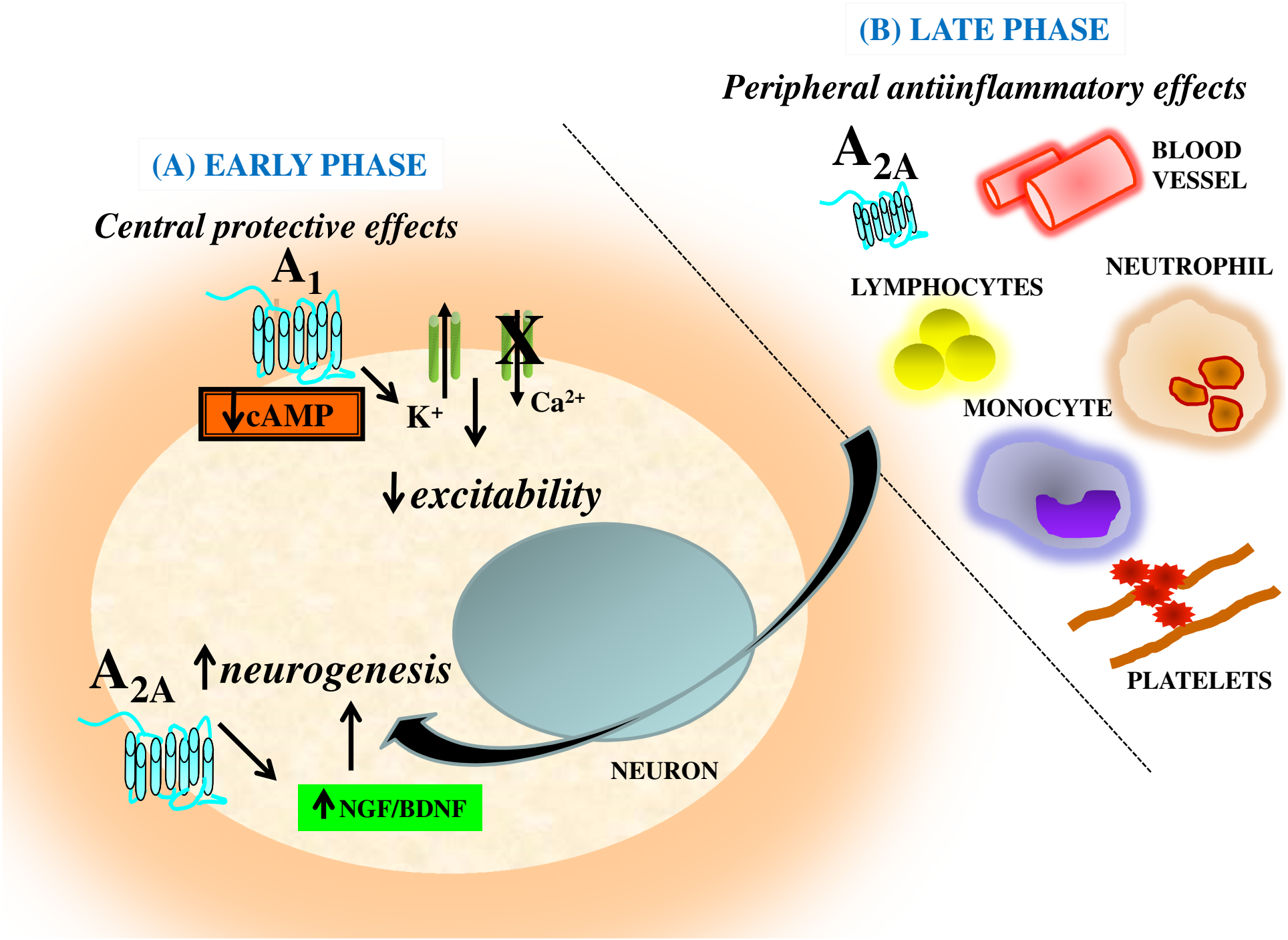
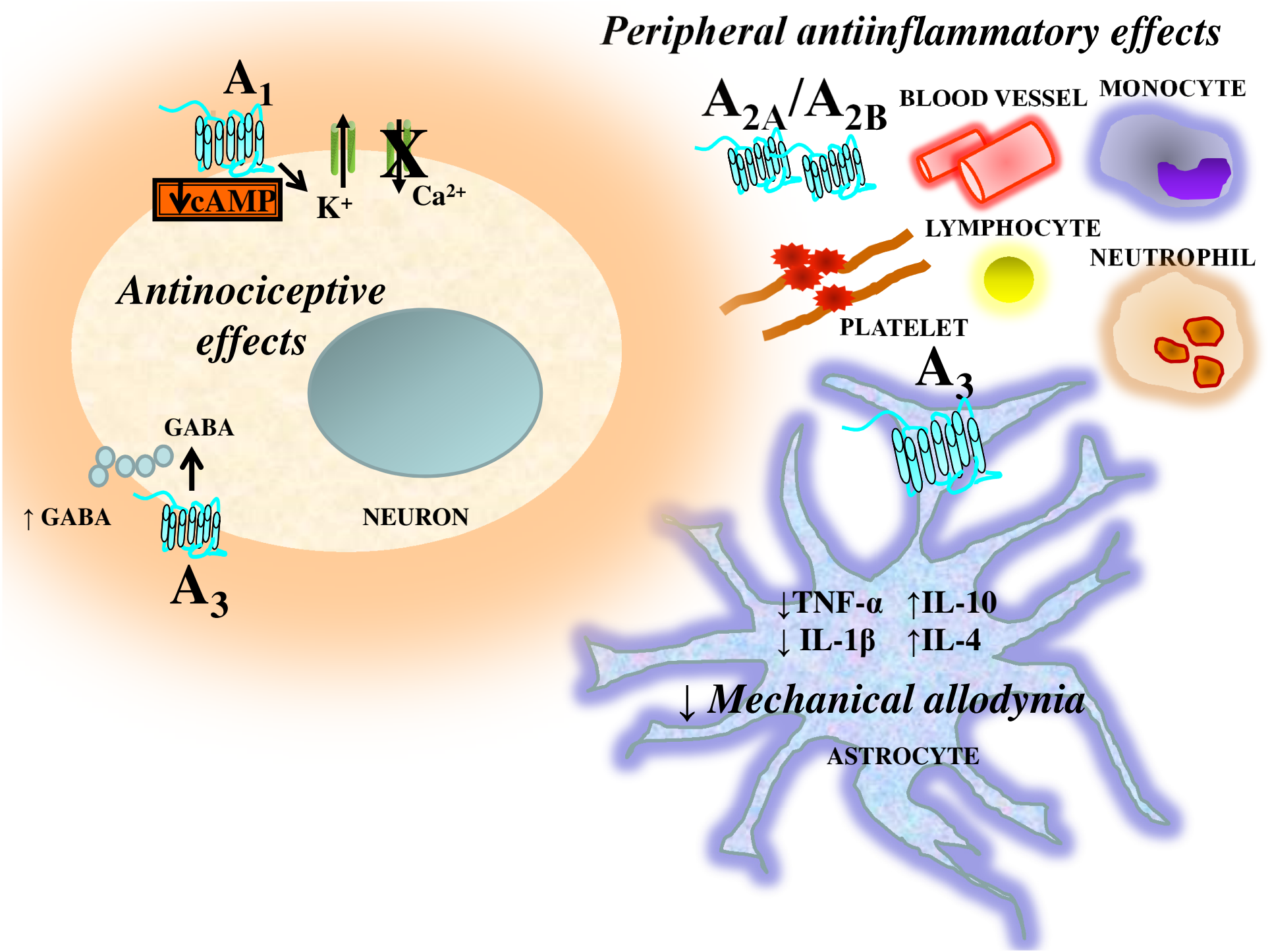
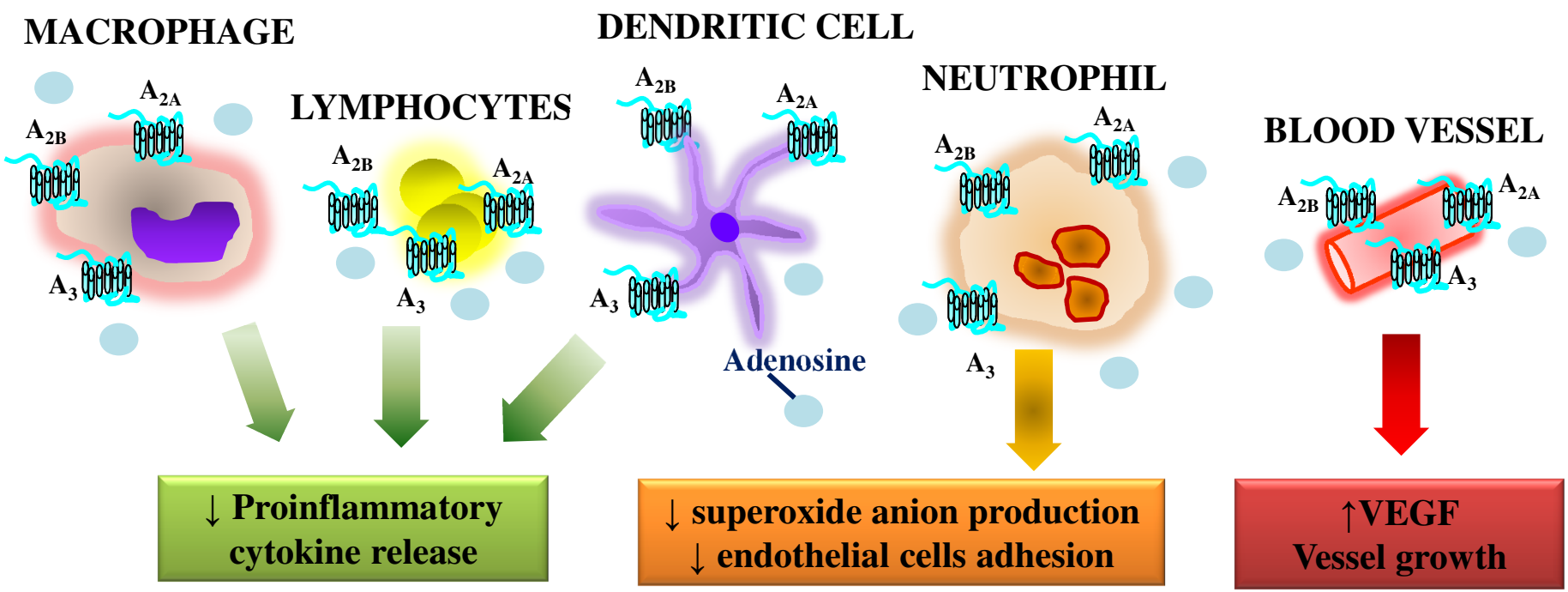
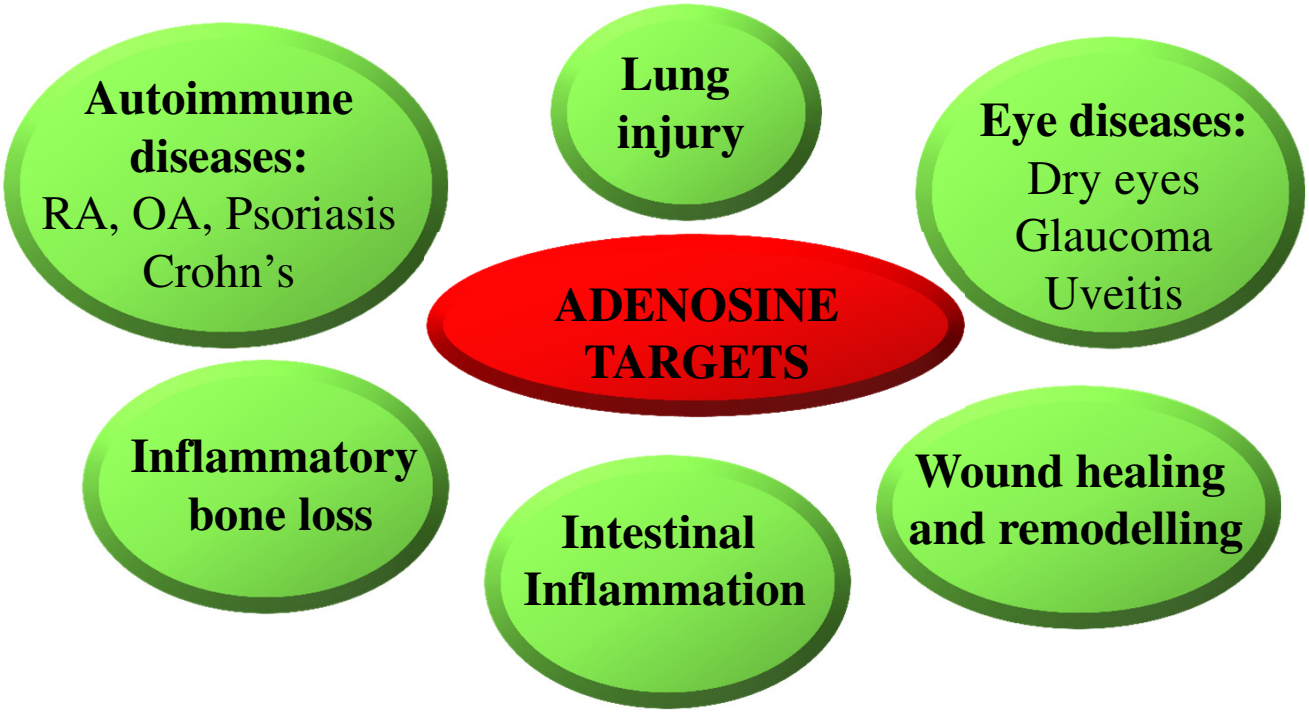
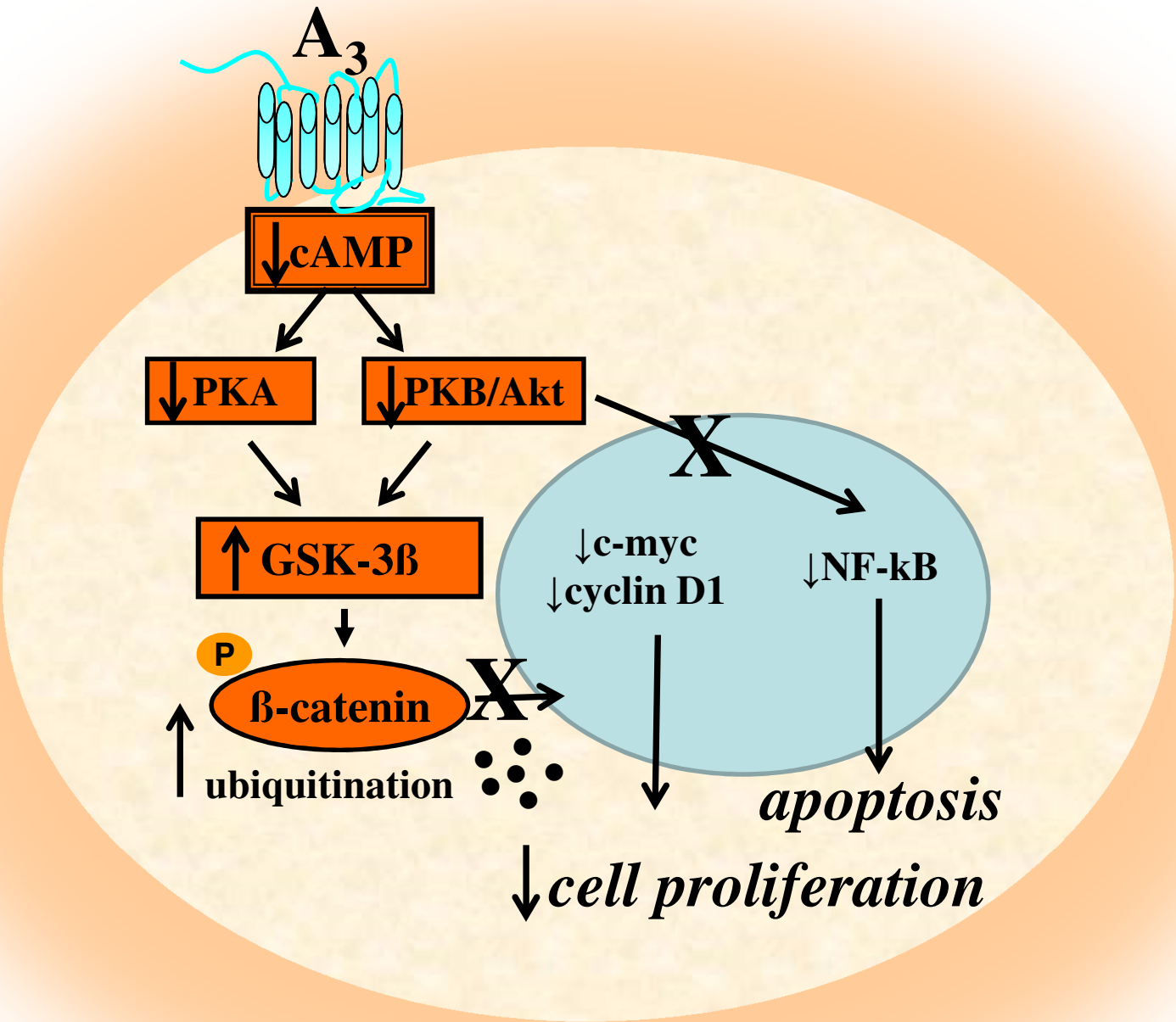
KD
Low Carb
High Fat
epileptogenesis


(A) EARLY PHASE
Central protective effects
A1
cAMP
K+
Ca2+
excitability
A2A
neurogenesis
NGF/BDNF
NEURON
(B) LATE PHASE
Peripheral antiinflammatory effects
A2A
BLOOD VESSEL
NEUTROPHIL
LYMPHOCYTES
MONOCYTE
PLATELETS


Peripheral antiinflammatory effects
A1
cAMP
K+
Ca2+
Antinociceptive effects
GABA
↑ GABA
NEURON
A3
A2A/A2B
BLOOD VESSEL
MONOCYTE
LYMPHOCYTE
NEUTROPHIL
PLATELET
A3
↓TNF-α ↑IL-10
↓ IL-1β ↑IL-4
↓ Mechanical allodynia
ASTROCYTE


MACROPHAGE
LYMPHOCYTES
DENDRITIC CELL
NEUTROPHIL
BLOOD VESSEL
A2A
A2B
A3
Adenosine
↓ Proinflammatory cytokine release
↓ superoxide anion production
↓ endothelial cells adhesion
↑VEGF
Vessel growth


Autoimmune diseases:
RA, OA, Psoriasis
Crohn's
Lung injury
Eye diseases:
Dry eyes
Glaucoma
Uveitis
ADENOSINE TARGETS
Inflammatory bone loss
Intestinal Inflammation
Wound healing and remodelling


A3
↓cAMP
↓PKA
↓PKB/Akt
X
↑GSK-3ß
↓c-myc
↓cyclin D1
↓NF-kB
P
ß-catenin
X
ubiquitination
apoptosis
↓cell proliferation

Glossary

Ischemic preconditioning: a process where repeated short, sublethal insults protect the tissue against a subsequent ischaemic damage

Reperfusion injury after ischemia: is the tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen (anoxia, hypoxia).

Chronic pain: defined as pain that lasts longer than 12 weeks.

Neuropathic pain: pain induced by injury or damage that concerns the sensory system

Nociceptive pain: pain caused by ongoing noxious stimuli, such as heat, cold and chemicals, or acute injury.

Inflammatory pain: pain associated with tissue injury and inflammation, autoimmune disease or exposure to irritating agents

Osteoblast: cell with a single nucleus that synthesizes bone

Osteoclast: a large multinucleate cell that is closely associated with areas of bone resorption

Outstanding questions

- Are synergistic approaches, for example those based on allosteric enhancers or prodrugs, more efficacious means of activating adenosine receptors with fewer side effects than conventional ligands?

- Is it possible to selectively activate A_1/A_3 adenosine receptor subtypes, and will this lead to better outcomes for patients affected by pain?

- Is it possible to selectively activate A_{2A} adenosine receptor subtypes with a view to improving inflammatory disease therapy?

- Could the activation of A_3 adenosine receptor in tumour cells be potentiated by agents able to overcome the A_{2A}-receptor-mediated immunosuppressive effect of adenosine on immune cells? In other words, could A_{2A} antagonists synergize with A_3 agonists in the fight against cancer?

- Can circulating levels of adenosine receptor subtypes play a useful role as biomarkers for the detection of disease development, thereby paving the way to individualized treatment?

- Is innovation in this field patentable and economically transferable? Is adenosinergic research likely to raise significant commercial interest in the future, fostering large-scale investment in the development of adenosine-based drugs?

Trends Box

- Adenosine is a ubiquitous nucleoside, an integral part of ATP, that acts as a homeostatic regulator through the activation of four GPCRs, A_1, A_{2A}, A_{2B}, and A_3, and through receptor-independent mechanisms.

- Adenosine levels increase in areas of inflammation and hypoxia, where it protects tissues by restoring the oxygen supply/demand ratio, as well as affecting preconditioning, exerting anti-inflammatory effects, and stimulating angiogenesis.

- Adenosine favours the resolution of pathologies like epilepsy, pain, ischemia, inflammation and cancer, in which it behaves like a guardian angel against cellular damage.

- New adenosinergic drugs for pain, inflammatory diseases and cancer are already in clinical development.