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ABSTRACTS

Abstracts from the 2017 Italian-German Purine Club Meeting

The pathophysiology and therapeutic potential of purinergic signalling

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There are exciting developments in the purinergic signalling field concerned with the development of purinergic compounds to treat a variety of diseases. In this talk, I will review information about diseases of the central nervous, cardiovascular, musculo-skeletal, immune, endocrine and reproductive systems, airways, special senses, gut, kidney, bladder, liver and skin, as well as pain. It is remarkable to think what our field is likely to contribute to clinical medicine.

Purinergic signalling: new ligands and structural insights

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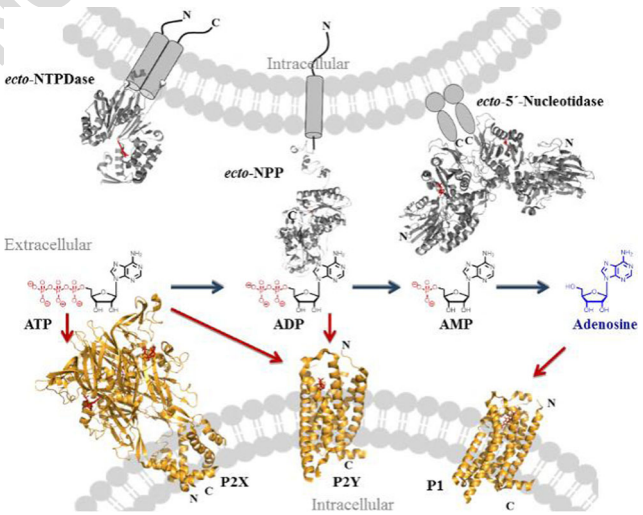
Nucleosides and nucleotides are important extracellular signalling molecules which activate different classes of cell membrane receptors, the co-called purine/pyrimidine P1 and P2 receptors. P1 (or adenosine) and P2Y receptors are G protein-coupled receptors (GPCRs), while P2X receptors are ligand-gated ion channels (see Figure) [1]. The concentrations of extracellular nucleosides and nucleotides are tightly regulated by ecto-enzymes, mainly by ectonucleotidases including ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases, CD39), ecto-nucleotide pyrophosphatases (ecto-NPPs) and ecto-5'-nucleotidase (CD73). Nucleoside and nucleotide signalling plays a major role in all parts of the body especially under pathological conditions, e.g. in inflammation, pain, immune reactions and cancer. Our group has focused (i) on the development and characterization of tool compounds and drugs for purine/pyrimidine receptors and ectonucleotidases, and (ii) on studies directed towards gaining structural information regarding protein-ligand interactions. Recent examples include antagonists for the uracil nucleotide-activated $P2Y_2$ [2] and $P2Y_4$ [3] receptor subtypes, allosteric modulators for P2X1 and P2X4 receptors [4], structural studies on A_{2A} and A_{2B} adenosine receptors (unpublished), and ligands for NPP1 [5,6].



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P2X7 receptor contributes to immunoparalysis of monocytes in human sepsis

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Sepsis remains the leading cause of death in critical-care units. Sepsis initiate with a hyperinflammatory response damaging different tissues and organs, which is then followed by an acute immunoparalysis due in part to a lack of aerobic metabolism in monocytes after the infection. We have characterized P2X7 receptor as an important molecular mechanisms responsible of the aerobic metabolism paralysis in monocytes from human sepsis patients of abdominal origin. We found that the cell surface expression of the ion channel P2X7 receptor increased in septic monocytes when compared with a control group of patients undergoing abdominal surgery but not developing sepsis. Despite this increase in P2X7 receptor, ATP failed to induce ASC aggregation and IL-1beta release in a fraction of septic patients. In these patients P2X7 receptor expression correlated with a lack of mitochondrial membrane potential. Also, this group of patients presented a high mortality during the septic episode. P2X7 receptor stimulation in human blood monocytes before LPS-priming, induced a decrease of mitochondrial membrane potential and impaired the respond of the monocytes to LPS and the engagement of the NLRP3 inflammasome. Our results suggest that during human sepsis P2X7 receptor expression increases in monocytes and could induce immunoparalysis in monocytes by damaging mitochondria.

Adult neurogenesis is enhanced in the hippocampus of mice null for the ADP-receptor $P2Y_{13}$

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The adult mammalian brain retains the capacity for lifelong de novo generation of neurons from stem cells within defined neurogenic niches. A multitude of hormones, growth factors, cytokines, and transcription factors govern adult hippocampal neurogenesis. Extracellular purine and pyrimidine nucleotides are involved in the control of both embryonic and adult neurogenesis [1]. Nucleotides are released from various cell types in the nervous system and act via purinergic receptors including ionotropic P2X or metabotropic P2Y and P1 receptors. Studies of the adult subventricular zone [2,3] and the dentate gyrus [4] provide strong evidence that ATP promotes progenitor cell proliferation in these stem cell rich regions. We have previously shown that the extracellular nucleotide-hydrolyzing enzyme NTPDase2, catalyzing the dephosphorylation of extracellular ATP to ADP and modulating extracellular nucleotide ligand availability and purinergic signaling, is highly expressed by adult neural stem and progenitor cells within both neurogenic niches [5]. Its deletion increases progenitor cell proliferation and expansion of the hippocampal stem and progenitor cell pool *in situ*. Using *in situ* hybridization we now allocate high expression levels of the ADP-sensitive $P2Y_{13}$ receptor specifically to microglia. Iba1-positive microglia are closely interrelated with all cell types of the neurogenic pathway in the dentate gyrus. We report that disruption of the receptor increased progenitor cell proliferation and long-term progeny survival as well as new neuron formation in the dentate gyrus. Increased progenitor cell proliferation and survival persisted in aged *P2ry13* knockout animals. Increased neuron formation was paralleled by increased CREB phosphorylation in immature neurons and enriched expression of the neuronal activity marker c-Fos in granule cells. Our data suggest that $P2Y_{13}$ receptor-expressing microglia constitutively attenuate hippocampal neurogenesis and activity of granule cells. This identifies a novel signaling pathway, whereby microglia via a nucleotide-mediated mechanism, contribute to the homeostatic control of adult hippocampal neurogenesis. Aberrant adult hippocampal neurogenesis is associated with neurological pathologies. Selective $P2Y_{13}$R antagonists could boost neurogenesis in pathological conditions associated with impaired hippocampal neurogenesis.

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Endogenous ATP increases the efficacy of a giant endbulb of Held synapse and promotes its functional development

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Synaptic refinement and strengthening are activity dependent processes that establish orderly arranged maps of the cochlear sensory epithelium throughout the central auditory system. The maturation of central auditory circuits is guided by action potentials (APs) arising from the inner hair cells in the developing cochlea [1]. The AP firing of developing central auditory neurons can be modulated by paracrine ATP signaling, as shown for the cochlear nucleus bushy cells and principal neurons in the medial nucleus of the trapezoid body [2,3]. Using slice recordings before hearing onset and in vivo recordings with iontophoretic drug applications after hearing onset we show that cell-specific purinergic modulation follows a precise tonotopic pattern in the ventral cochlear nucleus of developing gerbils. In regions activated by high-frequency sounds, ATP responsiveness diminished before hearing onset. In low-to-mid frequency regions, ATP modulation persisted after hearing onset in a subset of low-frequency bushy cells (CF < 10 kHz). Down-regulation of P2X2/3R currents along the tonotopic axis occurs simultaneously with an increase in AMPAR currents, thus suggesting a high-to-low frequency maturation pattern. Endogenously released ATP facilitates AP generation, raises firing frequency, and shortens EPSP-AP delay in vivo, thereby contributing to increased efficacy at the endbulb of Held synapse [3]. Combining recordings and pharmacology in vivo, in slices, and in HEK cells we could show that the above effects are mediated by the heteromeric P2X2/3R. Recent in vivo and slice experiments in P2X2/P2X3Dbl-/- mice demonstrate that the P2X2/3R is required for functional maturation of the glutamatergic endbulb of Held synapse during the period of early auditory experience.

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Activation of adenosine A_{2B} receptors and sphingosine kinase/sphingosine 1-phosphate signaling axis modulates the amplitude of outward K^+ currents and maturation of oligodendrocyte precursor cells

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Oligodendrocyte progenitor cells (OPCs) are present throughout the adult brain and spinal cord and can replace oligodendrocytes lost due to injury, aging or disease such as Multiple Sclerosis (MS), the most frequent demyelinating disease in the Central Nervous System (CNS). Remyelination does occur, but is limited especially in chronic disease stages. Multiple causes seem to contribute to such transient decline, including the failure of OPCs to differentiate and enwrap the vulnerable neuronal axons. Thus, OPCs are a viable target for MS clinical therapy. A number of pathways have been identified that may contribute to ameliorate/impair remyelination in MS lesions, among them adenosine and sphingosine kinase/sphingosine 1-phosphate (SphK/S1P) signaling axis. OPCs express each of the four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) at all maturational stage [1]. To date, a functional role has been attributed only to A_1 [1,2] and A_{2A} receptors [1,2,3], whose stimulation modulates OPCs proliferation, differentiation, migration and ionic channels activity. S1P, produced by the action of SphK (two isoforms: SphK1 and SphK2), is a bioactive lipid that regulates remyelination and cell injury. An unexpected finding is that fingolimod (FTY720), approved as orally active drug for relapsing MS, modulates S1P receptors. A relationship between SphK1 activity and A_{2B} adenosine receptor activation has been demonstrated in mouse and human normal and sickle erythrocytes in vitro [4]. In this work the role of adenosine A_{2B} receptors and SphK/S1Psignaling on oligodendrogenesis in rat cultured OPCs, at different times of maturation, was investigated. To this aim patch clamp experiments coupled to Real-time PCR and Western Blot analysis were carried out. Stimulation of A_{2B} receptors reduced the amplitude of outward currents elicited by a voltage ramp protocol. These currents were abolished when K^+ was replaced by equimolar Cs^+, indicating that, in OPCs, ramp-evoked outward currents are K^+ currents. In particular, BAY60-6583 (0.1-30 μM, n = 43), a selective A_{2B} agonist, reduced the amplitude of outward currents in a concentration dependent manner. This effect was prevented by the A_{2B} inverse agonist MRS1706 (10 μM, n = 5) and was reduced by VPC96047 (500 nM, n = 4), a pan-SphK inhibitor. Similarly, FTY720 phosphate (1 μM, n = 4), the active metabolite of FTY720, mimicked and partially occluded the effect of 10 μM BAY60-6583 on ramp-evoked current. In cultured OPCs, SphK1 phosphorylation was enhanced after acute (10 min) treatment with 10 μM BAY60-6583, demonstrating an interaction between SphK/S1P pathway and A_{2B} activation. Finally, chronic A_{2B} stimulation (incubation for 6 days with 10 μM BAY60-6583) reduced the expression of mature oligodendrocyte markers, as determined by Real-time PCR analysis, indicating an involvement of this pathway in OPC maturation.
Our findings reveal that novel pathways activated by adenosine A_{2B} and SphK/S1P are involved in the maturation of OPCs.

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The genetic inhibition of GPR17 affects myelination by influencing important players in oligodendrocyte maturation, including purinergic receptors

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Oligodendrocyte precursor cells (OPCs) are the primary source of myelinating oligodendrocytes in the central nervous system (CNS). Both OPCs and mature oligodendrocytes express purinergic receptors, and for some of them, their expression is restricted to specific differentiation stages, suggesting key roles in OPC maturation and myelination.
At early differentiation stages, OPCs also start to express GPR17, a P2Y-like G protein-coupled receptor activated by uracil-nucleotides and other non-purinergic ligands [1,2]. Although the endogenous ligands of GPR17 are still debated, in the last decade, we [3] and others [4,5] have contributed to highlight this receptor as a key actor in oligodendroglial differentiation and maturation. After reaching its maximal expression in pre-oligodendrocytes, GPR17 has to be downregulated to allow the correct expression of myelin proteins and cells' terminal maturation. Any interference in this time-regulated pattern result in myelination impairment [6]. This is also supported by the findings that in several models of disease, GPR17 is up-regulated, contributing to a blockade of OPC maturation program [3,7].
Here we aimed at identifying the genes downstream Of GPR17 to understand how the receptor influences oligodendrocyte differentiation. To this purpose, we transfected a specific GPR17 siRNA in rat OPCs during their differentiation ~~if~~ vitro in the absence of growth factors. In these conditions, significant reduction in the expression of the myelin oligodendrocyte glycoprotein (MOG) was found. We then analyzed the transcriptome of the cells by microarray hybridization and observed that 812 genes were significantly up- or down-regulated after GPR17 gene ablation. Results were clustered in gene ontology categories, pathways and biological processes. Among the most influenced pathways, we found the downstream signalling of the mammalian target of rapamycin (mTOR), important players in cytoskeletal rearrangements, several chemokines such as the stromal derived factor 1 (SDF1), which also act as a GPR17 ligand [8], and outward conductance potassium channels, previously described by us as effectors of GPR17 signalling [9]. Interestingly, also the expression of some purinergic receptors such as $P2Y_1$, $P2Y_{13}$, P2X2 and P2X3, was significantly altered, suggesting that different P2 receptors are recruited to complete maturation. Except for the $P2Y_1$ receptor, which is known to contribute to ADP-mediated OPC chemotaxis in vitro [10], the functional role of the other affected purinergic receptors in oligodendrocyte physiology is currently unknown.
Taken together, these data confirm that GPR17 absence profoundly affects the biological processes typical of oligodendrocyte differentiation and set the basis for a more detailed analysis of the interplay beteween distinct P2 receptors in mediating OPC maturation and myelination.

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Purinergic signaling in neuronal phenotype determination

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Intracellular calcium oscillations participate in many cellular processes. Spatial and temporal patterns of calcium spike and wave activity are important for fate determination in CNS development and neurotransmitter specification of differentiating neurons. As a suggested underlying mechanism for stem cell differentiation into defined phenotypes, a distinct calcium transient pattern in undifferentiated cells codes for the expression and activation of neural transcription factors, inducing expression of ion channels and other calcium-mobilizing mechanism characteristic for an advanced stage of differentiation. Any interference with this calcium transient activity affect the fate of differentiation. Purinergic receptors are already expressed at early development, and in vitro and in vivo data provided evidence for their participation in intracellular calcium transient signaling and control of neural differentiation. Using mouse embryonic stem (ES) cells as in vitro model for neuroectodermal differentiation into neurons and glial phenotype, we tracked calcium transient activity together with rhythmic neural transcription factor expression. Time-lapse imaging with calcium-sensitive fluorescent probes was combined with luminescence imaging of stable transfected cells with Mash-1 or neurogenin-2 promoter-protein fusioned to a luciferase reporter. Spontaneous calcium transients observed as spikes or waves exclusively depended on calcium mobilization from intracellular pools. Further, addition of ATP inducing increases in cytosolic calcium concentration by activation of ionotropic and metabotropic purinergic receptors augmented frequencies and amplitudes of intracellular calcium oscillations together with rhythmic changes in Mash-1 and neurogenin-2 expression levels. Pharmacological tools let us to conclude that ES cells pre-differentiated to neural stem cells or neural stem cells augment Mash-1 expression as a result of P2Y2 purinergic receptor activity, while expression rates of this neural transcription factor were reduced as consequence of P2X7 receptor activity. Mash-1 and neurogenin-2 oscillatory expression patterns depend on voltage-gated calcium channel, as probed by the time-lapse fluorescence and luminescence imaging technique. Using pharmacological tools to modulate purinergic receptors and L-type voltage gated calcium channels let us to conclude that neural stem cells showing augmented spike-like calcium oscillation frequency induced Mash-1 stable expression and consequently determination to GABAergic cell fate as a result of P2Y2 purinergic receptor activity. Overall, our studies show that temporal oscillations of Mash-1 and neurogenin-2 expression patterns code for the neural phenotype (differentiation into neurons or astrocytes) as well as for neurotransmitter specification, providing novel insights into mechanisms of neurogenesis.
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P2X7 Purinergic Signalling as a Therapeutic Target in Post-Menopausal Bone Loss

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Purinergic signalling in bone was first proposed in the early 1990s with the observation that extracellular ATP could raise intracellular calcium and induce secondary messenger activation [1]– events crucial to the normal functioning of the bone building cells osteoblasts. Since then the expression of nearly all the P2Y and P2X receptors by osteoblasts and osteoclasts (bone destroying cells) has been reported, mediating multiple processes including triggering intracellular calcium and the respective signalling cascades that direct the fate of bone cells and ultimately help control bone homeostasis. These observations have led to increasing interest in the therapeutic potential of P2 receptor signalling for the treatment of various bone diseases. Post-menopausal osteoporosis is a bone disease that affects millions worldwide and places a huge socio-economic burden on society. Previous research has shown an association of loss of function SNPs in the gene for the purinergic receptor P2X7R with low BMD, increased rates of bone loss and vertebral fractures in post-menopausal women [2-4]. In this study we used a mouse model of oestrogen deficiency-induced bone loss (to mimic osteoporosis) and the BALB/cJ P2X7R$^{-/-}$ to show that absence of the P2X7R resulted in increased cortical and trabecular bone loss. Osteoclast precursors were isolated from both BALB/cJ P2X7R$^{-/-}$ and BALB/cJ P2X7R$^{+/+}$ mice and then cultured in vitro to form mature resorbing osteoclasts. The BALB/cJ P2X7R$^{-/-}$ derived osteoclasts had slightly more osteoclasts but with a significantly reduced amount of resorption per osteoclast. Osteoclast activity was further increased in oestrogen deficient conditions in the absence of the P2X7R suggesting that both oestrogen and P2X7R regulate the activity and lifespan of osteoclasts. Finally using an anabolic intervention, we demonstrated that the increased oestrogen-deficient bone loss could be rescued with mechanical loading, even in the absence of P2X7R. This study provides further support for the role of P2X7R in pathological bone loss and paves the way for clinical intervention for women with post-menopausal osteoporosis and P2X7R loss of function polymorphisms.

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P2X7A and B as novel biomarkers of acute myeloid leukemia response to chemotherapy and innovative therapeutic targets for the disease

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The ATP gated ion channel P2X7 is attracting increasing attention for its role in oncogenesis as it is involved both in cancer cell transformation and in immune cells tumour crosstalk [1]. Nine different human P2X7 splice variants have been identified, among which the classical P2X7A receptor and the P2X7B isoform, which lacks the P2X7 large pore forming activity but still gives rise to a calcium channel and has been associated to cancer cell proliferation [2,3]. Overexpression of P2X7 and a pro-apoptotic role of ATP was recently demonstrated in the blasts of acute myeloid leukaemia (AML) patients [4]; however, a characterization of the different splice variants was still missing. Here we investigated the mRNA levels of P2X7A and B in 92 patients affected with AML or myelodysplastic syndrome (MDS), a pre-cancerous condition often leading to AML development. Interestingly, both P2X7A and B were overexpressed in AML firstly diagnosed patients as compared to MDS, suggesting that both receptors could be involved in the progression of the disease. Moreover, when subdividing the AML population in firstly diagnosed untreated (61), relapsing (11) and remitting (5) patients, we retrieved a differential expression of P2X7B versus P2X7A. In relapsing patients, which are presented with a return of the pathology after chemotherapy, while P2X7A tends to decrease, P2X7B is significantly increasing. On the contrary, when comparing de novo with remitting patients both P2X7A and B show a tendency to decrease. These data suggest that P2X7B expressing blasts could be resistant to chemotherapy and responsible for the relapse of AML. In accordance HEK293 cells expressing P2X7B showed increased resistance to death induced by danourubicin, possibly the most used chemotherapic in AML treatment, as compared to mock transfected or P2X7A expressing cells. In an AML xenograft model both daunorubicin and the P2X7 antagonist AZ10606120 significantly reduced tumour cell growth but the co-administration of the two drugs proved more efficacious. Of interest while danourubicin alone caused an increase in P2X7B expression in the tumour the co-treatment with the P2X7 blocking drug was able to reduce P2X7B levels. Taken together our data suggest that both P2X7A and P2X7B isoforms could be useful prognostic markers and potential therapeutic targets in AML.

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$P2X_7$ receptor activation severely improves the outcome of sepsis induced by α-haemolysin producing *Escherichia coli* in mice

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α-haemolysin (HlyA)-producing *Escherichia coli* commonly inflict severe urinary tract infections, including pyelonephritis, which comprises substantial risk for sepsis. *In vitro*, the cytolytic effect of HlyA is mainly imposed via ATP release through the HlyA pore and subsequent $P2X_1$/$P2X_7$ receptor activation. This amplification of the lytic process is not unique to HlyA but is by many other pore-forming proteins including also in complement-induced haemolysis. Since free haemoglobin in the blood is known to be associated to a worse outcome in sepsis one could speculate that inhibition of P2X receptors to ameliorate the course of sepsis. Surprisingly, this study demonstrates that $P2X_7^{-/-}$ and $P2X_4^{-/-}$ mice are exceedingly sensitive to sepsis with uropathogenic *E. coli.* These mice have markedly lower survival, higher cytokine levels and activated intravascular coagulation. Quite the reverse is seen in $P2X_1^{-/-}$ mice, which had markedly lower cytokine levels and less coagulation activation compared to controls after exposure to uropathogenic *E. coli*. The high cytokine levels in the $P2X_7^{-/-}$ mouse are unexpected, since $P2X_7$ is implicated in caspase-1-dependent IL-1β production. Here, we demonstrate that IL-1β production during sepsis with uropathogenic *E. coli* is mediated by caspase-8, since caspase-8 and RIPK3 double knock out mice show substantially lower cytokine during sepsis and increased survival after injection of TNFα. These data support that $P2X_7$ and $P2X_4$ receptor activation has a protective effect during severe *E. coli* infection.

Blockade of A_{2B} adenosine receptor inhibits fibroblast-derived CXCL12 impairing tumor angiogenesis

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Adenosine is an ATP metabolite, generated in the extracellular space by the ectonucleotidase CD73. In the tumor microenvironment adenosine impairs anti-tumor immunity, through the Gs-coupled receptors A_{2A} and/or A_{2B}, promoting tumor growth and survival [1]. Blockade of CD73 or A_{2A}R subtype

has been shown to improve the anti-tumor immune response [2,3]. Inhibitors of these targets are currently in Phase I clinical trials in cancer patients [NCT02503774 and NCT02655822]. Whilst $A_{2A}R$ is the most thoroughly characterized receptor involved in the immunosuppressive effects of adenosine, A_{2B} is emerging as a potential anti-cancer target. We have previously demonstrated that $A_{2B}R$ inhibition with PSB1115 significantly delayed tumor growth in melanoma-bearing mice, decreasing the accumulation of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment but not in non-tumoral peripheral organs [4]. Here we show that blockade of A_{2B} receptor reduced the number of fibroblast activation protein (FAP)-expressing cells within tumor tissue, that produce CXCL12. Inhibition of the CXCL12/CXCR4 pathway reversed the pro-angiogenic effect induced by A_{2B} receptor activation in tumor tissue, whilst it did not affect the accumulation of tumor MDSCs. These results suggest that pharmacological inhibition of A_{2B} receptor in mice inhibits the growth of tumor impairing the activation of melanoma-associated fibroblasts CXCL12 positive, that contribute to enhance tumor angiogenesis. Therefore data from animal studies support the therapeutic potential of A_{2B} blockers in melanoma.

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Adenosine A_{2A} receptor stimulation enhances survival of a murine model of Niemann Pick Disease Type C

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Niemann-Pick type C (NPC1) disease is a rare neurovisceral disorder due to intracellular accumulation of cholesterol and other lipids and characterized by progressive hepatosplenomegaly, neurodegeneration and premature death. Even though NPC1 is a visceral condition [1], the brain is the only organ in which progressive cell death ensues [2]. In particular, the cerebellum is affected by a selective and early loss of Purkinje neurons which contributes to the devastating neurological symptoms of the disease (motor impairment, ataxic gait and cognitive deficit). Since the therapeutic options for NPC1 are extremely limited (miglustat is the only drug approved for the treatment of neurological symptoms of NPC1 patients, and it has only limited effects) new therapeutic targets are highly warranted. In previous papers, we demonstrated that the stimulation of the adenosine A_{2A} receptor ($A_{2A}R$) by its selective agonist CGS21680 restores calcium homeostasis, mitochondrial membrane potential (mMP) and cholesterol content in both fibroblasts from NPC1 patients [3] and neural cellular models of NPC1 [4]. These findings strongly support the hypothesis that $A_{2A}R$ stimulation could represent a new approach to treat NPC disease and pave the way for the preclinical testing of $A_{2A}R$ agonists on mice models of the pathology. To this aim, the BALB/C $NPC1^{nih}$ mouse model was used as a model of NPC1 *in vivo*. Two groups of N = 9-11 mice were treated with the $A_{2A}R$ agonist T1-11 (50 mg/kg o.s.) or its vehicle starting from the pre-symptomatic stage of the disease (PN30) until death. The effects of the treatment were tested on body weight, motor impairment (coordination and ataxia), cognitive deficits, Purkinje degeneration, lipid accumulation in liver and spleen and, finally, mice survival. T1-11 was able to attenuate ataxic gait, cognitive deficit and peripheral lipid accumulation in NPC1 mice. Very interestingly, the treatment significantly reduced Purkinje neuron loss and prolonged mice survival. Considering that T1-11 is also an inhibitor of the adenosine transporter ENT1, we wanted to verify whether its effect onto NPC1 phenotype was mediated by the selective activation of $A_{2A}Rs$. Actually, the ability of T1-11 (10 μM) to reduce cholesterol accumulation in neuronal cell lines silenced for the expression of NPC1 protein was specifically mediated by $A_{2A}R$, since it was reverted by the $A_{2A}R$ antagonist ZM241385 (1 μM) but not by the A_1R antagonist DPCPX (1 μM). Taken together, our findings strongly suggest that stimulation of $A_{2A}R$ represents a valuable therapeutic strategy for NPC1. All efforts will be made in the future to obtain new agonists or positive allosteric modulators able to boost the stimulation of this receptor in the brain and, in general, suitable for clinical use.

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Role of A_{2B} adenosine receptor in the control of induced epithelial-mesenchymal transition in human epithelial lung cells

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The epithelial-mesenchymal transition (EMT) is a complex process in which the epithelial cells gradually lose intercellular contacts and undergo morphological changes to mesenchymal-appearing cells. The EMT dysregulation has been generally linked to the aggressiveness and metastasis of

several cancers [1]; however, it has been also involved in the etiophatogenesis of fibrosis [2]. In the last decade, several efforts have been devoted in understanding the mechanisms that trigger and sustain the transition process. Among the different extracellular stimuli, the inflammatory microenvironment (e.g. TNF-α, IL-6) and the presence of increased levels of different cytokines (e.g. TGF-β) seem to play a pivotal role in the EMT process. Adenosine is a purinergic signalling molecule that regulates various aspects of inflammation and has been implicated in the pathogenesis and exacerbation of chronic diseases, such as the idiopathic pulmonary fibrosis (IPF), which is defined by chronic, progressive, and irreversible interstitial fibrosis. Among the receptor for adenosine, the A_{2B} subtype has been emerging as the one that regulates many of the adenosine-driven remodelling responses in chronic lung disease. Moreover, the A_{2B} expression has been found elevated in patients with IPF [3]. However, the role of this receptor in the EMT is still unclear. In this respect, herein the effects of the inflammatory extracellular microenvironment on the A_{2B} expression and functionality were investigated in the human epithelial lung cells. Moreover, the link between the A_{2B} stimulation and the EMT progression was explored. The human epithelial lung cells were challenged with the inflammatory cytokine, TGF-β, in order to trigger the EMT. Then, the ability of the selective A2B adenosine receptor agonist, BAY 60-6583, and antagonist, MRS 1744 to modulate the transition was evaluated. EMT was monitored by the analysis of cell morphological changes using optical microscope and immunofluorescence staining, and by quantifying specific EMT markers and related intracellular signalling pathways by real time RT-PCR and western blot analysis. Challenging the human epithelial lung cells with TGF-β caused an increase in A_{2B} adenosine receptor expression, highlighting a link between these receptors and the EMT. The activation of the A_{2B}, per se, was not enough to induce the transition; however, the receptor activation with selective agonists potentiated EMT triggered by TGF-β. In parallel, the inhibition of A_{2B} adenosine receptor signalling obtained in the presence of the selective receptor antagonist, MRS, partially impaired the effect of the cytokine on EMT induction. These results could shed light on the role of the A_{2B} receptor in the EMT process, thus highlighting the receptor as a putative target to control EMT-related pathologies such as the IPF.

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The extracellular cAMP-adenosine pathway increases activation of brown adipocytes

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Brown adipose tissue (BAT) plays an important role in energy expenditure in mammals. Increasing abundance and activity of the tissue thus represents a self-evident pharmacological target to the worldwide obesity problem [1]. In recent studies we have demonstrated a stimulating effect of extracellular adenosine on energy expenditure of murine and as well of human brown adipocytes (BA). So far, the origin of adenosine in BAT is not completely clear. We could previously show that sympathetic nerve stimulation with an electrical field induces the release of adenosine together with norepinephrine (NE) and ATP from BAT [2]. Subsequently, we analyzed the metabolic conversion of ATP in the supernatant of BA and successfully confirmed its degradation to extracellular adenosine. However, not only ATP but also cAMP can function as a source for adenosine. Extracellularly, cAMP can be degraded to adenosine by ectoenzymes such as ectophosphodieseterases and CD73. Here we could show that BA release cAMP to the cell culture supernatant in response to acute NE-stimulation. Release of cAMP is mediated by ABCC transporters. We found that BA express these transporters and that treatment with specific inhibitors could significantly inhibit the NE-induced increase in extracellular cAMP. Our data show that cAMP can be secreted by BA, possibly functioning as a paracrine signaling molecule through the subsequent degradation to purine signaling molecules. In conclusion, our findings indicate the existence and importance of an extracellular cAMP-adenosine signaling pathway in brown adipocytes. Modulation of this pathway could be a possible target for increasing BA function.

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ATP and Kidney Physiology

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Extracellular nucleotides are important local signaling molecule in all organ systems. In the kidney, purinergic signaling is involved in numerous functions including the control of renal tubular transport. Purinergic receptors are expressed in all renal tubular segments and their stimulation commonly leads to transport inhibition. The luminal space is a restricted space for purinergic signaling and the ambient concentrations of ATP in the luminal fluids are sufficiently high to inflict a tonic inhibition of renal tubular absorption via apical P2 receptors. Apical $P2Y_2$ receptors play a prominent role in this process. ATP is released continuously into the tubular lumen. The release is augmented in response to an increase of tubular flow and after stimulation of G-protein-coupled receptors. The primary cilium appears necessary for flow-stimulated luminal ATP release. Tubular ATP secretion may occur via non-junctional channels and vesicular release. Local purinergic signaling provides an inhibitory tone for renal tubular transport and thus is an endogenous diuretic regulation system. Blocking components of this system leads to tubular hyper-absorption, volume retention and elevated blood pressure.

P2Y1 receptor-expressing interneurons drive network activity in the olfactory bulb

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The olfactory bulb is the first relay station in the olfactory sensory pathway. Here, the incoming signals from olfactory sensory neurons of the nasal epithelium are processed before being transmitted to the centres of odour perception in the cortex. Although it has been shown that the sensory neurons use ATP as a cotransmitter together with glutamate [1], little is known about the impact of the released ATP on the neurons of the neuronal network. The axons of the sensory neurons contact local interneurons and mitral cells in specialized processing compartments, the glomeruli. Mitral cells integrate the different contributions and create the output of the olfactory bulb. In the study presented we used laser-controlled photolysis of caged-ATP to rapidly release ATP confined to a single glomerulus. We discovered a distinct subgroup of juxtaglomerular interneurons which responded directly to ATP via P2Y1 receptor activation. Those interneurons were able, in turn, to induce a population response of the entire neuronal network. Beyond this, we could demonstrate actions of endogenously released ATP. Using electrical stimulation of olfactory sensory axons and thereby mimicking incoming odour signals we showed that the afterhyperpolarization of mitral cells is dependent on activation of P2Y1 receptors. However, ATP seemed not only to be released upon activity of sensory neurons. In the resting network, mitral cells undergo rhythmic fluctuations of excitability, reflected as membrane potential up and down states, which were modulated by tonically released ATP via activation of P2Y1 receptors. In summary, we have evidence that ATP impacts the physiology of different neuronal constituents of the neuronal network in the olfactory bulb. Therefore, we assume that purinergic signalling is able to modulate the processing of sensory information in the olfactory pathway. Supported by the DFG (HI 1288/3).

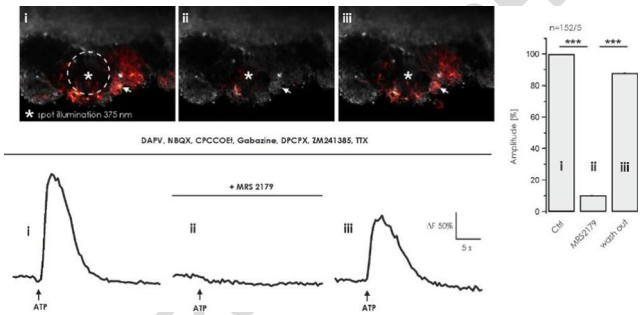

Fig 1. Juxtaglomerular cells responding selectively to photorelease of ATP confined to a single glomerulus with calcium signals (i). The response is abolished in MRS217, an antagonist for P2Y1 receptors (ii) and recovers after washout of MRS2179 (iii).

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Basal adenosine modulates the functional properties of AMPA receptors in mouse hippocampal neurons through the activation of A_1R $A_{2A}R$ and A_3R

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Adenosine is a widespread neuromodulator within the CNS and its extracellular level is increased during hypoxia or intense synaptic activity, modulating pre- and postsynaptic sites. We studied the neuromodulatory action of adenosine on glutamatergic currents in the hippocampus, showing that activation of multiple adenosine receptors (ARs) by basal adenosine impacts postsynaptic site. Specifically, the stimulation of both A_1R and A_3R reduces AMPA currents, while $A_{2A}R$ has an opposite potentiating effect. The effect of ARs stimulation on glutamatergic currents in hippocampal cultures was investigated using pharmacological and genetic approaches. A3R inhibition by MRS1523 increased GluR1-Ser845 phosphorylation and potentiated AMPA current amplitude, increasing the apparent affinity for the agonist. A similar effect was observed blocking A_1R with DPCPX or by genetic deletion of either A_3R or A_1R. Conversely, impairment of $A_{2A}R$ reduced AMPA currents, and decreased agonist sensitivity. Consistently, in hippocampal slices, ARs activation by AR agonist NECA modulated glutamatergic current amplitude evoked by AMPA application or afferent fiber stimulation. Opposite effects of AR subtypes stimulation are likely associated to changes in GluR1 phosphorylation and represent a novel mechanism of physiological modulation of glutamatergic transmission by adenosine, likely acting in normal conditions in the brain, depending on the level of extracellular adenosine and the distribution of AR subtypes.

Adenosine A_1 receptors modulate information processing in the olfactory bulb

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Astrocytes in the olfactory bulb respond to neuronal activity with calcium signalling and release ATP that is degraded to adenosine [1,2]. While in other brain regions the effect of adenosine on neuronal performance is well described, no information exists how adenosine affects information processing in the olfactory bulb. We studied the effect of adenosine on the resting membrane potential, synaptic transmission and information processing of mitral cells, one type of principle neurons in the mouse olfactory bulb, using acute mouse brain slices. Bath application of 100 μM adenosine evoked a hyperpolarization in wildtype, but not in A_1 receptor knockout mice and in the presence of DPCPX, an antagonist of A_1 receptors (Fig. 1). We tested the effect of several potassium channel blockers on the adenosine-evoked hyperpoparization, upon which only blockers of two-pore domain potassium channels significantly reduced the hyperpolarization by adenosine. In addition, adenosine reduced voltage-gated calcium currents of the N- and P/Q-type in mitral cells, leading to reduced glutamate release at reciprocal dendrodendritic synapses and hence attenuation of recurrent inhibition. Synaptic input from olfactory sensory neurons (OSN), in contrast, was not affected and bursts of action potentials triggered by OSN stimulation remained constant in the presence of adenosine. The spontaneous (resting) firing frequency, however, was reduced by the adenosine-mediated hyperpolarization. The unaffected stimulation-evoked firing pattern (signal) at reduced spontaneous firing (noise) upon adenosine application resulted in an improved signal-to-noise ratio of the input-output relation of information processing. The results demonstrate that A_1 receptors hyperpolarize olfactory bulb mitral cells by activation of two-pore domain potassium channels, attenuate reciprocal synaptic inhibition and increase the signal-to-noise ratio of stimulation-evoked action potential firing, thereby modulating information processing.
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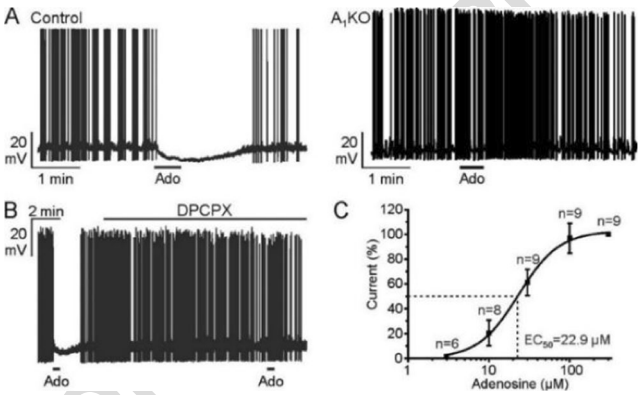

Figure 1. A) Adenosine (100 μM) hyperpolarizes mitral cells in wildtype (control), but not A_1 knockout mice (A_1KO). B) The adenosine-evoked hyperpolarization is blocked by 1 μM DPCPX. C) Dose-response curve.

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Selective adenosine A_3 receptor stimulation inhibits voltage-dependent Ca^{2+} currents and Ca^{2+}-activated K^+ conductances in rat dorsal root ganglion neurons

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Interest has been focused in recent years on the analgesic effects exerted by adenosine in different *in vivo* models of acute and chronic pain. Adenosine is an endogenous neuromodulator that acts on four metabotropic receptors: A_1 and A_3 receptors coupled to adenylyl cyclase inhibition, and A_{2A} and A_{2B} coupled to adenylyl cyclase stimulation. Preclinical and clinical studies demonstrate that A_1 receptor and its agonists exert antinociceptive effects. However, the therapeutic utility of these compounds is limited by adverse cardiovascular and central side effects. Recent preclinical observations also indicate that A_3 receptors (A_3R), which are known to be free from cardiovascular side effects, exerts a powerful analgesic action in *in vivo* rodent models of experimental neuropathic pain, such as spinal nerve ligation or chemotherapy-induced peripheral neuropathy [1,2]. However, the cellular and molecular basis of A_3R-mediated antinociception are still unknown.
In this study we investigate whether the A_3R agonist Cl-IB-MECA modulates excitability in dorsal root ganglion (DRG) neurons, which are the primary sensory peripheral afference of pain. Dissociated rat DRG neurons were tested for their responsiveness to the selective A_3R agonist Cl-IBMECA and to the endogenous ligand adenosine (Ado) in the absence or presence of different A_3R antagonists.

Patch clamp recordings from primary cultures of rat DRG neurons were performed as described [3]. Exogenous application of Cl-IB-MECA concentration dependently (0.1-100 nM) inhibited voltage-gated outward currents evoked by a ramp protocol (from +80 mV to -120 mV, 800 ms duration) in medium- and small-sized DRG neurons. The I-V relationship of Cl-IB-MECA-inhibited current, investigated by applying a voltage step protocol (13 steps from -40 to +80 mV, 200 ms, Vh = -80 mV) was consistent with the inhibition of non-inactivating and depolarization-activated K^+ currents (as confirmed by Cs^+-replacement experimetns). Cl-IB-MECA effect was mimicked by adenosine (Ado: 30 μM) and prevented in the presence of the selective A_3 antagonists MRS1523 and VUF5574 (100 nM) but not by MRS1220 (0.1-1 μM). On the other hand, adenosine-mediated K^+ current inhibition was only partially blocked either by MRS1523 or VUF5574. In the presence of 1 mM extracellular Cd^{2+}, which inhibits Ca^{2+} entry from voltage-operated Ca^{+2} channels (VOCCs), the effect of Cl-IB-MECA was completely prevented, thus demonstrating that the K^+ conductances inhibited by the A_3R agonist belong to Ca^{2+}-activated K^+ channel (K_{Ca}) family.

In order to verify whether the A_3R agonist directly inhibits K_{Ca} or blocks Ca^{2+} entry from VOCCs and, in turn, decreases outward K_{Ca} currents, we blocked all K^+ channels by replacing intra- and extracellular K^+ by equimolar Cs^+. Under these experimental conditions, a Cd^{2+}-sensitive inward current appeared in the ramp traces at voltages from -30 to +40 mV, coherently with VOCCs gating. This inward Ca^{2+}-mediated current was inhibited by 100 nM Cl-IB-MECA application.

Present data demonstrate that adenosine A_3R activation inhibits Ca^{2+} entry from VOCCs and, in turn, decreases outward K^+ currents evoked by a voltage ramp protocol in rat DRG neurons. These effects might represent the molecular basis of A_3-mediated antinociceptive effects observed *in vivo*, since Ca^{+2} current inhibition would result in hampering of nociceptive neurotransmission from DRG to CNS.

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2',3'-*O*-Substituted ATP derivatives as potent antagonists of purinergic P2X3 receptors and potential analgesic agents

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Compounds able to block membrane currents induced by the stimulation of the purinergic P2X3 receptor localized on nociceptive sensory fibres may be useful as novel and promising tools to treat chronic pain conditions including neuropathic pain, migraine, and inflammatory pain. Inhibitors of these receptors have been reported in literature, among which the ATP analogue 2',3'-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) represent a reference tool with nanomolar potency at the P2X3. With the aid of molecular modelling studies performed at P2X4-based homology models of the human, rat, and mouse P2X3 receptor [1-3], we analysed the binding mode of the TNP-ATP at this protein and designed novel adenosine nucleotide analogues ("PF compounds", Figure 1) bearing alkyl or arylalkyl substituents of various hindrances and replacing the trinitrophenyl moiety of TNP-ATP. These compounds were synthesised and then functionally evaluated on native P2X3 receptors from mouse trigeminal ganglion (TG) sensory neurons using patch clamp recordings under voltage clamp configuration. The results show that these molecules are not endowed with agonist activity at the P2X3 while they are able to inhibit with nanomolar potency currents evoked by α,β-methyleneATP-induced stimulation of this protein. The P2X3 inhibition is reversible and its potency appears influenced by the volume of the 2',3'-*O*-substituent introduced in the ATP moiety. Finally, these molecules do not show any apparent effect on trigeminal $GABA_A$ and 5-HT_3 receptors that are expressed by the same neurons [4]. The activity of these compounds was also analysed at the recently published crystal structures of the P2X3 receptor [5].

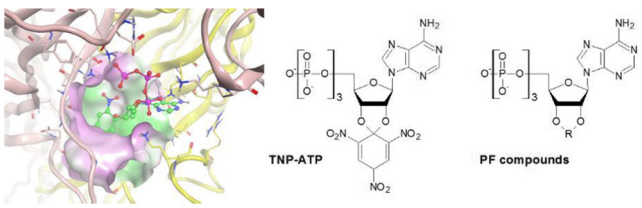

Figure 1. Left. Binding mode of TNP-ATP at the P2X3 receptor representing the mechanism of action of this molecule as P2X3 inhibitor. Right. Molecular structure of the same compound and the molecules (PF compounds) designed, synthesised, and tested in this study.

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A new type of pain-relevant, ligand-gated cationic channel; the ASIC3/P2X3 tandem receptor

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The P2X3 receptor (R) is a ligand-gated cationic channel, which is activated by extracellular ATP. The acid sensing ion channel 3 (ASIC3) belongs to the ENaC/degenerin family and isgated by extracellular protons. Despite their different amino acid sequences both ion channels share the same structure and pore architecture, by i.e. consisting of three identical subunits. Besides, they are both located at partially overlapping subpopulations of dorsal root ganglia neurons and are implicated in acidic pain signaling. Consequently, their physical interaction in the cell membrane or even the formation of heteromeric receptor channels from P2X3 and ASIC3 subunits has to be taken into consideration. We transfected rat (r)P2X3R and rASIC3 constructs individually or together in a ratio of 1:1 into CHO cells. We further used the whole cell patch clamp technique to analyze the current responses either elicited by the application of α,β-methylene-ATP (α,β-meATP) or by a decreasein the extracellular pH value.After co-transfection of both ion channels,the concentration-response relationships of both α,β-meATP and protons were largely inhibited in the CHO cells, in spite of no agonist induced activation of the co-expressed receptor-channel. However, a pH-shift from 7.4 to 6.7 caused a rapidly desensitizing current response and a subsequent strong potentiation of the α,β-meATP-induced current. An even larger potentiation was achieved after a decrease of the pH value to 6.5. By contrast, the pH-induced ionic currents failed to become facilitated in the presence of α,β-methylene ATP (α,β-meATP). Thus a unilateral facilitatory interaction was observed after the stimulation of tightly associated ASIC3/P2X3Rs by acidification. Multiple evidence suggests that ASIC3 and P2X3 receptors form a tandem receptor rather than a heterotrimer: (1) The receptor-channels show complex functional interaction with each other; (2) The proton-binding site is essential for this interaction; (3) They switch their ionic permeability during co-activation, but do not conduct a homogenous macroscopic current; (4) Both recombinant and naïve receptors in DRG neurons interact, excluding the influence of their over-expression in CHO cells; (4) The effect is not limited to ASIC3 as the partner of P2X3, but is valid also for ASIC2a; (5) Co-immunoprecipation proves the close association of the two protein structures; (6) Human recombinant ASIC3 and P2X3Rs show a similar positive interaction.

Insights in receptor Biology derived from identification of adenosine A_1-A_{2A} receptor heteromerization and heteromer-mediated function

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Identification of direct interactions between adenosine receptors: A_1, which is coupled to Gi, and A_{2A} receptor, which is coupled to Gs, was unexpected. Soon after the discovery in an heterologous expression system, the physiological relevance of the A_1-A_{2A} heteroreceptor complex was demonstrated. In fact glutamatergic terminals arriving from the cortex to the striatum express a high percentage of A_1-A_{2A} receptor heteromers that mediate the efcts of adenosine on glutamate release. In fact the A_1-A_{2A} heteroreceptor complex acts as a sensor of adenosine concentration in such a way that low concentrations and high concentrations of the nucleoside leads to opposite regulatory actions [1]. A completely similar scenario was found in astrocytes where adenosine regulates the activity of GAT GABA transporters. Modulation of GAT activity occurs through A_1-A_{2A} heteroreceptor complexes that may signal via two different G proteins: activation of Gs enhances GABA uptake, whereas activation of Gi inhibits it [2]. More recent studies have shown that the minimal signaling unit in an heterologous expression system is constituted by a tetramer composed of two A_1 and two A_{2A} receptors coupled to one Gs and one Gi protein. Computer modeling, aided by bioluminescence resonance energy transfer assays have served to predict the interacting interfaces and propose a quaternary structure of the GPCR tetramer in complex with two G proteins [3]. The putative mechanisms by which activation of A_{2A} receptor blocks activation of Gi in the overall complex will be presented.
Work on this subject started around 2004 and has been supported by grants from ad hoc Spanish Research funding bodies (for instance by MINECO; some of them may include FEDER funds) and from La Marato de TV3.

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Biochemical evidence that a trimeric structure is common to full-length members of P2X and ENaC/ASIC families of ligand-gated ion channels

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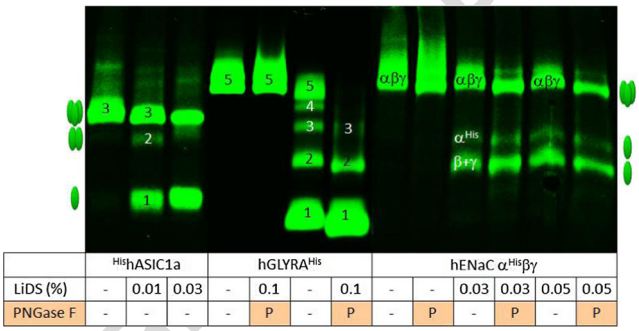
In 1994, two adjacent Nature papers reported the cloning of the first two members of the P2X family, P2X1 and P2X2. By blue native polyacrylamide gel electrophoresis (BN-PAGE) and chemical cross-linking we obtained strong evidence, that P2X1 and P2X3 receptors unexpectedly function as homotrimers [1], as shown later for the entire P2X family [2]. The epithelial Na^+ channel (ENaC) and the related acid-sensing ion channels (ASICs) were cloned around the same time and found to share with P2X receptors the same topology of two transmembrane segments flanking a large ectodomain without any sequence homology to P2X receptors. Biochemically and functionally ENaC channels were identified to be tetramers [3]. However, while the trimeric architecture of P2X receptors was verified by X-ray crystallography [4], it turned out as a surprise when an ASIC1 deletion mutant crystallized as a homotrimer [5]. The crystal structure of ENaC channels has not yet been reported.
A recent study casts doubts that ASIC channels operate as trimers by showing that non-deleted functional ASIC1a migrates in the SDS-PAGE predominantly as tetramers following chemical cross-linking [6]. Here we purified human ASIC1a and ASIC2a and human hENaC ($\alpha\beta\gamma$ and $\delta\beta\gamma$) by Ni NTA chromatography in mild detergents such as DDM and digitonin. We found the cell surface forms of hASIC1a and hASIC2a to co-migrate with the human P2X3 receptor in the BN-PAGE gel; in their fully glycosylated state, the protomers of hASIC1a, hASIC2a and hP2X3 have very similar masses. A homotrimeric and a heterotrimeric state of hASIC1a and the $\alpha\beta\gamma$ hENaC, respectively, could be further verified by comparison with the migration pattern of the partially dissociated homopentameric glycine receptor in the BN-PAGE gel (Fig. 1). Altogether, our data strongly support the current view that full-length P2X receptors and ASIC/ENaC channels share a trimeric architecture.



	^{His}hASIC1a			hGLYRA^{His}				hENaC α^{His}βγ					
LiDS (%)	-	0.01	0.03	-	0.1	-	0.1	-	-	0.03	0.03	0.05	0.05
PNGase F	-	-	-	-	P	-	P	-	P	-	P	-	P

Figure 1. Blue native PAGE (4-16 % acrylamide) evidence for a trimeric structure of the cell surface forms of hASIC1a and hENaC $\alpha\beta\gamma$. The homopentameric glycine receptor hGlyR is shown in the non-denatured and partially denatured state as a mass ruler (52 kDa per N-glycosylated protomer).

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Mapping the allosteric antagonist binding pocket of the human P2X7 receptor for ATP

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The human P2X7 receptor (hP2X7R) provides a therapeutic target for a range of conditions including inflammatory and neuropathic pain, epilepsy, neurodegenerative diseases and transplant rejection. Antagonists of the receptor are predicted to have a low side effect profile as the high concentrations of ATP required to activate the receptor are often only found following cellular damage or in disease states. To investigate the mode of action of hP2X7R antagonists we have used a combination of mutagenesis and molecular modelling to provide validated models of drug action. Molecular docking identified a range of potential antagonist binding modes with the main

clusters found in the orthosteric pocket and an allosteric pocket formed at the subunit interface at the apex of the receptor. Several P2X7R antagonists show high selectivity for the receptor with limited effects at the hP2X1R as well as other P2XR subtypes. The P2X7 receptor has several unique features, including an ~7 amino acid insertion (residues 73-79 when compared to the P2X1R), a four amino acid deletion in the dorsal fin as well as several individual unique/variant residues. We generated a series of chimeric receptors (replacing sections of the hP2X7R with the corresponding region of the hP2X1R) to identify regions of the receptor that were important for high affinity antagonist action. Subsequent point mutants were then generated and characterized and highlight residues within the allosteric pocket that are important for high affinity antagonist binding. These mutagenesis data were used in conjunction with molecular docking to provide validated docking poses for P2X7R antagonists. Interestingly many of the residues that were important for high affinity action were either conserved or similar between P2XR subtypes. This suggests that variations in the structure/access/dimensions of the allosteric pocket play an important role in antagonist selectivity. For the antagonist AZ10606120 we show that the 73-79 insertion and P2X7 unique residues T90 and T94 are important. Molecular dynamics simulations suggest that these features are important for determining the size of the allosteric pocket and thus access of the antagonist. Characterization of the residues involved in binding of other P2X7 receptor antagonists show some common features but also some differences in the mode of selectivity.

Identification of a specific residue that confers BzATP sensitivity to P2X2 receptors

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P2X receptors (P2XR) are trimeric ATP-gated cation channels involved in the fast signal transduction in many cells. Significant advances in the molecular understanding of the P2XR function that have been achieved by the availability of high-resolution structures of the zfP2X4R and, recently, hP2X3R and pdP2X7R [1-4]. However, little is known regarding the molecular determinants of the subtype-specific potency and efficacy of orthostericP2XR ligands. We used docking analyses performed athomology models of the rP2X2R and rP2X7R [5,6] to analyze the ligand-binding of ATP and the potent P2X7R agonist 2'(3')-*O*-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) that is a weak and partial agonist at the P2X2R. The difference between ATP and BzATP is the 4-benzoylbenzoyl (Bz) moiety present at the ribose of the nucleotide in BzATP. We identified two residues K^{212} and $S^{284}$of the P2X2R that spatially correspond to the P2X7R-specific aromatic residues F^{218} and $F^{288}$of the dorsal fin and left flipper domains, respectively. Residues at these positionsmay coordinate the ribose and Bz moiety of ATP and BzATP, respectively, and determine the different potencies of these ligandsat the P2X2R.

The K^{212} and $S^{284}$of the P2X2R were substituted by phenylalanine residues to yield $rP2X2R^{K212F}$ and $rP2X2R^{S284F}$ andwere analyzed by TEVC electrophysiology following expression in *X. laevis* oocytes. Mutating S^{284} to F in rP2X2Rdid not affect the potency for ATP or BzATP. By contrast, the introduction of an aromatic moiety in position 212 as realized in the $P2X2R^{K212F}$ mutant significantly reduced and strongly increased the potency for ATP and BzATP, respectively. BzATP exhibited a higher potency and efficacy than ATP at the $rP2X2R^{K212F}$.

Our results clearly indicate that an aromatic moiety facing the water-exposed surface in the α_3-helix of the P2X2R determines BzATP potency and efficacy, but weakensthe potency and efficacy of ATP.

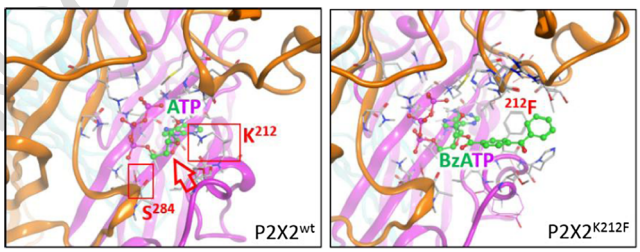

Figure 1. ATP-binding site of the rP2X2R homology models with docked ATP or BzATP. (Left) The $P2X2R^{wt}$ K^{212} and S^{284} residues that spatially correspond to the P2X7R-specific aromatic residues F^{218} and $F^{288}$are indicated. The arrow points to the ribose moiety of ATP to which the 4-benzoylbenzoyl(Bz) moiety of BzATP is attached. (Right) The K^{212}F substitution of the $P2X2R^{K212F}$ coordinates theBzmoiety of BzATP.

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Ligand diversity in P2Y receptors - the $P2Y_6$ case

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The classical P2Y receptors bind nucleotides and are encoded by eight genes with limited sequence homology. Based on their amino acid sequences P2Y receptors can be subdivided into at least two groups. One comprises $P2Y_{1, 2, 4, 6, 11}$, while the second group contains $P2Y_{12\text{-}14}$. P2Y receptors cluster in the d subfamily of rhodopsin-like GPCR together with leukotriene, lipid, and short fatty acid receptors such as the leukotriene receptor CysLT1R, the succinate receptor GPR91, and the 2-oxoglutarate receptor GPR99. It is therefore not surprising that binding of non-nucleotide ligands to classical P2Y receptors was anecdotally observed. Previous studies identified prostaglandin E2 glyceryl ester (PGE2-G) as signaling molecule involved in inflammation. Thus, PGE2-G mobilizes Ca^{2+} and activates protein kinase C and ERK, suggesting the involvement of a GPCR. In a broad screening approach, we found PGE2-G as agonist for $P2Y_6$. We show that PGE2-G and UDP are both agonists at P2Y6, but they activate the receptor with extremely different EC50 values of ~1 pM and ~50 nM, respectively. We speculate that $P2Y_6$ integrates the two different chemical signals related to cell damage into a common intracellular response.

Optimization of Protein Cross-Linking Mass Spectrometry to Identify P2X7R Interacting Proteins

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The P2X7 receptor (P2X7R) belongs to the P2X receptor family of trimeric, ATP-gated, non-selective cation channels. Within the family of purinergic P2X receptors, the P2X7R differs from all other P2X receptors, by its extended cytoplasmic C-terminal domain consisting of 240 amino acids. This long C-terminus was shown to be essential for the receptors distinctive abilities to form large pores and to induce membrane blebbing and is supposed to be crucial for interactions with other proteins. Triggering of P2X7R leads to the activation of caspase-1 and subsequent release of the mature proinflammatory cytokines Interleukin-1β and Interleukin-18 and has been shown to contribute to a variety of pathologies, such as neurodegenerative processes and inflammatory bowel disease. Due to its involvement in different pathologies, the receptor is increasingly coming into focus as a promising target for the development of novel drugs. Despite its importance as a drug target, the proteins involved in its localization, signalling, and functional modulation are still unclear. Great efforts have been undertaken to identify interacting proteins. However, the common used method is co-immunoprecipitation (Co-IP) followed by mass spectrometry or western blot analysis and might disregard transient interactions that are lost due to harsh protein extraction conditions or extensive washing during the purification process. An approach to stabilize labile interactions prior to the purification process and improve chances to identify protein-protein interactions with high dissociation rates is the use of chemical cross-linkers. Here we used the membrane permeable homo-bi-functional NHS-ester disuccinimidyl suberate (DSS) to analyze the P2X7R interactome via protein cross-linking coupled mass spectrometry (XL-MS). To allow the tissue-specific purification of interacting proteins we generated a BAC transgenic mouse model, in which the receptor is fused via a streptag-heptahistidyl-linker to an EGFP reporter protein that allows expression of the fusion protein at moderate over-expression levels under the control of its endogenous promotor. Expression patterns and levels of P2X7R-EGFP correlate with the endogenous protein and glycosylation analysis as well as immuno-histochemistry data indicate its complex glycosylation, correct folding, and proper transport to the plasma membrane. Purification of the P2X7R-EGFP fusion protein with nanobodies against EGFP leads to co-purification of the endogenous P2X7R subunits, confirming their coassembly. Initial biochemical experiments show that P2X7Rs can be *in situ* cross-linked with DSS, which leads to the formation of stable di- and trimers of the endogenous P2X7R and the P2X7R-EGFP fusion protein. The purified P2X7R complexes can be efficiently enriched for visualization on coomassie gels and subsequent analysis by mass spectrometry. The optimization of cross-linking conditions, especially to allow ~~*in-vivo*~~ cross-linking, is ongoing and will be presented. Besides the identification of interaction partners, XL-MS experiments bear the potential to map interaction domains and to provide an insight into the structural organization of the P2X7R signaling complex.

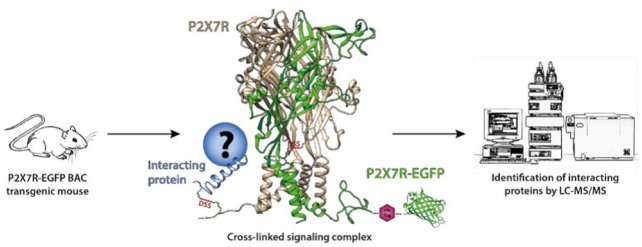

Figure 1. Approach to map the P2X7R interactome by protein cross-linking mass spectrometry using the membrane permeable homo-bi-functional NHS-ester disuccinimidyl suberate (DSS).

Structural Studies on Nucleotide pyrophosphatases/phosphodiesterases

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Nucleotide pyrophosphatases/phosphodiesterases (NPPs) are a family of ectophosphodiesterases comprising seven members in vertebrates [1]. NPPs are glycoproteins and able to hydrolyze a wide range of molecules involved in different signaling pathways (e.g. in purinergic signaling). Whereas NPP1 and 3 are specific for nucleotides and dinucleotides, the natural substrates of NPP2, NPP5 and NPP7 are phospholipids. NPP1-3 include besides the catalytic domain a nuclease-like domain, which has no catalytic activity. Furthermore at the N-terminus of NPP1-3 two consecutive cysteine-rich somatomedin B (SMB)-like domains are located, which are involved in substrate binding (NPP2) and membrane anchoring (NPP1 and 3). NPP4-7 are only contain the catalytic domain. Apart from NPP2 all NPP family members are membrane associated [2]. Based on their involvement in many physiological functions and diseases NPPs are regarded as attractive drug targets. Structures have now been determined for NPP1-4, NPP6 and NPP7 [3, 4]. We report on the structure and function of NPP enzymes, in particular concerning nucleotide specificity and the mechanism of phosphoryl transfer.

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Gate and pore of P2X7 receptors

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The P2X7 receptor (P2X7R) belongs to the P2X family of ATP-gated cation channels. P2X7Rs are expressed in epithelial cells, leucocytes and microglia, and they play important roles in immunological and inflammatory processes. P2X7Rs are obligate homotrimers, with each subunit having two transmembrane helices, TM1 and TM2. Structural and functional data regarding the P2X2 and P2X4 receptors indicate that the central tri-helical TM2 bundle forms the intrinsic transmembrane channel of P2X receptors. Here, we studied the accessibility of single cysteines substituted along the pre-TM2 and TM2 helix (residues 327–357) of the human P2X7 receptor (hP2X7R) using as readouts (i) the covalent maleimide fluorescence accessibility of the surface-bound hP2X7R and (ii) covalent modulation of macroscopic and single-channel currents using extracellularly and intracellularly applied methanethiosulfonate (MTS) reagents. We found that the channel opening extends from the pre-TM2 region through the outer half of the tri-helical TM2 channel. Covalently adducted $MTSEA^+$ ($MTS\ ethylammonium^+$) strongly increased the probability that the channel was open by delaying channel closing of seven of eight responsive hP2X7R mutants. Structural modeling, as supported by experimental probing, suggested that resulting intraluminal hydrogen bonding interactions stabilize the open-channel state. The additional decrease in the single-channel conductance by $MTSEA^+$ in five of the seven positions identified Y336, S339, L341C, Y343 and G345 as the narrowest part of the channel lumen. The gate and the ion-selectivity filter of the hP2X7R could be co-localized at and around residue S342. None of our results provided any evidence for dilation of the hP2X7R channel upon sustained stimulation with ATP^{4-}.

Dynamics of adenosine receptor activation studied in real time by FRET-based biosensors

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Recently published crystal structures of the adenosine A_1 and A_{2A} receptor in the inactive and active state (for the A_{2A} receptor) have revealed static endpoints of the conformational changes associated with the activation process. To investigate the activation dynamics of different adenosine receptor subtypes we used fluorescence resonance energy transfer (FRET) measurements of a modified A_1 and A_{2A} receptor construct (A_1R, $A_{2A}R$). Those optical probes where designed by fusion of the cyan fluorescent protein (CFP) to the C-terminus of the receptor and insertion of the fluorescein arsenical hairpin binder (FlAsH) motif into the 3rd intracellular loop. Based on the ligand binding pocket revealed from the crystal structure 10 optical probes including individual mutations were created for each receptor. To compare A_1R and $A_{2A}R$ dynamics, we established HEK293 cell lines stably expressing these optical probes and investigated the signal amplitude and the receptor activation kinetics in living cells. We indentified 3 different effects of these mutations. One class causes problems in membrane localization of the A1R but not the A2AR. The 2nd group is involved in binding of the ribose moiety and has

stronger effects in the A1R compared to the A2AR. The 3rd class consists of the mutants that are involved in binding of the adenine moiety and have similar effects for adenosine and theophylline binding for the A2AR. Thus, our study provides evidence that amino acids serve different functions within the A1R and A2AR ligand binding pocket. In summary the different signal amplitudes and different activation kinetics are indicative for a different activation behavior of the A_1R and A_2AR and the data from the receptor mutants support these findings and gives new insight into the A_1R- structure.

P2X7 receptor activation modulates inflammation and autophagy in amyotrophic lateral sclerosis mouse microglia

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Neuroinflammation plays a crucial role in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS), where degenerating motor neurons contribute to produce danger signals that activate microglia to initiate and propagate a cytotoxic cascade culminating in muscle weakness, atrophy, spasticity, compromised breathing and finally death in patients. Extracellular ATP performs as one of such endogenous alarm signal by signaling through purinergic P2 receptors, particularly the P2X7 ionotropic subtype [1, 2]. Activation of P2X7 in ALS-microglia exacerbates pro-inflammatory responses exemplified by modulation of NF-κB, Rac, NADPH oxidase ($gp91^{phox}$), ROS, TNF-α, COX-2 and MAPKs levels, and culminating into cytotoxicity towards neurons [3-5]. In the SOD1-G93A mouse model of ALS, the disease onset is significantly anticipated and the disease progression is worsened in mice genetically lacking P2X7, thus suggesting that the receptor plays beneficial roles at least during a precise, but still undefined, phase of the disease [6]. Despite of this, the P2X7 antagonist Brilliant Blue G administered to SOD1-G93A mice from late pre-onset, enhances motor neuron survival and reduces microgliosis in lumbar spinal cord, moreover ameliorates the disease progression [7]. In mouse microglia and muscle cells, as well as in human monocytes/macrophages and epithelial cells, P2X7 has been reported to differently shape autophagy by acting as a context-specific either positive or negative regulator. The purpose of this work was thus to establish if P2X7 participates to ALS pathogenesis by directly modulating the autophagic flux of SOD1-G93A microglia. Our results identify the modulation of autophagy as a novel mechanism by which P2X7 activates ALS-microglia, perhaps to be considered as a strategy to counteract ALS progression.

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P2X7-blocking nanobodies ameliorate inflammation

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The ATP-gated P2X7 ion channel drives inflammation by promoting the release of interleukin-1β. P2X7 is an important therapeutic target, yet there remains a need for drugs with better selectivity and fewer side effects. Unlike small molecules, antibodies are highly selective for their targets, yet most available antibodies fail to antagonize ion channel functions. Nanobodies are single domain antibody fragments derived from the heavy chain antibodies naturally ocurring in llamas and other camelids (Figure 1). We hypothesized that nanobodies offer a means to develop specific biologics to the P2X7 ion channel. From P2X7-immunized llamas, we selected nanobodies that effectively block (13A7) or potentiate (14D5) gating of mouse P2X7 [1]. Systemic injection of nanobody 13A7 ameliorated experimental glomerulonephritis, while injection of nanobody 14D5 made disease significantly worse. Ex vivo analyses showed that prominent populations of kidney leukocytes express P2X7 at very high levels. These include cells found only at low frequencies, if at all, in lymphatic tissues, i.e. tissure resident memory cells (Trems) and natural killer T cells (NKT). Systemic injection of P2X7-specific nanobodies resulted in complete opsonization and functional modulation of P2X7 on these cells. Our results underscore the utility of the Nanobody technology to generate antibody therapeutics against ion channels and confirm P2X7 as a therapeutic target for inflammatory disorders.

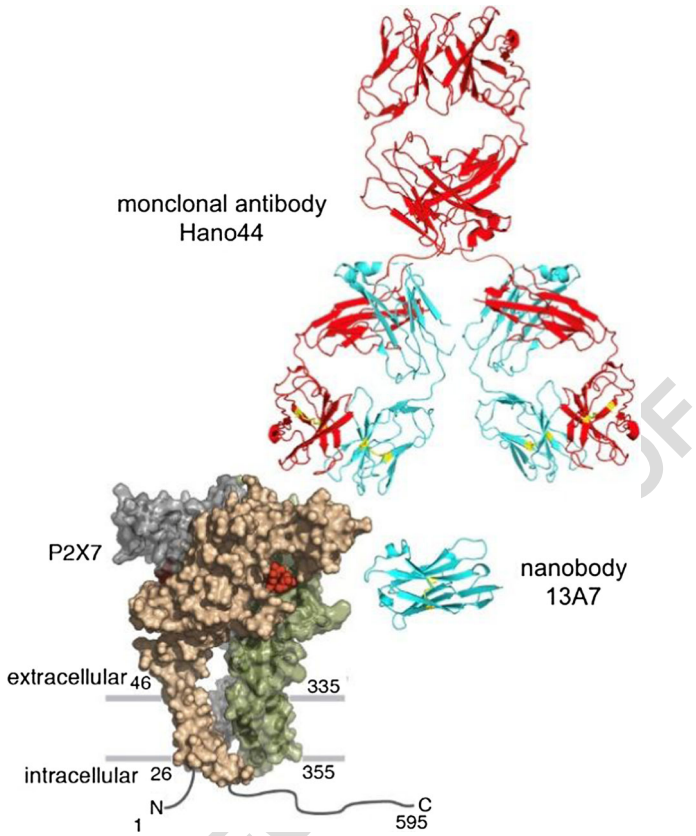

Figure 1. Schematic diagram of P2X7 interacting with a conventional monoclonal antibody (Hano44) and a nanobody (13A7).

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Cross-talk between microglia and oligodendroglial progenitors in cerebral ischemia: implications for new purinergic strategies to brain repair

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Oligodendrocytes, the myelin-forming cells in the brain, are severely affected by ischemia [1], contributing to stroke-associated deficits. The possibility to implement spontaneous post-injury repair mechanisms by targeting myelin still represents an unexplored field. In this respect, GPR17, a P2Y-like receptor transiently expressed on Oligodendrocyte Progenitor Cells (OPCs) has emerged as a target to implement stroke repair through stimulation of OPC maturation [2]. Results obtained by fate-mapping analysis using the conditional GPR17-iCreERT2xCAG-eGFP transgenic mice showed that the subpopulation of adult OPCs expressing GPR17 (GFP^+-cells) represents "a reserve pool" that is maintained for repair purposes after brain damage [3]. In particular, we recently demonstrated that, after brain ischemia, GFP^+-cells actively respond to injury increasing their proliferation rate and migratory capacity. However, at later stages, only a low percentage of these cells undergoes maturation [4]. This limited post-stroke repair is likely due to local unfavourable inflammatory milieu mediated by macrophages and resident microglia, which participate to post-ischemic inflammation assuming both detrimental and beneficial phenotypes.

Here, we aimed at: (i) characterizing the spatio-temporal distribution of GFP^+-cells in relation to microglia/macrophage polarization in transgenic mice after middle cerebral artery occlusion (MCAo); (ii) exploring the cross-talk between microglia and OPCs, by assessing how vesicles released extracellularly (EVs) by microglia, polarized toward a pro- or anti- inflammatory state, influence OPC behaviour.

In vivo studies showed that GFP^+-cells accumulate at the border of the ischemic lesion starting from 72 h after ischemia, when microglia and macrophages show both pro- and anti-inflammatory features. One week after stroke, the absolute number of pro-inflammatory cells increases, while myeloid cells with pro-regenerative phenotype do not significantly change. *In vitro* studies pointed out that EVs produced by pro-inflammatory microglia only slightly limit OPC proliferation, whereas EVs produced by pro-reparative microglia tend to increase it. Preliminary data showed that all types of

EVs (from unstimulated, pro-inflammatory or pro-regenerative microglia) are able to induce OPC migration, indicating that EVs provide attractive guidance cues independently of the activation state of donor microglia. Interestingly, EVs from pro-regenerative microglia have a higher chemotactic effect on the subpopulation of cells expressing GPR17, suggesting that EVs may also contain purinergic signals able to influence OPC migration via GPR17. Finally, exposure to EVs from either pro- or anti-inflammatory microglia (but not resting cells) promote OPC maturation. However, only EVs released by pro-regenerative cells significantly foster myelin deposition in an *in vitro* system of OPCs co-cultured with DRG neurons. Shedding light on these signals is important for developing combined therapeutic interventions where a purinergic approach, aimed at implementing recovery after stroke, is potentiated by agents promoting a better microglia phenotype with pro-regenerative effects on OPCs.

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Prostaglandin E_2 impairs P2Y receptor signaling in astrocytes

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Prostaglandin E2 (PGE2) is one of the main bioactive lipids accumulated after tissue damage or inflammation due to the rapid expression of cyclooxygenase 2. This bioactive lipid activates specific membrane receptors belonging to the G-protein coupled receptor family: EP1-EP4 and mediates pro- or anti-inflammatory actions depending on cell-context. Nucleotides can also be released in these situations and even contribute to PGE2 production. In previous studies we described a reverse interaction, the selective impairment of P2Y nucleotide receptors by PGE2 in macrophages and fibroblasts [1,2]. In both models, the effect of the prostaglandin was independent on PG receptors, involving the activation of PKC and PKD. Considering that macrophages and fibroblasts contribute to the regulation of inflammatory response and tissue remodeling, a similar aligned mechanism involving P2Y signaling could take place in astrocytes in order to resolve neuroinflammation and brain repair.

In the present study we have analyzed the modulation of $P2Y_2$/$P2Y_4$ receptors by PGE_2 in rat cerebellar astrocytes. We demonstrated that PGE_2 inhibited $[Ca^{2+}]_i$ increases elicited by UTP in single cell. Using different approaches we proved that the inhibition of UTP calcium responses by PGE_2 was independent on membrane receptors. Besides, the inhibition of P2Y signaling had an impact on the astrocyte migration elicited by the nucleotide. With the purpose of figuring out the intracellular signaling pathway triggered by the prostaglandin, we studied the PGE_2 effect on the activation of two proteins targeted by P2Y nucleotide receptors, such as ERKs and Akt proteins. The results showed that PGE_2 acting through nPKC negatively modulates ERK and Akt phosphorylation induced by UTP. We are currently investigated the intracellular mechanisms responsible for the inhibition of UTP responses. We hypothesize that $P2Y_2$ or $P2Y_4$ receptors could be the target of PGE_2-activated nPKC/PKD. PGE_2 could promote the internalization of P2Y receptors as have been described in fibroblast transfected with COX-2 [2].

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Glial cells and neuroinflammation: the adenosinergic perspective

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The hallmark of neuroinflammation is the activation of microglia, the immunocompetent cells of the central nervous system, releasing a number of proinflammatory mediators, such as reactive oxygen and nitrogen species, as well as cytokines including interleukin IL-6, tumor necrosis factor TNF-α, strongly associated to the pathogenesis of several neuronal pathologies, spanning from Alzheimer's and Parkinson's diseases to multiple sclerosis [1]. Therefore modulating the mediators of inflammation produced by activated microglia may help to attenuate the progression of neuroinflammatory and neurodegenerative diseases [2]. Adenosine is an ubiquitous autacoid exerting neuroprotective and immune-modulatory functions by regulating several microglia activities, through the stimulation of four receptor subtypes, named A_1, A_{2A}, A_{2B} and A_3 adenosine receptors [3,4]. An overview of adenosine-induced modulatory effects on different microglia cell functions, focusing on cytokines, cellular signaling, proliferation and activation, will be given. These findings add a new important piece of knowledge on the role of adenosine receptors in microglia activation and neuroinflammation and suggest that adenosine receptors represent good targets to suppress inflammatory events in microglia [5,6].

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Proteomic analysis of ectosomes and exosomes provides new clues on microglia response to ATP

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Extracellular ATP is among molecules promoting microglia activation and inducing the release of extracellular vesicles (EVs), which are potent mediators of intercellular communication between microglia and the microenvironment. We previously showed that EVs released from microglia under ATP stimulation (ATP-EVs) induce a robust inflammatory reaction in cultured glial cells and propagate microglia activation in mice with subclinical neuroinflammation [1]. However, the proteomic profile of ATP-EVs has not yet been elucidated.
In this study we applied a label free proteomic approach to analyse the protein composition of the two main populations of EVs released constitutively or upon ATP stimulation from rat primary microglia, i.e. quite large ectosomes, shed from the plasma membrane and exosomes originating from the endosomal compartment. To separate ectosomes from exosomes we used a classical differential ultracentrifugation protocol [2]. To avoid cell damage, microglia were exposed to ATP for only 1 h and EVs were isolated from medium conditioned by microglia for this time period. Due to the shortness of the protocol and the limited expansion of primary microglia, small EV batches could be generated, finally limiting detection of low abundant EV proteins and quantitative analysis of EV proteome.
We found that exosomes and ectosomes have a set of specific proteins but also share a substantial fraction of proteins. Proteome overlap may derive, at least in part, from the isolation procedure, which does not allow net separation of exosomes from ectosomes. Analysis of biological processes of constitutive EVs by Gene Ontology (GO) term database revealed "response to molecules" (~32 %), "response to environmental changes" (~20 %), "cytoskeletal dynamics" (17 %), "innate immune response" (~10 %), as predominant categories. The most significant pathways identified using the Kyoto Encyclopedia of Genes and Genome (KEGG) analysis were "phagosome", "antigen processing and presentation", "lysosomes" and "complement and coagulation". These categories and pathways are consistent with the surveying function of unstimulated microglia and their role in antigen presentation and degradative activity following phagocytosis. Besides immune molecules (C1q, galectin-3, CD14, Lysozyme C) constitutive EVs also contained anti-inflammatory mediators, such as the leaderless proteins Annexin A1 and A2, which may balance the pro-inflammatory action of immune factors.
About ~60 % of proteins of ATP-EVs were dependent on ATP, not being present in constitutive EVs. They included a set of proteins implicated in cell adhesion, extracellular matrix organization, in autophagy-lysosomal pathway and antigen processing. The functional properties of ATP-specific proteins may reflect enhancement of degradative pathways to meet increased synaptic pruning and phagocytosis in response to ATP and possible enhancement of EV movement and interaction with target cells. However, the most significant change in the proteome of ATP-EVs was related to cell metabolism. ATP-specific proteins included several enzymes involved in glycolysis, the oxidative branch of the pentose phosphate pathway, pyruvate metabolism, glutamine metabolism, and fatty acid synthesis. Panther GO pathway classification showed an increase in glycolysis, in pentose phosphate pathway, in fatty acid synthesis rather than of beta-oxidation, and in glutamine metabolism, which may serve to replenish levels of TCA cycle metabolite. Changes of metabolic proteins in ATP-EVs suggest a metabolic shift of microglia from oxidative phosphorylation to anaerobic glycolysis. This metabolic alteration may accommodate the increased energy requirements to enhance routine ATP-dependent cellular behavior of microglia such as process scanning and phagocytic activity [3]. Of note, the augmented sorting of metabolic enzymes in EVs also suggest that EVs secreted from ATP-activated microglia may influence metabolism in receiving cells.

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Obesity and enteric inflammation: role of adenosine in intestinal neuromotor dysfunctions

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Adenosine A_{2B} receptors (A_{2B}R) regulate several enteric functions. However, their role in the pathophysiology of intestinal dysmotility associated with high fat diet (HFD)-induced obesity has not been elucidated. We investigated the expression of A_{2B}R in mouse colon and their role in the mechanisms underlying the development of enteric dysmotility associated with obesity.

Wild type C57BL/6 J mice were fed with HFD (60 % kcal from fat) or normocaloric diet (NCD, 18 % kcal from fat) for 8 weeks. Colonic $A_{2B}R$ localization was examined by immunofluorescence. The role of $A_{2B}R$ in the control of colonic motility was examined in functional experiments on longitudinal muscle preparations (LMPs). Groups of mice fed with NCD or HFD were treated with clodronate encapsulated into liposomes to deplete macrophages.
In NCD mice, $A_{2B}R$ were predominantly located in myenteric neurons; in HFD animals their expression increased throughout the neuromuscular layer. Functionally, the $A_{2B}R$ antagonist MRS1754 enhanced electrically-induced NK_1-mediated tachykininergic contractions in LMPs from HFD mice, while it was less effective in tissues from NCD mice. $A_{2B}R$ stimulation with BAY 60-6583 decreased tachykininergic contractions in LMPs from NCD-fed mice, while resulting in an increased efficacy in tissues from HFD animals. Both $A_{2B}R$ ligands did not affect contractions elicited by exogenous substance P. Macrophage depletion in HFD-mice normalized $A_{2B}R$ immunopositivity, while reverting the inhibitory control of $A_{2B}R$ on colonic contractions.
Obesity is related with a condition of colonic inflammation, leading to an increase of $A_{2B}R$ expression. $A_{2B}R$, modulating the activity of excitatory tachykininergic nerves, participate to the enteric dysmotility associated with obesity.

Purinergic signaling shapes microglial functions in Alzheimer's disease

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Genome-wide association studies have highlighted the importance of microglia, the main immune cells of the CNS, in Alzheimer's disease (AD), implicating microglia as a promising target for AD treatment. Microglial cells in mouse models of AD show signs of functional impairment. Whether alterations in purinergic signaling, which under physiological conditions is critically involved in shaping microglial functions, contribute to this phenomenon and whether microglial dysfunction is reversible by targeting purinergic signaling has remained unclear.
Plaque-related microglia from APP/PS1 mice showed impaired phagocytic capacity and cell motility in intravital 2-photon microscopy and acute cerebral slice phagocytosis assays. Transcriptional analysis of microglial cells harvested by laser-microdissection revealed significantly altered expression of ectonucleotidases and purinoreceptors in plaque-associated cells. Importantly, short-term treatment of microglia with purinoreceptor agonists or pharmacological blockage of ectonucleotidases rapidly normalized phagocytic and dynamic function of plaque-associated microglia in acute cerebral slices from APP/PS1 mice, suggesting that microglial dysfunction indeed is reversible upon treatment.
In summary, we have identified altered purinergic signaling as a potential cause for the impairment of cellular dynamics, morphology and phagocytic capacity of plaque-associated microglia in the APP/PS1 model. Thus, targeting microglial purinergic signaling may represent a promising novel approach for the treatment of AD.

$A_{2A}R$ blockade prevents memory impairment caused by cannabinoids in mice

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Cannabinoid-mediated memory impairment is a concern in cannabinoid-based therapies [1,2]. Caffeine exacerbates cannabinoid CB1 receptor (CB1R)-induced memory deficits through an adenosine A_1 receptor-mediated mechanism [3,4]. We herein report recently published [5] data from a study where we evaluated how chronic or acute blockade of adenosine A_{2A} receptors ($A_{2A}Rs$) affects long-term episodic memory deficits induced by a single injection of a selective CB1R agonist. Long-term episodic memory was assessed by the novel object recognition (NOR) test. Mice received an intraperitoneal (i.p.) injection of the CB1/CB2 receptor agonist WIN 55,212-2 (1 mg/kg) immediately after the NOR training, being tested for novelty recognition 24 h later. Anxiety levels were assessed by the Elevated Plus Maze test, immediately after the NOR. Mice were also tested for exploratory behaviour at the Open Field. For chronic $A_{2A}R$ blockade, KW-6002 (istradefylline) (3 mg/kg/day) was administered orally for 30 days; acute blockade of $A_{2A}Rs$ was assessed by i.p. injection of SCH 58261 (1 mg/kg) administered either together with WIN 55,212-2 or only 30 min before the NOR test phase. The involvement of CB1Rs was assessed by using the CB1R antagonist, AM251 (3 mg/kg, i.p.). WIN 55,212-2 caused a disruption in NOR, an action absent in mice also receiving AM251, KW-6002 or SCH 58261 during the encoding/consolidation phase; SCH 58251 was ineffective if present during retrieval only. No effects were detected in the Elevated Plus maze or Open Field Test. The finding that CB1R-mediated memory disruption is prevented by antagonism of adenosine $A_{2A}Rs$, highlights a possibility to prevent cognitive side effects when therapeutic application of CB1R drugs is desired.
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A_{2A} receptor dysregulation: an instrumental role in Alzheimer's disease?

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Consumption of caffeine, a non-selective antagonist of adenosine A_{2A} receptor (A_{2A}R), mitigates cognitive decline during ageing, reduces Alzheimer's disease (AD) risk in humans, and also decreases amyloid and Tau pathologies in AD transgenic mouse models [1]. In line, selective A_{2A}R blockade improves memory and pathology in transgenic models mimicking Tau and amyloid lesions [2-4]. These data support A_{2A} receptor as a valuable target in AD. Beneficial effects of caffeine and A_{2A}R antagonists are presumably ascribed to the normalization of dysregulated A_{2A}R activity. Indeed, brains of aged and AD individuals but also AD models are characterized by an abnormal upsurge of A_{2A}Rs [5], being of neuronal and astrocytic origin [3,4,6,7]. However, impact of neuronal and astrocytic A_{2A}R dysfunction towards development of cognitive deficits and AD lesions remains largely unknown.
At the meeting, we will provide new insights on the impact of neuronal and astroglial gain of A_{2A}R function towards cognition and AD lesions. Indeed, we have developed a conditional models allowing A_{2A}R overexpression specifically in forebrain CaMKII-positive neurons or GFAP-positive astrocytes. We used these new models to evaluate the consequences of cell-specific A_{2A}R dysregulation towards cognition and hippocampal plasticity. In addition, to uncover impact of A_{2A}R dysfunction towards AD pathology, these models were crossed with Tau transgenic developing progressive Tau pathology associated with a cognitive decline.
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The role P2X7 receptors in animal models of neurodevelopmental psychiatric disorders

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Maternal immune activation is a principal environmental risk factor contributing to neurodevelopmental psychiatric disorders, including autism spectrum disorder (ASD) and schizophrenia (SZ). Maternal infection is associated with the later emergence of ASD and SZ compromising foetal brain development at critical periods of pregnancy, and the elevation of maternal pro-inflammatory cytokines has been causally linked to perinatal brain reprogramming [1,2]. However, the molecular signalling pathway that converts maternal immune activation to pathologically relevant neurodevelopmental abnormalities in the offspring has been unclear until now. The NLRP3 inflammasome signalling pathway, triggered by the co-activation of P2X7 purinergic receptors is one of the major signalling routes that mediates the conversion of innate immune response to inflammation in response to exogenous and endogenous danger signals [3]. Recently we have reported that both genetic deletion and pharmacological inhibition of P2X7 receptors alleviate schizophrenia-like behavioral alterations in a rodent, phencyclidine (PCP)-induced animal model of SZ [4]. We have also functionally identified purinergic receptors regulating the activity of subplate neurons of the developing rat cortical plate, which is a transient neuronal subpopulation critical in shaping of the development of cortical networks. Here we report that activation of maternal P2X7 receptors is necessary and sufficient to transduce maternal immune activation (MIA) to autistic phenotype in the offspring. We show that whilst maternal immune activation by poly(I:C) injections to pregnant wild-type mouse dams elicits autism-like phenotype in their offspring, including social deficit, impairment of sensorimotor coordination, repetitive behaviours, atrophy of cerebellar Purkinje cells and destruction of synapses, no such alterations are observed in mice genetically deficient in P2X7 receptors. The effect of P2X7 gene deficiency could be reproduced by a single maternal treatment with specific P2X7 receptor antagonist JNJ7965567 (20 mg/kg i.p.). Inhibition of maternal P2X7 receptors effectively prevented the induction of IL-6 in the maternal plasma and

foetal brain. Interestingly, postnatal P2X7 receptor inhibition was also effective in the alleviation of certain behavioural and morphological alterations in the offspring. Our results suggest that P2X7 receptor signaling contributes to pathophysiology of neurodevelopmental disorders and offer a therapeutic possibility for early detection and prevention of ASD, the increasingly prevalent psychiatric disorder in children.

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Dissecting P2RX7 expression and function using a humanized mouse model

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The purinergic P2X7 receptor (P2X7R) has attracted considerable interest as a potential target for various central nervous system pathologies including affective and neurodegenerative disorders [1]. The comprehension of the distribution and cellular localization of the P2X7R in the brain is a prerequisite for understanding its contribution to brain disease. Therefore, we generated a genetic mouse model by introducing the human P2X7R into the mouse as mammalian model organism. We demonstrated its functionality and revealed species-specific characteristics of the human receptor, compared to the murine ortholog, regarding its receptivity to activation and modulation. This humanized *P2rx7* allele is accessible to spatially and temporally controlled Cre recombinase-mediated inactivation. In contrast to previously generated knockout (KO) mice, none of the described *P2rx7* splice variants evade this null allele. By selective disruption and assessment of human *P2RX7* expression in different brain regions and cell types, we were able to demonstrate that the P2X7R is specifically expressed in glutamatergic pyramidal neurons of the hippocampus. Also, P2X7R is expressed in major non-neuronal lineages throughout the brain, i.e., astrocytes, oligodendrocytes and microglia [2]. Thus, this novel multifunctional allele provides the means to test compounds targeting the P2X7R under *in vivo* conditions and to address its function by more precise approaches since it avoids compensatory mechanisms and other caveats accompanying constitutive KO mice. Moreover, we used this humanized P2X7R mouse line as an appropriate control for the *in vivo* interrogation of a mood disorder-associated non-synonymous single-nucleotide polymorphism (SNP rs2230912). The SNP results into a glutamine (Gln) by arginine (Arg) substitution at codon 460 of the P2X7R. Using a similar strategy as described above, we generated a second mouse line in which we substituted the mouse P2X7R by the human P2X7R-Gln460Arg variant. The P2X7R-Gln460Arg variant per se is not compromised in its function. However, heterologous expression of P2X7R-Gln460Arg together with wild-type P2X7R has recently demonstrated to impair receptor function [3]. Here we show that this also applies to humanized mice co-expressing both human P2X7R variants. Primary hippocampal cells derived from heterozygous mice showed an attenuated calcium uptake upon agonist stimulation. While humanized mice were unaffected in their behavioral repertoire under basal housing conditions, mice but also humans that harbor both P2X7R variants showed alterations in their sleep parameters resembling signs of a prodromal disease stage. The predisposition of heterozygous humanized mice to develop symptoms reflecting endophenotypes of mood disorders manifested when exposed to chronic social defeat stress. These results indicate that heterozygosity for the wild-type P2X7R and its mood disorder associated variant P2X7R-Gln460Arg represents a genetic risk factor, which in conjunction with stress is able to convey susceptibility to mood disorders.

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New therapeutic strategy to prevent the onset of dyskinesia in models of Parkinson's disease

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Adenosine A_{2A} antagonists have emerged as new class of very promising non dopaminergic drugs in the treatment of Parkinson's Disease (PD). Indeed, the development of new highly selective adenosine A_{2A} antagonists, and their encouraging anti-parkinsonian responses in animal models of PD, has provided a rationale for clinical trials to evaluate the therapeutic potential and the safety of these agents in PD patients [1-3]. Specifically, adenosine A_{2A} antagonists, significantly increase L-dopa efficacy in PD without exacerbating dyskinetic-like behavior [1-3]. The pre-sinaptic serotonin 5-$HT_{1A/1B}$ receptors modulate the release of dopamine formed from L-Dopa in serotoninergic neurons after dopamine denervation [4]. In line with this finding, serotonin 5-$HT_{1A/1B}$ receptor agonist eltoprazine suppressed dyskinetic-like behavior in animal models of PD, but at the same time, it reduced L-dopa-induced motility [5]. On this basis, we hypothesize that the early combined administration of preladenant and eltoprazine may produce reduction and prevention of the onset of L-dopa-induced-dyskinesia in a rodent model of PD.

Unilateral 6-OHDA-lesioned L-dopa-non primed rats, were treated for 2 weeks with preladenant (0.3 mg/kg) and/or eltoprazine (0.6 mg/kg), alone or in combination with L-dopa (4 mg/kg), and abnormal involuntary movements (AIMs) as index of dyskinesia, were evaluated. Moreover, induction of immediate-early gene *zif-268 (*an index of long-term changes correlated with dyskinesia) was evaluated [6].
Results show that combined administration of L-dopa plus preladenant plus eltoprazine significantly counteracted and prevented dyskinetic-like behaviors induced by L-dopa, without impairing the efficacy of L-dopa in relieving motor symptoms.
Preliminar results showed that *zif-268* was increased in striatum of rats treated with L-dopa and L-dopa plus preladenant compared with vehicle. In contrast, rats treated with eltoprazine (with or without preladenant) had lower *zif-268* activation after treatment with L-dopa.
Results suggest that combination of L-dopa with preladenant and eltoprazine may be a promising therapeutic strategy for treating motor symptoms, delaying, at the same time, the onset of dyskinesia in PD.

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Signaling pathways promoting nitric oxide production involved in mnesic effects of guanine

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Growing evidence indicates that some guanine-based purines (GBPs), in particular Guanosine (GUO), exert important neuroprotective [1,2] and neuromodulatory roles in CNS, by acting as throphic [3], antiapoptotic [4] and anticonvulsant [5] agents. In contrast, the effects elicited by Guanine (GUA) are poorly known, although it has been reported that GUO and GUA affect memory and learning in opposite manner. Indeed GUO, systemically administered to rats before training session, caused amnesic effects in inhibitory avoidance task [6,7], whereas GUA prevented the amnesic effect provoked by the inhibition of NOS activity and, once administered after training, showed positive effects also on memory consolidation process [7]. In this context, it has to be stressed that GUO and GUA undergo a fine functional interplay, which is regulated by the activity of purine nucleoside phosphorylase that converts GUO into GUA also at extracellular level [8], and by specific trans-membrane transporters of these compounds. Since several effects elicited by GUO or GUA still persist in the presence of inhibitors of these transporters [2,4], it seems reasonable that these compounds may also act at extracellular level, likely by interacting with putative trans-membrane sites. For this reason we investigated, in cultured SH-SY5Y neuroblastoma cells, the signal transduction pathways responsible for the previously mentioned GUA-mediated "in vivo" effects on learning and memory. GUA increased Ask1, cJUNK and p38 phosphorylation through a prevalent intracellular activity, since these effects were mainly prevented by cell pre-treatment with the inhibitors of purine uptake. At the same time, GUA caused a time- and dose-dependent Akt and ERK1/2 phosphorylation, associated with an increased production of nitric oxide (NO). These effects were reduced by about 50 % when GUA uptake was inhibited as well as when cells were pretreated with pertussis toxin. This suggests that, in addition to an intracellular effect, GUA behaves as signalling molecule, likely acting through a G protein-coupled receptor. Cell pretreatment with the PI3K-Akt inhibitor, LY294002, significantly reduced Akt phosphorylation, induced by GUA, and NO production. Conversely, cell pretreatment with inhibitors of PKC or HemeOxigenase (HO), GF102903X or zinc protoporphyrin-IX respectively, able to inhibit the PKC-HO-CO pathway and, consequently, cGMP production known to be responsible for the GUO-induced neurite outgrowth in PC12 cells [9], did not influence the effects of GUA, in particular the NO/cGMP production. In summary, these results indicate that the PI3K-Akt downstream effectors, involved in the positive effects of GUA on learning and memory, are different from those responsible for the protective, neuritogenic and amnesic effects elicited by GUO. The possibility that the effects of GUA may also be mediated by a so far unidentified membrane receptor needs further investigation.

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The role of P2Y receptors during seizures

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Epilepsy, characterized by recurrent seizures, has an incidence of ~1-2 % and affects ~60 million people worldwide. Pharmacoresistance in epilepsy, however, remains as high as 30 %, probably in part due to the relatively narrow spectrum of mechanisms targeted by available anti-epileptic drugs (AEDs) (e.g. Na^+, Ca^{2+} or K^+ channels and GABA or glutamate receptors). An increasing recognition of the importance of neuroinflammation in epileptogenesishas spurred interest in targeting inflammatory pathways for developing AEDs with a non-classical mechanism of action. Adenosine 5'-triphosphate (ATP),released at millimolar concentrationsin the brain in response to insults such as neuronal hyperexcitation, inflammation and cell death, mediates the release of gliotransmitters, influences synaptic structure and neuroinflammatory cascades via the activation of purinergic P2 receptors. This includesthe fast acting P2X cationic channels and the slower-acting, metabotropic, G-protein-coupled P2Y receptors. While the expression and function of P2X receptors has been well-established in experimental status epilepticus and epilepsy, the P2Y receptor subfamily has received minimal prior attention. In our studies we used different mouse models of status epilepticusand brain tissue from temporal lobe epilepsy (TLE) patients to examine the expression responses and the *in vivo* effects of P2Y receptor agonists on acute seizures and hippocampal pathology. Our present data demonstrates a specific induction and expression profile of the P2Y receptor family after prolonged, damaging seizures and during epilepsy and identifies P2Y receptors as possible new targets for the treatment of status epilepticus and drug-refractory epilepsy.

Immune modulatory and cardio-stimulatory effects of the adenosine A_{2B} receptor after myocardial infarction

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Antiinflammatory adenosine originates from the hydrolysis of proinflammatory ATP by CD73 activity in the ischemic environment. After myocardial infarction (MI) the rate of extracellular ATP degradation to adenosine on immune cells is increased. The role of the adenosine A_{2B} receptor ($A_{2B}R$) among the four G-protein-coupled adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) in cardiac remodeling is a controversial issue. This study therefore aimed to investigate the role the $A_{2B}R$ *in vitro* as well as *in vivo* using A_{2B} and A_{2A} receptor knockout mice subjected to 50 min ischemia followed by reperfusion.
Analysis of all four adenosine receptors on cardiomyocytes isolated from WT mice 3 days after MI revealed that in WT mice the A1 is the most prominent receptor expressed and that MI resulted in an overall decline in adenosine receptor expression with the exception of the $A_{2B}R$ which was significantly increased. On immune cells isolated from MI hearts we observed a similar effect: T cells, granulocytes and APCs expressed the $A_{2B}R$ only after infiltrating the infarcted myocardium suggesting an important role of the $A_{2B}R$ in cardiac remodeling. In $A_{2B}R$ deficient mice, the MI-induced decrease in ejection fraction (measured by MRI at 9.4 T) was surprisingly similar to that of WT mice, but was associated with an increase in infiltrating immune cells to the infarcted myocardium including increased regulatory T cells, which might have compensated for the loss of the antiinflammatory signal delivered by the $A_{2B}R$. In line with this interpretation, specific activation of the $A_{2B}R$ *in vivo* by BAY 60-6583 in mice lacking the $A_{2A}R$ (these mice solely depend on the $A_{2B}R$) significantly increased the ejection fraction when applied 2 days after MI. Under these conditions, the application of BAY 60-6583 resulted in a decline of the serum level of several proinflammatory cytokines such as IL3, IL13 or MIP-1alpha. *In vitro* activated $CD4^+$ T cells showed an upregulation of the $A_{2B}R$ transcript while the expression of $A_{2A}R$ was reduced. When such activated T cells were cultured in the presence of the unspecific adenosine receptor agonist NECA the secretion of the proinflammatory cytokine IFNγ was significantly reduced which was partially rescued by blocking the $A_{2B}R$ with PSB-603.
In summary the observed upregulation of adenosine $A_{2B}R$ expression on cardiomyocytes and the *de novo* expression of $A_{2B}R$ on immune cells after MI, strongly suggests a functional role of the $A_{2B}R$ in cardiac remodeling. This includes a positive inotropic effect and a general antiinflammatory activity when pharmacologically activated.

HDAC inhibitor sodium butyrate attenuates ATP-induced angiogenic responses to hypoxia and enhances barrier properties of pulmonary artery (PA) vasa vasorum endothelial cells

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Vasa vasorum (VV) is a microcirculatory network in the adventitia and media of large blood vessels and is an important player to the pathogenesis of vascular diseases. Using cell and animals models of pulmonary hypertension (PH), we demonstrated that extracellular ATP plays an autocrine/paracrine role in hypoxia-induced pulmonary vascular remodeling and angiogenic expansion of the VV network [1,2]. Protein acetylation has also proved a key contributor to the pathogenesis of PH, and HDAC inhibition is beneficial, via anti-proliferative effects in vascular cells [3,4]. Butyrate is a 4-carbon fatty

acid, with HDAC activity, and a metabolic substrate for intracellular Acetyl-CoA production [5]. However, the effects of butyrate on VV angiogenesis remain unexplored. In this study, we showed that sodium butyrate (SB, 1 mM) inhibited ATP-induced DNA synthesis (Fig. 1A) and migration of VVEC isolated from chronically hypoxic calves, suggesting an anti-angiogenic effect. However, SB pretreatment (24 hr) potentiated ~~ATP- induced~~ VVEC migration and increased basal and uncoupled mitochondrial respiration rates (OCR) (Fig. 1B). In respiration assays, we found that acute SB treatment (5-500 μM) increased OCR at mitochondrial complex II, whereas prolonged in treatment (2 mM, 24 hr) stimulated elongation of mitochondrial reticulum (Fig. 1C). We also found that chronic hypoxia decreased histone H3 acetylation level in VVEC, but both butyrate and ATP (100 μM) increased it. In addition, treatment with SB (1, 2, and 5 mM) for 2 hr enhanced VVEC barrier function (measured as Trans Endothelial Resistance, TER) and potentiated a barrier-protective effect of adenosine. Using Sprague Dawley rat model of PH and lectin angiography for VV, we demonstrated that supplementation with SB (500 mg/kg) prevented right ventricular hypertrophy and improved PA VV barrier function in chronically hypoxic hypertensive rats. Thus, our data suggest that purinergic receptor- and nutrigenomic-based therapies may represent novel treatments for PH and other cardiovascular diseases.
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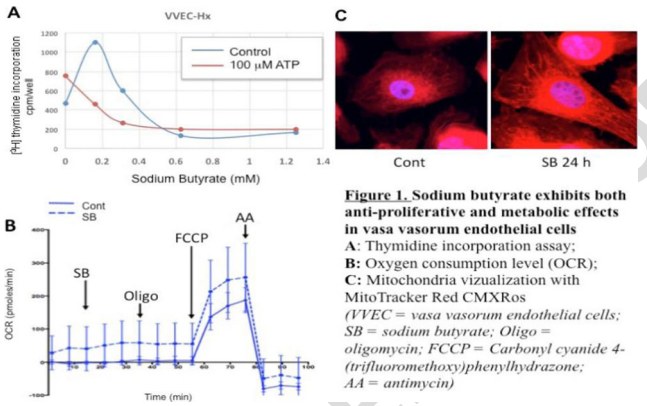

Figure 1. Sodium butyrate exhibits both anti-proliferative and metabolic effects in vasa vasorum endothelial cells
A: Thymidine incorporation assay;
B: Oxygen consumption level (OCR);
C: Mitochondria vizualization with MitoTracker Red CMXRos
(VVEC = vasa vasorum endothelial cells; SB = sodium butyrate; Oligo = oligomycin; FCCP = Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; AA = antimycin)

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Purinergic-dependent contraction of small intrapulmonary veins: role in a Pulmonary Arterial Hypertension rat model

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Objectives / background - For a long time, the vasoactivity of pulmonary veins has been debated. Increasing evidences about the role of pulmonary veins to the total pulmonary vascular resistance has been particularly well supported by studies associated to development of fetal and neonatal pathology. In contrast, the vasoactivity of pulmonary veins in adult mammalians has been more controversial and largely unexplored. Nevertheless, the alterations of the vascular tone of pulmonary veins are believed to play an important role during the development of cardiovascular diseases including Pulmonary Arterial Hypertension (PAH). In the lung, nucleotides are released from the cytoplasm of many cells including endothelial, smooth muscle and epithelial cells under physiological and pathological conditions. Particularly, release of ATP and UTP has been found elevated under certain pulmonary diseases. This extracellular ATP and UTP binds to P2Y2/4 receptors, widely expressed in blood vessels, attributing a pivotal role in the control of vascular tone. However, there are no studies on either the effects of ATP and UTP on small intrapulmonary vein (SPV) contraction or the mechanisms that couple purinergic signalling to PAH. Here we have used 'living' lung slices and phase-contrast video microscopy to investigate, for the first time, purinergic-dependent dynamic changes in SPV contraction in PAH rats.
Methods and Results - Lung slices (150 μm thick), from healthy and Monocrotaline (MCT)-induced PAH *Sprague Dawley* rats (200gr), in a vibratome were performed. ATP and UTP-induced SPV contraction was recorded using phase contrast video microscopy. Statistical differences ($p < 0.05$) were performed using non-parametric tests. After 21 days of a single subcutaneous injection of MCT, (60 mg/Kg) the rats develop PAH, including right ventricle hypertrophy. Also, in PAH rats there was an exacerbated venous constriction in response to UTP (EC_{50} = 6,9 ± 2,45 μM) versus healthy rats (EC_{50} = 19 ± 5,01 μM). Similarly, ATP-dependent vasoconstriction was strongest in PAH (EC_{50} = 14,3 ± 1.9 μM) in comparison with healthy rats (EC_{50} = 28.6,3 ± 1.1 μM). ATP and UTP-induced SPV contraction was strongly inhibited by Suramin, a non specific antagonist of purinergic receptors.

Significant $P2Y_2$ inhibition (41.5 ± 9.0 %) with ARC118925XX was predominant only in healthy rats, but not PAH rats, suggesting a main role of $P2Y_4$ in PAH. The presence of $P2Y_2$ and $P2Y_4$ receptors in co-localization with smooth muscle cells was demonstrated by indirect immunofluorescence. Conclusion - These results suggest a novel mechanism involving $P2Y_4$ receptor in exacerbated vasoconstriction observed in PAH. The study of purinergic therapies to improve survival and quality of life of PAH patients is promising.
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Epicardium-derived cells are a source of ATP, adenosine, IL-1β, and IL-6, which can modulate cardiac remodeling after infarction

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Epicardium-derived mesenchymal cells (EPDC) play a pivotal role in heart development by differentiating into coronary vascular precursors, fibroblasts and cardiomyocytes. In the adult heart, EPDC are reactivated by myocardial infarction (MI) and are considered as endogenous cell source involved in cardiac remodeling. Inflammasome activation is essential for cardiac remodeling after MI and has been reported for cardiomyocytes, fibroblasts, and smooth muscle cells. The NLRP3 inflammasome is the most clinically implicated inflammasome type. A typical activator of this inflammasome is extracellular ATP signaling via the receptor $P2X_7$. Since EPDC are a unique cell population formed after MI, we explored inflammasome activation in cultured adult EPDC, isolated from rat hearts 5 days after ischemia/reperfusion.
Quantitative RT-PCR analysis revealed that EPDC expressed $P2X_7$ and the three NLRP3 inflammasome components: the pattern recognition receptor NLRP3, the adaptor protein ASC, and the IL-1β generating effector enzyme caspase-1. Stimulation with the stable ATP analog BzATP induced the release of IL-1β and IL-6 from EPDC as measured by Bio-Plex. The $P2X_7$ inhibitor A740003 blocked the BzATP-triggered IL-1β secretion. HPLC analysis showed that EPDC rapidly metabolize extracellular ATP to adenosine via the extracellular purinergic enzyme cascade. Adenosine-A_2B signaling strongly induced IL-6 secretion, but did not activate inflammasomes like ATP. However, adenosine-A_2B signaling stimulated the release of nucleotides from a Brefeldin A-sensitive intracellular pool, providing ATP as endogenous inflammasome activator.
Our data demonstrate that the NLRP3 inflammasome is present in EPDC and can be activated by extracellular ATP via $P2X_7$. EPDC can degrade ATP to adenosine, but also release additional ATP in response to adenosine-A_2B signaling. EPDC formed in response to MI appear to be a source of ATP, adenosine, IL-1β, and IL-6, which together are likely to play a role in cardiac inflammation and remodeling.

P2X7 receptor is a key modulator of energy cell metabolism

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The P2X7 Receptor (P2X7R or P2RX7) is widely considered a cytotoxic nucleotide receptor, but is also increasingly recognized to play a central role in cell survival and proliferation. Recently, there has been an increase in efforts to understand the mechanism by which P2X7R supports cells growth or survival [1]. We previously showed that P2X7R expression increases mitochondrial Ca^{2+} concentration, mitochondrial membrane potential, density of the mitochondrial network and overall intracellular ATP content, while on the contrary pharmacological P2X7R stimulation causes mitochondrial potential collapse and fragmentation. These findings point to major role for P2X7R in the modulation of mitochondrial metabolism [2]. To clarify the molecular basis of this effect we investigated mitochondrial oxygen consumption, ATP synthesis and P2X7 mitochondrial localization in WT and P2X7-deleted cells. Our data show that lack of P2X7R decreases basal respiratory rate, ATP-coupled respiration, maximal (FCCP)-uncoupled respiration, resting mitochondrial potential and mitochondrial matrix Ca2+ level. This activity depends on P2X7R ability to localize to the mitochondrial membrane, as shown by confocal microscopy analysis and fractionation studies. Reduced respiratory rate in mitochondria from P2X7R-less cells is specifically dependent on block at Site I of the respiratory chain since supplementation of succinate restores near normal oxygen consumption.
Furthermore we investigated structure and function of heart mitochondria, a tissue strongly dependent on oxidative phosphorylation, isolated from P2X7-KO and WT mice. Mitochondria from heart of P2X7-KO mice were not apparently altered, but morphometric analysis revealed that they are smaller and show lower basal and stimulated respiratory rates than mitochondria from P2X7-WT mice. Based on this indication, we exposed P2X7R-KO and WT mice to rotarod test, and observed that P2X7R-KO mice have a statistically significant worse performance perform compared to WT. In conclusion these data suggest that the P2X7R is key determinant of cellular energy metabolism.

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Cell adhesion, migration and neuroimmunoregulatory role of astrocyte-specific ecto-5'-nucleotidase/CD73 expression and activity

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Purinergic signaling builds complex messaging network in CNS, involving not only nucleotides and nucleosides and their specific receptors, but their hydrolyzing enzymes which maintain the fine homeostasis of the system. The balance is impaired in states of

neuroinflammation, ischaemia, injuries and neurodegenerative diseases by the diverse changes in the release of nucleotides and the expression of the purinergic system components. Speaking of the balance, it is well known that the ecto-5'-nucleotidase (eN/CD73) is the main switch between extracellular ATP/ADP/AMP and adenosine, the pro- and anti-inflammatory mediators, respectively. It has been previously described in the rat model of cortical stab wound injury that CD73 has two-phase regulation firstly being downregulated on neuronal cells, followed by the upregulation on astrocytes [1,2]. The studies on the EAE model for multiple sclerosis showed similar results, the increased expression of the CD73 mainly on reactive astrocytes in the lumbar spinal cord, while the CD73 expression was fairly present on microglial cells. The significal increase in total AMP hydrolysis was measured during the peak of the disease, which could be attributed to the additional presence of the second CD73 protein variant (probably due to differences in glycosylation) [3].

Since our previous studies showed the astrocytes to be important effectors in the sense of CD73 expression and activity, and needless to mention their role in glial scar forming, the reactive response to microglia-derived arsenal of cytokines and chemokines, and overall part in purinergic signaling, we have conducted several *in vitro* studies on primary rat cortical astrocyte cultures. Our research shows the expression and activity of two CD73 protein variant being specifically regulated by single or combined effect of TNF-α, IFN-γ, ATP [4,5] and IL-1β, IL-6, with less impact of IL-10 and adenosine. The addition of ATP in culture medium reflects the *in vivo* extracellular milieu, occurring when cells are metabolically or mechanically injured. Thus, we paid special attention on treating the cultured astrocytes with different ATP concentrations, showing their both reactive and protective responses [6]. Having previously in mind, it was interesting to explore the overall impact of ATP treatment on astrocyte migration and to investigate the role of the CD73 in the cellular adhesion and migration, with particular reference to direct interactions of CD73 with ECMs, such as tenascin C, laminin or fibronectin. By applying the anti-CD73 antibodies, the specific inhibitor of CD73 activity (α,β-metADP, APCP) and CD73-siRNA, we have estimated the astrocytes migratory capacity, in parallel with its mRNA expression and enzyme activity. The approach of inhibiting the CD73 activity and/or modifying its adhesive characteristics has become important tool in cancer biology (including glioma tumors), thus certainly imposing the need to describe thoroughly both phenomenon in healthy and diseased nervous system.

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Synthesis and characterization of AR-C118925 - a useful antagonist for studying $P2Y_2$ receptors

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The G_q-coupled purinergic receptor $P2Y_2$, which is activated by ATP and UTP, is a potential therapeutic target for diseases such ascystic fibrosis, osteoporosis, inflammation, atherosclerosis and cancer [1-3]. However, discovery of drugs for this target has been mitigated by the lack of well characterized potent and selective ligands as pharmacological tools. AR-C118925, a thiouracil derivative, is one of the most potent and selective antagonist for the $P2Y_2$ receptor although it has not been thoroughly studied [4]. Also, until recently, AR-C118925 was not commercially available for pharmacological studies. We therefore synthesized and characterized AR-C118925 at $P2Y_2$ receptors expressed in 1321 N1-astrocytoma, and in CHO-K1 cell lines, respectively, by intracellular calcium mobilization and β-arrestin recruitment assays. AR-C118925 was found to be a competitive antagonist with pA_2 values for the human $P2Y_2$ ~~receptorof~~, 37.2 nM in the calcium assay and 51.3 nM in the β-arrestin assay. We show that AR-C118925 is highly selective for the $P2Y_2$ receptor against an array of P2Y and P2X receptor subtypes and ectonucleotidases. AR-C118925 was found to be slightly permeable in the Caco2 cell assay indicating limited oral bioavailability. The $P2Y_2$ antagonist

was highly stable in both human and mouse liver microsomes. In conclusion, AR-C118925 represents a useful pharmacological tool for both in vitro and in vivo studies, and is currently thegold standard for $P2Y_2$ receptor studies [5].

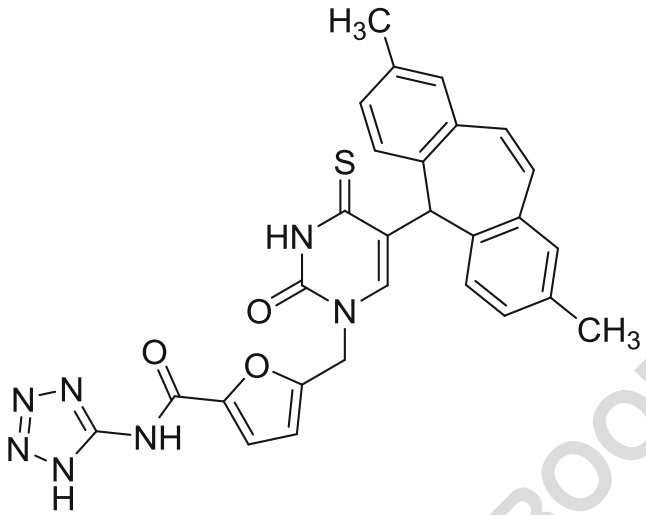

Figure 1: Structure of AR-C118925.

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Neurochemical findings in the MPTP model of Parkinson's disease

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It is known that purinergic signaling modulates both physiological and pathophysiological dopaminergic neurotransmission. In this study, we investigated the regulation of neurotransmitters and neuromodulator levels by P2X7 and $P2Y_{12}$ purinergic receptors in the dopamine depleted striatum using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-model of Parkinson's disease.
We examined the neurochemical changes of mice striatum induced by acute regimen (4x20 mg/kg with 2 h i.p. injections) of MPTP. Experiments were performed using wild-type, P2X7-and $P2Y_{12}$-receptor deficient mice coupled with on-line column-switching liquid chromatographic analysis.
MPTP treatment provoked a massive reduction (88.9 ± 1.4 %) in dopamine (DA) content of P2X7R knock-out mice striatum, when compared to wild-type mice. However, this acute treatment caused a moderate DA-decrease (66.6 ± 2.3 %) in $P2Y_{12}$R knock-out mice. Decreased level of dopamine showed significant difference ($F3,49 = 14.55$, $p < 0.000001$) by repeated measures ANOVA between the two knock-out lines. Acute regimen of MPTP caused similar reduction on ATP/ADP ratio of striatum in both P2X7- and $P2Y_{12}$-knock-out mice. After MPTP treatment the glutamate (Glu) content was lower in the striatum of both receptor knock-out lines, but the degree of changes showed significant differences ($F3,49 = 19.87$, $p < 0.000001$) by repeated measures ANOVA. Effect of MPTP showed increase of anandamide (AEA) content, but had a significant effect only on $P2Y_{12}$-receptor knockout mice striatum ($F3,49 = 13.55$, $p < 0.000001$) by repeated measures ANOVA. There was a tendency of elevation in 2-arachidonoylglycerol content (41.8 ± 5.5 vs. 23.5 ± 1.5 pmol/mg protein) after MPTP treatment in P2X7-receptor knock-out mice; however this change was not significant.
Finally, we highlight possible therapeutic strategies that might be important to slow down the progression of Parkinson's disease.

Mapping the Binding Site for the P2X7 Receptor Antagonist AZ11645373

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The P2X7 receptor (P2X7R) is a ligand gated ion channel opened by the binding of extracellular ATP. The receptors are mainly localized on immune cells and glia within the central and peripheral nervous system, where they mediate inflammatory cytokine release and apoptosis [1]. P2X7R antagonists are promising drug targets for disease states including treatment of inflammation, pain and depression. Recently crystal structures of the P2X7R with five different antagonists, A740003, A804598, AZ10606120, GW791343 and JNJ47965567 have been solved. All of these antagonists bind to an inter-subunit allosteric siteat the apex of the receptor [2]. AZ11645373 (AZ116) is a selective antagonist for the human P2X7R (hP2X7R) (IC_{50} = 90nM). However, it is > 500 fold less effective at the rat P2X7 receptor (rP2X7R) (less than 50 % inhibition at 10 μM) [3]. A previous study showed that variation at position 95, Phenylalanine (F)-aromatic amino acid to Leucine (L) non-polar amino acid in the rP2X7R, contributed to the species difference in antagonist sensitivity [4]. This residue is at the base of the allosteric pocket. In the current study a range of point mutations at this residue have been used to determine their effects to understand the importance of side chain properties at this position. To determine the contribution of other residues lining the allosteric pocket, several chimeras were made swapping regions with the hP2X1R that is 1000 fold less sensitive (≤ 10 % inhibition occurring at 10 μM of AZ116) [3]. Compared with the hP2X7R, there was no significant difference between AZ116 action at the hP2X7-105-114, hP2X7-122-128, hP2X7-164-168 and hP2X7-295-310 receptors. However, the hP2X7-81-84 chimera was approximately 18 fold more sensitive to AZ116 than the hP2X7R, while the hP2X7-85-88 chimera was approximately 8 fold less sensitive to AZ116 antagonist. At the hP2X7-89-94 chimera 1 μM AZ116 had no effect on ATP responses indicating that the receptor is insensitive to the antagonist. Point mutations within these chimeras are being tested to determine which residues contribute to high affinity antagonist binding.

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Neuroinflammation in Wild-Type and P2X7R knock out Mice in a Subchronic PCP-Induced Mouse Model of Schizophrenia

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Phencyclidine (PCP) is an NMDA receptor (NMDA-R) antagonist used to mimic schizophrenia in rodents, fitting the hypo-glutamatergic hypothesis of schizophrenia. We recently reported that the P2X7R KO mice and a specific BBB-permeable P2X7R antagonist attenuates PCP-related behavioral effects using acute treatment, and that PCP upregulates the functional responsiveness of P2X7R in the prefrontal cortex (PFC). In addition, alterations in the mRNA expression of schizophrenia related genes was also subject to regulation by P2X7R in the PFC [1]. The translational relevance of the PCP induced schizophrenia model partly relies on the assumption that PCP exerts its action between the presynaptic glutamatergic terminal of principal-cells and the spines of the GABAergic parvalbumin positive (PV+) fast-spiking somato-projecting interneurons in the prefrontal cortex. In PCP-treated animals an acute and reversible vacuolization of the principal cells is detected in the retrosplenial cortex, which undergoes rapid tolerance; PCP is less, but still neuro-toxic in other non-PFC areas, and display different brain pattern activities upon the extent and duration of the treatment. The sub-chronic PCP treatment induce a long lasting induction of the immediate early genes in the posterior cingulate and retrosplenial cortex (RSC), as well as in other hippocampal and thalamic regions. Whilst the PV+ sub-population, accounting for the synchronization of intra-cortical microcircuits, is a minority of the GABAergic neurons in the rodent's PFC, they represent a prominent sub-population in the RSC. In addition, activation of microglia in the RSC but not PFC have been reported following PCP treatment. RSC in mice is a massive cortex respect to the human's correlative, and it is implicated in several cognitive domains, strictly related and somehow overlapping those of frontal cortices. RSC in humans represent the central part of the Default Mode Network, which is reported to be altered in many psychiatric diseases, comprised schizophrenia. To gather data upon the hypothesis on the "smoldering inflammation", which is likely to be triggered upon cellular stressful conditions, we decided to study behavioral phenotype, cytokine content and microglial population both in the prefrontal and the retrosplenial cortex in the sub-chronic PCP-induced schizophrenia model in mice and we found genotype- and region-dependent alterations in the above parameters.

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Adenosine A_{2B} receptors stimulation inhibits oligodendrocyte maturation by interacting with sphingosine kinase/sphingosine 1-phosphate signalling axis in primary purified oligodendrocyte cultures

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Multiple Sclerosis (MS) is the most frequent demyelinating disease in the Central Nervous System (CNS). Remyelination does occur, but is limited especially in chronic disease stages. Despite effective immunomodulatory therapies reduce the number of relapses, the progressive disease phase cannot be prevented. Therefore, promotion of neuroprotective and repair mechanisms, such as remyelination, represents an attractive additional treatment strategy. A number of pathways have been identified that may contribute to ameliorate the impaired remyelination in MS lesions; among them adenosinergic signalling and sphingosine kinase/sphingosine 1-phosphate signalling axis (SphK/S1P). Oligodendrocyte development involves progression from oligodendrocyte progenitor cells (OPCs) to mature oligodendrocytes. Oligodendrocytes express each of the different adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) at all maturational stage. Interestingly, treatment of OPCs in culture with adenosine promotes maturation, an effect that is A_1 receptor mediated [1]. Activation of A_1 receptors also stimulated OPC migration, without adverse effects on cell viability [2]. Treatment of OPCs in culture with selective A_{2A} agonists reduces cell maturation [3,4]. A very recent study demonstrated that Fingolimod (FTY720), that has been approved as orally active drug for relapsing MS, modulates S1P receptors. A relationship between SphK1 activity and A_{2B} adenosine receptor (ADORA2B) activation has been demonstrated in mouse normal and sickle erythrocytes in vitro [5].
The interaction between ADORA2B and S1P/SphK signalling on oligodendrocyte maturation in rat cultured OPCs, at different times of maturation, was investigated performing Real-time PCR and Western Blot analysis.
In cultured OPCs, SphK1 phosphorylation, that is a hallmark of the activation state of the enzyme, was enhanced after a 10 min treatment with BAY60-6583 (10 μM), a selective ADORA2B agonist, thus demonstrating an interaction between ADORA2B activation and SphK1. Chronic application (7 days) of BAY60-6583 (1-10 μM) or of the newly synthetized ADORA2B agonist, P453 (50-100 nM) in cultured medium reduced OPC differentiation, as indicated by the decrease of the two genes target MAG (myelin-associated glycoprotein) and Mbp3 (myelin basic protein 3), typically expressed by mature oligodendrocytes. FTY720 phosphate (1 μM), the active metabolite of FTY720, mimicked the effect of 10 μM BAY60-6583 on OPC maturation. On the contrary, VPC96047 (0.5 μM), a pan-SphK inhibitor, and VPC96091 (0.5 μM) a selective SphK1 inhibitor, increased MAG and Mbp3 levels. These effects were abolished in the presence of 10 μM BAY60-6583.
Our findings reveal a novel signalling network i.e. ADORA2B and SphK1 regulating remyelination process in rat cultured OPCs.

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Adenosine A_3 receptors mediates modulation of neurotransmitter release in hippocampus

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Adenosine is an important endogenous modulator of brain function and exerts multiple effects through several adenosine receptors in physiological and pathological conditions. Adenosine receptors have been also shown to mediate the activity of chemokines, chemoattractant molecules involved in neuroinflammation, neurodevelopment and neuromodulation. In particular, modulation of synaptic transmission recently emerged as crucial activity during pathological and physiological conditions. CXCL16 is a chemokine normally expressed in the brain, where it exerts neuroprotective activity against glutamate-induced damages through cross communication with astrocytes and the involvement of the adenosine receptor type 3 and the chemokine CCL2. We demonstrated for the first time that, under physiological conditions, CXCL16 exerts a modulatory activity on excitatory and inhibitory synaptic transmission in CA1 area via presynaptic mechanisms. We found that CXCL16 increases the frequency of the mIPSCs and the PPR of eIPSCs suggesting a presynaptic modulation of the probability of GABA release. In addition, CXCL16 increases the frequency of mEPSCs and reduces the PPR of evoked excitatory transmission, indicating that the chemokine is also able to modulate and enhance the release of glutamate at presynaptic level. These effects were not present in the A_3R KO mice and in WT mice treated with the selective A_3 antagonist MRS1523, confirming the involvement of A_3 receptors as key mediators of the modulatory activity of CXCL16 on neurons.

Determining the structure and dynamics of the intracellular regions of hP2X1 using cysteine reactive crosslinking

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P2X1 receptors (P2X1R) are members of the family of ATP-gated ion channels involved in a wide array of physiological processes, such as regulating cardiovascular tone and platelet responsiveness. Advances have been made to understand the molecular structure and function of these ion channels, including the recent crystallization of human ~~P2X3with~~ and without ATP bound [1], providing structural information about the intracellular portions of the receptor for the first time. However, these structures only give snapshots of the conformations of the receptor and resulted in no intracellular structure observed when the receptor was in the closed or desensitized states.
We have used cysteine scanning mutagenesis in combination with a range of crosslinking molecules, with two cysteine specific groups and variable spacer lengths, and Western blotting to probe the intracellular portions of hP2X1Rs immediately before the first transmembrane domain (TM) and after the second TM. Single cysteine point mutations were introduced at positions R25 to G30, H355 to R360 and transiently transfected into Freestyle

HEK293 cells. Transfected cells were then incubated in cysteine reactive crosslinkers, with different spacer lengths between the thiol binding groups, and hP2X1Rs identified by Western blot. If there was any crosslinking then this would be shown as an increase in molecular weight from monomer (~55 kDa) to dimer (~110 kDa). The different spacer length of the crosslinkers allows the estimation of distance between the mutated cysteine residues, e.g. if no dimerization is seen with a given crosslinker then the residues would not come within that particular distance. The cells were tested in the presence of either apyrase or ATP to test the closed and desensitized conformations of hP2X1Rs.
In this study we determined whether three cysteine-cysteine reactive crosslinkers, 1,2-ethanediyl bismethanethiosulfonate (MTS-2-MTS), 1,4-bismaleimidobutane (BMB) and 1,8-bismaleimido-diethyleneglycol ($BM(PEG)_2$) with spacer lengths 5.2 Å, 10.9 Å and 14.7 Å respectively, could cause molecular weight shifts with the hP2X1Rs cysteine mutants detailed above. A distinct pattern of dimerization appeared on the blots, with only twoof the six mutants in the carboxyl portion and two of the six mutants in the amino portion showing a robust shift in molecular weight with the crosslinkers tested, in both the presence of apyrase and ATP. This distinct pattern suggests that the intracellular portions of hP2X1Rs do adopt specific conformational arrangements in the closed and desensitized states. These data were then incorporated into *in silico* simulations of hP2X1R to assist in the modelling of the intracellular portions of the receptor.

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The selective block of adenosine A_{2B} receptors protects synaptic transmission from damage induced by oxygen and glucose deprivation in the CA1 rat hippocampus

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During hypoxia or ischemia the extracellular concentrations of adenosine significantly increase reaching ~~micromolarconcentrations~~ that activate all adenosine receptor subtypes so far identified: A_1, A_{2A}, A_{2B}, and A_3 [1]. The A_{2B} receptor is the most enigmatic among all different adenosine receptor subtypes; no data about its involvement in cerebral ischemia are so far available. In this work we characterized ~~therole~~ of adenosine A_{2B} receptors during oxygen and glucose deprivation (OGD) in the CA1 region of rat hippocampus. To this purpose, two selective antagonists of the A_{2B}receptor subtype, MRS 1754 (N-(4-Cyanophenyl) -2-[4-(2,3,6,7-tetrahydro-2,6-dioxo- 1,3-dipropyl-1H-purin-8-yl) phenoxy] -acetamide) and PSB 603 (8-[4-[4-(4-Chlorophenzyl) piperazide-1-sulfonyl) phenyl]] -1-propylxanthine) were used. Extracellular recordings of CA1 field excitatory post-synaptic potentials (fEPSPs), obtained byelectrical stimulation of Schaffer collaterals, were performed. Application of 7 min OGD induced the appearance of a marked depolarization, known as anoxic depolarization (AD), an unambiguous sign of neuronal damage, in all hippocampal slices recorded (n = 20). Furthermore, after 7 min OGD, fEPSPs did not recover their amplitude after return to normoxic condition. The A_{2B} antagonists MRS 1754 or PSB 603 were applied 15 min before, during and 5 min after OGD. MRS 1754 (500 nM, n = 9) did not modify synaptic transmission under basal conditions, but completely prevented the appearance of AD in5 out of the 9 slices examined. In the remaining 4 slices, a significant delay in AD development was observed. Furthermore, MRS 1754 allowed a substantial recovery of fEPSPs in all the hippocampal slices underwent to severe OGD, also in the slices in which AD was recorded. Similar results were obtained in the presence of PSB 603 (50 nM, n = 8). The compounddid not affect synaptic transmission before OGD, but significantly delayed the appearance of AD induced by 7 min OGD in 4 out of the 8 slices recorded. In the remaining 4 slices no AD was detected. In any case, in all slices a significant recovery of fEPSPs amplitude was recorded.
In order to characterize the role of adenosine A_{2B} receptors on AD development, we prolonged the duration of the OGD from 7 to 30 min; this longer duration is invariably associated with tissue damage [2,3]. We compared the time of the appearance and the magnitude of depolarizing d.c. shift, in the absence or in thepresence of the two A_{2B} adenosine receptor antagonists. Thirty minutes OGD ~~elicitedthe~~ appearance of AD in all untreated OGD slices, with a ~~meanpeak~~ amplitude of -7.3 ± 0.48 mV (n = 18) and a mean latency of 6.2 ± 0.16 min (n = 18). When OGD was applied in the presenceof 500 nM MRS 1754 (n = 2) or 50 nM PSB 603 (n = 2), the d.c. shifts were ~~alwaysdelayed~~ although theAD amplitude was not ~~significantlychanged~~. Data demonstrate for the first time that the selective adenosine A_{2B} receptor antagonism delays the occurrence of AD and improves neuronal survival following severe OGD in the CA1 hippocampus, as demonstrated by the significant recovery of an otherwise disrupted neurotransmission.

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Signaling pathways converging at Sp1 transcription factor control P2X7 receptor expression in neural cells

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In the nervous system, purinergic P2X7 receptors (P2X7R) are ATP-gated cation channels involved not only in physiological functions such as cell growth, differentiation or apoptosis, but also in brain pathologies including neurodegenerative diseases and cancer [1,2]. Indeed, P2X7R are highly expressed by nearly all human cancers so far investigated, including neuroblastoma cells from both primary tumours and cell lines [3], although little is known about how *P2rx7* gene expression is controlled. Here we report the first molecular and functional evidence that specificity protein 1 (Sp1) transcription factor plays a pivotal role in the transcriptional regulation of P2X7R in neural cells [4]. We delimited a minimal region in the murine P2rx7 promoter containing four SP1 sites, two of them being highly conserved in mammals. The functionality of this SP1 sites was confirmed by site-directed mutagenesis, and Sp1

overexpression/downregulation. Using P2rx7-EGFP transgenic mice that express enhanced GFP under the control of P2rx7 promoter (including functional SP1 sites), we found a high correlation between reporter expression and Sp1 brain levels. Blockade of P2X7R resulted in increased neuritogenesis in neuroblastoma cells cultured in serum-free medium, whereas P2X7R overexpression significantly reduced the formation of neurites [5]. Moreover, serum deprivation induced a significant increase in P2X7 transcript and protein levels, circumstance that facilitates proliferation of neuroblastoma cells in the absence of trophic support [6]. The increase in P2X7R expression was dependent on PI3K/Akt signaling and required the activation of EGF receptor at the cellular membrane. Interestingly, nuclear Sp1 levels were strongly reduced by inhibition of PI3K/Akt pathway, and blockade of Sp1-dependent transcription with mithramycin A prevented upregulation of P2rx7 gene expression following serum withdrawal. Furthermore, atypical PKCζ was also implicated in the regulation of P2X7R expression by preventing Akt activation [6]. In summary, these data show that Sp1 is a key element in the transcriptional regulation of P2X7R and support the involvement of this receptor in the maintenance of neural cells in a non-differentiated state, promoting a pro-survival outcome.
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The role of P2X7 receptor in maternal Poly(I:C) Evoked Rodent Model of Autism Spectrum Disorder

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Autism Spectrum Disorder (ASD) is a neurodevelopmental condition as a result of interaction of large set of genes and environmental factors. Symptoms are variable but most frequently include individualized behaviours from deficits of social-emotional reciprocity, verbal and non-verbal communication, and deficits of development and maintenance of relationships. Typical symptoms are stereotype and repetitive motor movements, ritual behaviour patterns, fixated interests with abnormal intensity and hypo- or hyper reactivity to sensory input of the environment. Comorbidities are various from GI disturbances, increased seizure susceptibility, sleep disorders, anxiety, motor and sensory impairment, reduced nociception, altered mitochondrial dynamics, hypoimmunoglobulinaemia, neuroinflammation, Purkinje cell loss etc. [1-3].
Recent studies have revealed that Suramin, a broad spectrum P2 receptor antagonist relieves the majority of symptoms in different animal models of autism. Our aim was to measure the role of P2X7 receptor in ASD utilizing behavioural, neurochemical and anatomical parameters. We measured the effect of prenatal Poly(I:C) (PIC) treatment in 8-weeks-old C57Bl/6 mice and P2X7R KO mice respectively. As a confirmation we investigated the effect of pharmacological blockade of P2X7 receptor both as maternal pretreatment and postnatal treatment. We performed social preference test, rotarod test, self-grooming and marble-burying tests. After behavioural experiments animals were sacrificed; para-sagittal sections of the cerebellar vermis were cut and Purkinje cells were counted. Half-brain synaptosome fractions were examined by electron microscopy. PIC treated animals showed decreased sociability and impaired motor coordination. Maternal Poly(I:C) treated animals showed increased repetitive behaviour in the marble-burying and in the self-grooming tests. Quantitative Purkinje cell dropout was found in PIC treated mice and electron microscopy of half brain synaptosome fractions revealed ultrastructural abnormalities in them. Mice lacking P2X7 receptor did not show the above mentioned changes and pharmacological blockade had significant facilitating effect on PIC induced autistic behaviour and morphological changes.
Our results suggest that P2X7 receptor has a role in neuronal development and the formation of ASD. Based on our findings it seems that selective blockade of P2X7 receptor has anti-autistic action not just as preventive but as postnatal treatment too.

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NTPDase2 is downregulated by white matter fibrous astrocytes in two distinct models of demyelination

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Extracellular ATP is metabolized by coordinated action of ectonucleotidase enzymes. Specifically, ectonucleoside triphosphate diphosphohydrolase1 (NTPDase1/CD39) and ectonucleoside triphosphate diphosphohydrolase 3 (NTPDase3) hydrolyze ATP or ADP to AMP, while ecto-5'-nucleotidase (eN/CD73) catalyzesthe conversion of AMP to adenosine Yet another ectonucleotidase, with restricted expression in the brain and strict substrate affinity is NTPDase2. In rat brain, the enzyme is mainly expressed by white matter fibrous astrocytes and it strictly hydrolyzes ATP to ADP, generating the ligand for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. Thus, astrocyte-expressing NTPDase2 directly modulates ATP-receptor mediated signaling and cell communication in distinct brain regions. Our preliminary results show that NTPDase2 is strongly downregulated in spinal cord tissue during neuroinflammation. Therefore, present study was undertaken in order to compare expression profiles of NTPDase2 in two distinct models of demyelination – immune-mediate experimental autoimmune encephalomyelitis (EAE) and cuprizone neurotoxic model. We have shown that NTPDase2 is comparably downregulatedby spinal white matter in EAE and in cerebral commissure corpus callosum, in 7-day cuprizone model. Double immunofluorescence labelling demonstrate that white matter fibrous astrocyte account for the observed downregulation. Since expression analysis demonstrated similar downregulation of $P2Y_1$ and $P2Y_{12}$ receptor-mRNAs, obtained results imply that demylination may be associated with reduced activity of ADP-mediated signaling.

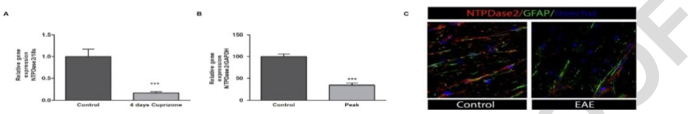

Figure 1. NTPDase2 downregulation in corpus callosum of mice treated with 0.25 % cuprizone 4 days (A) and in the lumbar spinal cord of EAE rats at the peak of the disease (B). NTPdase2 is strongly downregulatedfromspinal cord fibrous astrocytes in EAE (C).

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Pilocarpine-induced status epilepticus increases the sensitivity of P2X7 receptors to nucleotides at neural progenitor cells of the juvenile rodent hippocampus

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Adult neural progenitor cells (NPCs) have been identified in the subgranular zone (SGZ) of the hippocampal dentate gyrus [1,2]. It has been reported that pilocarpine-induced status epilepticus of rodents increases the number of NPCs, fosters their migration and ectopic settlement/maturation in the hilus hippocampi and thereby causes recurrent spontaneous seizures after a certain lag time. In brain slices prepared from the hippocampus of transgenic (Tg) nestin/enhanced green fluorescence protein (EGFP) mice, we could identify NPCs under the fluorescence microscope. Dibenzoyl-ATP (Bz-ATP), a prototypic P2X7 receptor (R) agonist initiated inward current in NPCs and the selective antagonist A-438079 reversed this effect. We induced status epilepticus (SE) by the intraperitoneal application of pilocarpine in Tg(nestin/EGFP) mice and prepared 24 h later slices from their hippocampus for electrophysiological experiments. We found that when hippocampalslices were taken from mice subjected to pilocarpine treatment, the current responses to Bz-ATP largely increased. Interestingly, 1-h incubation with pilocarpine also facilitated the Bz-ATP currents, although a 2-min incubation had no effect. Moreover, co-incubation with tetrodotoxin (TTX) for 1 h eliminated the potentiation by pilocarpine. We concluded that pilocarpine, a muscarinic stimulant known to evoke SE, increased the frequency of action potentials in hippocampal circuits and thereby fostered the sensitivity of P2X7Rs against their agonists ATP and Bz-ATP. Adult neurogenesis starts early in postnatal life and continues afterwards, although with an age-dependent decrease in efficiency. In order to clarify whether P2X7Rs are present only at the SGZ NPCs of young mice studied hitherto, we also investigated the NPCs of older animals. We found that such NPCs also possessed functional P2X7Rs which mediated Bz-ATP-induced current responses. These currents could be reversibly blocked by the selective P2X7R antagonist A-438079; 1-h incubation with pilocarpine potentiated the current amplitudes suggesting similarity of purinergic control of hippocampal NPCs in the two age groups of mice. Eventually, we excluded the possibility that hilar glutamatergic or GABAergic interneurons are involved in the spontaneous seizure activity following the SE. On the basis of these experiments we hypothesize that P2X7R activation by ATP released in the consequence of SE counteracts the development of temporal lobe epilepsy otherwise initiated by the massive proliferation of NPCs and the subsequent integration of their neuronal progeny into pathological circuits in the hippocampal hilus [3].

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A_{2A}R agonists/A_3R antagonists: design, synthesis, and biological evaluation of new ligands with dual activity

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Adenosine receptors and in particular the A_{2A} and A_3 (A_{2A}R and A_3R) receptor subtypes control a range of key physiological processes during inflammation in different conditions. Potent and selective A_3AR agonists/antagonists could provide tools for the characterization of the role of these receptors and for the development of new drugs having anti-inflammatory, anticancer, and cardioprotective effects [1,2]. The A_{2A}R crystallographic structures furnished important information about the interaction of the receptor with the co-crystallized ligands [3]. In particular, some polar interactions between the 6-amino group of adenosine and the residue Asn253 and Glu169 and hydrophobic (π-stacking bonding) interaction of the aromatic scaffold of adenosine and Phe168 are formed [4]. The A_{2A} and A_3 AR sequence comparison analysis shows that, among these residues, only Phe168 and Asn253 are conserved while Glu169 of A_{2A}AR is replaced by Val169 in the A_3AR [5]. Furthermore, it has been found that replacement of the hydoxymethyl group of the ribose moiety of 2-alkoxyadenosine with a C-tetrazolyc function led to compounds endowed with high A_{2A}R potency [6].
On these bases, known A_{2A} ligands modified through the introduction of an N^6-amino or N^6-alkylamino group were designed and synthesized. Additionally, 5'-C-tetrazolic-adenosine derivatives, bearing analogue chains in 2 position of the first series, were prepared. The new compounds were tested in binding and functional studies at human A_1, A_{2A}, A_{2B}, and A_3 receptors transfected in CHO cells. Preliminary results show that the synthesized derivatives present pronounced affinity and potency at the A_3AR subtype and reduced interaction with the A_{2A}R. In addition, the newly synthesized Ado derivatives are endowed with a dual pharmacological activity, resulting potent agonists at A_{2A}R and antagonists at A_3R.

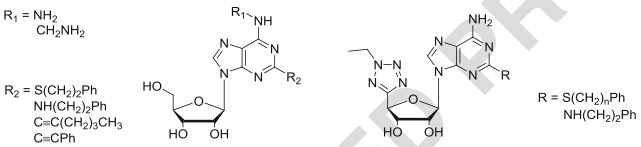

Figure 1. General structures of new synthesized AR ligands.

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Potentiation of P2X3R current responses by ASIC3 opening indicates the functional interaction of two pain relevant ion channels

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The P2X3 receptor (R) is a purinergic receptor that acts as a ligand-gated ion channel and is activated by extracellular ATP. The acid sensing ion channel 3 (ASIC3) belongs to the ENaC/degenerin family and is gated by extracellular protons. Despite their different amino acid sequences both ion channels share the same structure and pore architecture, by i.e. consisting of three identical subunits. They are located at partially overlapping subpopulations of dorsal root ganglia (DRG) neurons and are implicated in acidic pain signaling. We transfected recombinant rat (r)P2X3R and rASIC3 constructs into CHO cells. The whole cell patch clamp technique was used to analyze their current responses elicited by either α,β-methylene-ATP (α,β-meATP) or a decrease in the extracellular pH value. After co-transfection of both ion channels, already non-activated P2X3Rs markedly inhibited the ASIC3-mediated effects. However, a pH-shift from 7.4 to 6.7 or 6.5 caused a rapidly desensitizing current response and a subsequent strong potentiation of the rP2X3R specific current by rASIC3 activation. These findings were confirmed, when the same measurements were performed incultured rat DRG neurons. Interestingly, aninverse interaction did not take place, i.e. the activation of P2X3Rs failed to alter the pH-induced current amplitude. There are three possibilities for this interaction: (1) 1 subunit of ASIC3 and 2 subunits of P2X3 form a heteromeric receptor; (2) ASIC3 and P2X3Rs are tightly associated in the plasma membrane and form an interacting multiprotein complex; and (3) the two receptors communicate with each other by a Ca^{2+}-dependent mechanism. The reversal potential of the ASIC3 and P2X3R currents were around +40 mV and 0 mV, respectively. Co-activation of the receptors by protons and α,β-meATP resulted in a biphasic current, the early and late components of which had the expected reversal potentials of the two agonists. The pre-application of protons before the superfusion of α,β-meATP, however, led to a switch of the agonist reversal potentials probably indicating a corresponding switch in ionic conductance. To evaluate whether the mechanism of interaction is Ca^{2+}-dependent, we modified the free extra- or intracellular Ca^{2+} concentration. Ca^{2+} was omittedcompletely or doubled in the extracellular medium, or the intrapipette concentration of EGTA was decreased or EGTA was replaced by BAPTA. In cells individually transfected with the receptor-channels, all ionic manipulations mentioned above

decreased the effect of protons, but had no impact on the potentiation of α,β-meATP currents. In cells co-expressing rP2X3R/rASIC3, both a reduction and an increase of $[Ca^{2+}]_o$ potentiated the effect of acidification on the α,β-meATP current. By contrast, changes in $[Ca^{2+}]_i$ eliminated the normally occurring facilitation. In conclusion, the receptor subunits do not appear to form a heteromeric channel, but tightly associate with each other, mediating unidirectional potentiation and in addition cross-talk via Ca^{2+}-dependent mechanisms.

Pharmacological characterization of the GPR17 receptor dual profile

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The GPR17 receptor is a G protein-coupled receptor (GPCR) that seems to respond to two unrelated families of endogenous ligands: nucleotide sugars (UDP, UDP-galactose, and UDP-glucose) and cysteinyl leukotrienes (LTD4, LTC4, and LTE4), with significant affinity at micromolar and nanomolar concentrations, respectively [1]. In fact, it interacts with LTD4 (EC_{50} 3.15 nM) and UDP (EC_{50} 1,779 nM), a cysteinyl-leukotriene and a purinergic receptor agonist, respectively. GPR17 activation leads to both adenylyl cyclase inhibition and intracellular calcium decrease. This receptor, which is highly expressed in organs typically undergoing ischemic damage, plays a key role in the progression of brain injury and cell damage repair. GPR17 has been proposed as a potential pharmacological target for the treatment of multiple sclerosis and traumatic brain injury in humans.
In order to pharmacologically characterize the dual profile of GPR17, a new compound designed and synthesized to be able to interact with both the binding sites present in the receptor was tested in comparison with known nucleotides and cysteinyl-leukotriene agonists and antagonists. The experiments were performed using the GloSensor™ cAMP assay that allows to monitor GPCR activity through change in the intracellular cAMP concentration (Figure 1).

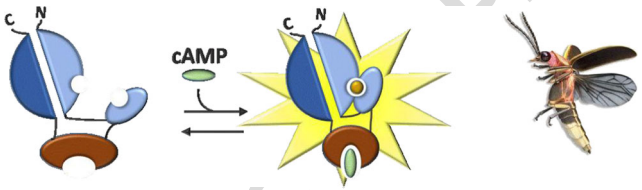

Figure 1. Overview of the GloSensorTM cAMP assay: Conformational change upon binding to cAMP that produces an increase of light output.
Human embryonic kidney (HEK293) L9-2 cells, stable transfected with the biosensor and transiently with human GPR17 receptor, were used to detect the activity of compounds under study [2]. Results obtained with different concentrations of the new ligand co-incubated with reference antagonists, montelukast and PF4, showed that the designed compound is really able to interact with both sites expressed in the receptor, so behaving as a dual ligand of GPR17.

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High-throughput screening of approved drugs and bioactive compounds identifies aurintricarboxylic acid as a nanomolar potent antagonist of the P2X3 receptor

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Introduction: P2X receptors are ATP-gated cation channels involved in fast signal transduction in many cell types. The P2X3 receptor (P2X3R) plays a role in sensory neurotransmission and nociception and is thus a promising drug target for the treatment of chronic pain. To overcome the bias that results from fast desensitization of the P2X3R, we have generated a 1321 N1 astrocytoma cell line, expressing theslowly desensitizing S^{15}V-rP2X3 mutant that was shown to be suitable for automated fluorescence-based screening of molecule libraries [1].
Objectives: The present study aimed to identify potent P2X3R ligands by academic high-throughput screening (HTS) of a collection of 2,000 approved small molecule drugs andbioactive compounds (Spectrum Collection, Discovery Systems, Inc.).
Material and Methods: We used anautomated Fluo-4-based Ca^{2+} measurement assay in 384-well plates to screen and validate the whole Spectrum Collection regarding the ability to affect ATP-induced (500 nM, ≈EC_{85}), P2X3R mediated responses of S^{15}V-rP2X3 1321 N1 cells. Additional hit validation was performed by conventional two-electrode voltage-clamp electrophysiology at the S^{15}V-rP2X3R expressed in *X. laevis* oocytes.
Results: HTS identified 27 compounds that, in a concentration of 10-20 μM, markedly blocked ATP-induced P2X3R mediated responses. Hit validation by Ca^{2+} imaging-based concentration response analysis identified six compounds that blocked P2X3R mediated responses with an estimated IC_{50} value

of < 5 μM. Aurintricarboxylic acidwas identified to inhibit ATP-induced S^{15}V-rP2X3R-mediated responses with aK_i value of 180nM. In *X. laevis* oocytes, aurintricarboxylic acid blocked the S^{15}V-rP2X3R with a K_i value of 29 nM.
Conclusion: HTS screening and hit validation identified aurintricarboxylic acid as a nanomolar potent antagonist of the rP2X3R.

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P2X7 receptors drive spine synapse plasticity in the learned helplessness model of depression

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Major depressive disorder is characterized by structural and functional abnormalities of cortical and limbic brain areas, including a decrease in spine synapse number in the dentate gyrus (DG) of the hippocampus [1]. Recent studies highlighted that both genetic and pharmacological invalidation of the purinergic P2X7 receptor (~~P2rx7~~) leads to antidepressant-like phenotype in animal experiments, however, the influence of ~~P2rx7~~ on depression-related structural changes in the hippocampus is not clarified yet [2, 3]. Our present study aims to explore how genetic deletion of ~~P2rx7~~ affects depressive–like behaviour and spine synapse density in the DG using the learned helplessness mouse model of depression. We demonstrate that in wild type animals inescapable footshocks (IES) lead to learned helplessness behaviour reflected in increased latency and number of escape failures to subsequent escapable footshocks. This behaviour is accompanied with downregulation of mRNA encoding ~~P2rx7~~ and decrease of spine synapse density in the DG as determined by electron microscopic stereology. In addition, a decrease in synaptopodin but not in PSD95 and NR2B/GluN2B protein level was also observed under these conditions. Whereas the absence of ~~P2rx7~~ was characterized by escape deficit, no learned helpless behaviour is observed in these animals. Likewise, no decrease in spine synapse number and synaptopodin protein levels are detected in response to IES in ~~P2rx7~~ deficient animals.
In conclusion, our findings suggest the endogenous activation of ~~P2rx7~~ in the learned helplessness model of depression and decreased plasticity of spine synapses in ~~P2rx7~~ deficient mice might explain the resistance of these animals to repeated stressful stimuli.

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Targeting of P2X7 on brain microglia and renal leukocytes by optimized nanobodies

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Nanobodies, a new class of therapeutic proteins, are the smallest antigen-binding domains derived from heavy chain antibodies, naturally occurring in Camelids.
The ATP-gated P2X7 ion channel is expressed by immune cells and has an important role in promoting inflammatory processes. We have generated nanobodies that effectively antagonize P2X7 ion channel and show clinical benefit in animal models of inflammation [1]. The clinical efficacy of this new class of biologic antagonists is thought to reflect their excellent in vivo tissue penetration. However, it is not known whether nanobodies can penetrate the endothelial barriers of solid tissues such as kidney or brain as well as those of lymphatic tissues such as lymph nodes and spleen. It has been reported that increasing the isoelectric point (pI) of nanobodies improves their capacity to cross the blood-brain barrier (BBB) [2].
In this study we reformatted and modified the chemical properties of nanobodies in order to improve tissue penetration. Furthermore, we developed a sensitive immunological detection system for monitoring binding of intravenously injected nanobodies to P2X7 on the cell surface of immune cells in different tissues. We raised the pI of the antagonistic-nanobody 1c81 by introducing two basic amino acid residues by site-directed mutagenesis and by C-terminal fusion to a high-pI epitope tag. Similarly, five mutations were introduced into the framework in order to increase the stability of the nanobody at high concentration. Furthermore, by genetic fusion via (Gly4Ser)n peptide linkers, we dimerized 1c81 to increase its binding affinity and added an albumin-specific nanobody to increase its half-life *in vivo*. Tissue penetration *in vivo* and nanobody binding to P2X7 expressing cells on renal leukocytes and brain microglia was monitored at various time points after intravenous administration of 1c81. The results show that within 4 h after injection, 1c81 fully occupied P2X7 on renal tissue resident memory T cells and natural killer T cells, suggesting complete translocation through the renal endothelium. In contrast, P2X7 on brain microglia was only partially occupied by 1c81 indicating only partial penetration through the highly restrictive brain endothelium.

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P2Y1 receptor-mediated modulation of neuronal activity in the mouse olfactory bulb

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Purine nucleotides such as ATP and ADP modulate the communication between cells throughout the nervous system. In the mouse olfactory bulb (OB), the first olfactory relay station, ATP is released from sensory axons together with glutamate as a neurotransmitter and stimulates calcium signaling in glia cells. It has recently been shown that ATP also evokes neuronal network activity in the OB [1], though the origin of this effect could not be identified so far. We used spatiotemporal defined photolysis of caged ATP or caged ADP in acute mouse OB slices to further dissect purinergic modulation in the olfactory bulb. Therefore, we mimicked incoming odor signals by releasing ATP or ADP locally restricted to a glomerulus, a specific processing unit in the OB and recorded the response in output neurons (mitral cells and external tufted cells) conveying to this glomerulus by whole-cell patch clamp.
The release of ATP led to a P2Y1 receptor-dependent increase in synaptic activity in mitral/tufted cells (MC/TC) and a prominent depolarization of MC/TCs independent of glutamatergic and GABAergic neurotransmission. This depolarization was strongly reduced in TTX, suggesting that the major ATP response of MC/TCs is indirect and mediated via a different population of neurons by a yet unknown mechanism. By means of confocal calcium imaging we identified a population of juxtaglomerular neurons selectively activated by ATP via P2Y1 receptors, possibly being the trigger of the ATP-evoked increase of neuronal activity in the OB.
Our results show, that purinergic signaling is present in one of the main processing units in the OB and presumably is able to modulate the processing of odor information.
Supported by the DFG (HI1288/3-1).

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Pharmacological characterization of new highly potent A_{2A} adenosine receptor inverse agonists with antinociceptive activity

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Despite opioids remain the most effective drugs for the treatment of pain, their use is often associated with numerous side effects highlighting a need for the development of new analgesics [1]. The study of A_{2A} adenosine receptor and its pharmacological modulation is a growing area of research where A_{2A} antagonists and inverse agonists open new therapeutic frontiers for the treatment of neurodegenerative diseases and pain [2,3]. In this work we describe the pharmacological characterization of novel A_{2A} adenosine receptor inverse agonists by using in vitro and in vivo assays. In competition binding experiments the new compounds emerged as outstanding ligands showing two affinity values for the A_{2A} receptor with the high affinity K_i value (KH) in the femtomolar range. The in vitro functional activity assays, performed by using cyclic AMP experiments, assessed that they behaved as potent inverse agonists at the A_{2A} receptor, but not at the other adenosine receptor subtypes. The novel compounds were evaluated for their anti-nociceptive activity in acute experimental models of pain such as writhing and hot water tail immersion tests. The acetic acid-induced writhing response was performed after intraperitoneal injection of 10 ml/kg of 0.6 % acetic acid solution where a writhe is indicated by stretching of the abdomen followed by the extension of the hind limbs [4]. The warm-water tail immersion assay was performed using a water bath with the temperature maintained at 52 °C. Interestingly, the novel A_{2A} inverse agonists showed an anti-nociceptive effect equal to or major than morphine in the writhing test as well as in the hot water tail immersion test. Moreover they revealed a more evident analgesic effect than the typical A_{2A} antagonist/inverse agonist ZM 241385. Overall, these novel inverse agonists might represent potential drug candidates for an alternative approach to the management of pain.

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Prostaglandin E_2 glyceryl ester is an endogenous agonist of the nucleotide receptor $P2Y_6$

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Cyclooxygenase-2 catalyses the rate-limiting step of prostaglandin biosynthesis from arachidonic acid but also of prostaglandin E2 glycerol ester (PGE_2-G) from 2-arachidonoylglycerol [1]. Despite its rapid degradation, PGE_2-G is detectable following activation of different macrophage cell lines [2-4] and is

present in rat paw after treatment with carrageenan [5]. This implicates PGE_2-G as potential mediators of pain, inflammation and the innate immune response but still very little is known about its biological function. Previous work suggests that PGE_2-G activates a G protein-coupled receptor (GPCR) in the murine macrophage-like cell line RAW264.7 and the human lung adenocarcinoma cell line H1819 [6,7]. The fast Ca^{2+} response observed with both cell lines indicates specific signal transduction via a G_q- and/or G_i protein-coupled receptor. Interestingly, these studies revealed an extremely low EC_{50} value in the range of 1 pM for PGE_2-G. Physiologically, this seems reasonable because PGE_2-G occurs in low amounts and is rapidly hydrolysed to PGE_2. To identify the endogenous receptor for PGE_2-G, we performed a subtractive approach where mRNA from PGE_2-G response-positive and -negative cell lines was subjected to transcriptome-wide RNA sequencing analysis. We found several GPCRs that are only expressed in the PGE_2-G responder cell lines. Using a set of functional readouts in heterologous and endogenous expression systems, we identified the UDP receptor $P2Y_6$ as the specific target of PGE_2-G. We showed that PGE_2-G and UDP are both agonists at $P2Y_6$, but they activate the receptor with extremely different EC_{50} values of ~1 pM and ~50 nM, respectively. Identification of the PGE_2-G receptor is a first step toward characterizing the physiological function of PGE_2-G and to pharmacologically manipulate this signaling system.

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Searching novel therapeutic targets for scleroderma: P2X7-receptor is up-regulated and promotes a fibrogenic phenotype in systemic sclerosis fibroblasts

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Objectives. Systemic sclerosis (SSc) is a connective tissue disease characterized by generalized fibrosis of the skin and internal organs, for which no effective treatments are currently available. Increasing evidence indicates that the P2X7 receptor (P2X7R), a nucleotide-gated ionotropic channel primarily involved in the inflammatory response, may also play a pivotal role in the development of tissue fibrosis in different body districts. Aim of this study was to investigate P2X7R expression and function in promoting a fibrogenic phenotype in dermal fibroblasts from SSc patients, also analyzing putative underlying mechanistic pathways. Methods. Fibroblasts were isolated by skin biopsy from 7 SSc patients and 6 healthy controls. P2X7R expression, and function (cytosolic free Ca^{2+} fluxes, collagen release, cell migration) were studied. Moreover, the role of cytokine production (interleukin-1β, interleukin-6) and extracellular signal-regulated kinases (ERK) activation in mediating P2X7R-dependent pro-fibrotic effects in SSc fibroblasts was evaluated. Results. P2X7R expression and Ca2+ permeability induced by the selective P2X7R agonist 2'-3'-O-(4-benzoylbenzoyl)ATP (BzATP) was markedly higher in SSc than control fibroblasts. Moreover, increased cell migration and collagen release were observed in lipopolysaccharides-primed SSc fibroblasts after BzATP stimulation. While P2X7-induced cytokine changes did not affect collagen production, it was completely abrogated by inhibition of the ERK pathway. Conclusion. In SSc fibroblasts, P2X7R is overexpressed and its stimulation induces Ca^{2+}-signaling activation and a fibrogenic phenotype characterized by increased migration and collagen production. These data point to the P2X7R as a potential, novel therapeutic target for controlling exaggerated collagen deposition and tissue fibrosis in patients with SSc.

The purinergic P2X7 receptor sustains inflammation in Multiple Sclerosis

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Multiple sclerosis (MS) is characterized by an increase of macrophages and inflammatory infiltrates into the CNS contributing to demyelination. Because purinergic P2X7 receptor (P2X7R) is known to be copiously expressed in cells of the hematopoietic lineage and nervous system, we have assessed its phenotypic expression in MS and experimental autoimmune encephalomyelitis (EAE) pathogenesis. By quantitative reverse transcription polymerase chain reaction, ~~western~~ blotting, confocal microscopy and flow cytometry analysis, we have compared P2X7R expression in cerebral cortex from secondary progressive MS cases, moreover in human peripheral blood mononuclear cells from stable and acute phases, finally in rat monocytes from EAE. Purified human and rat monocytes were also challenged *in vitro* with pro-inflammatory stimuli such as the lipopolysaccharide (LPS), or the P2X7R preferential agonist 2'3'-O-(4-benzoyl-benzoyl) adenosine5'-triphosphate (BzATP). We have demonstrated that P2X7R is differently expressed in MS cerebral cortex on monocytes and astrocytes, but totally absent from microglia/macrophages. Moreover, the receptor is found down-regulated *in vivo* in

peripheral monocytes from acute phase of MS and from rat EAE, and *in vitro* after inflammatory stimulation. By dissecting the P2X7R dynamics, we expect to gain insights into the molecular mechanisms of MS and to identifyP2X7R as promising marker for MS, and its modulators as suitable pharmacological tools against the disease.

Plasma P2X7 receptor correlation with plasma C-Reactive Protein in infectious diseases

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Adenosine triphosphate (ATP) is an extracellular signalling mediator that stimulates P2 receptors and is found to accumulate at sites of tissue injury and inflammation. P2X7 receptor (P2X7R) is expressed by virtually all cells of innate and adaptive immunity and seems to be the P2 receptor most involved in inflammation and immunity regulation [1]. P2X7R activation by ATP is one of the most potent stimuli for NLRP3 inflammasome activation thus leading to the production of interleukin-1β (IL-1β) [2] which is recognized as one of the earliest and most potent pro-inflammatory agents synthesized and released in response to infectious agents and injuries, and therefore central to both septic and sterile inflammation. P2X7R is known to be expressed on cell membrane but a soluble form has not been investigated yet. Increased P2X7R expression of peripheral blood monocytes was shown to be associated with high serum CRP, TNF-α, and IL-1β levels [3].
The present study aimed to assess the presence of P2X7R in blood, and the possible relationship between blood levels of P2X7R and CRP in healthy and unhealthy subjects. P2X7R was measured by ELISA in sera of healthy control subjects and subjects with known CRP whose samples were taken randomly from our ~~Lab~~.
Results showed significant correlation between serum levels of P2X7R and CRP. Serum P2X7R was higher in patients with infectious diseases compared to patients with other types of diseases and healthy subjects. These findings indicate that P2X7R in blood may be involved in the pathological changes of patients with inflammatory disease, and might be considered as a novel marker for inflammation.

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Nucleotide alteration in the mouse model of mammary 4T1 carcinoma under high-fat diet treatment

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There is growing evidence that obesity may affect cancer development and progression by alterations of cellular energy and signaling pathways, epitelial-mesenchymal transformation as well as inflammation [1]. High-fat diet is known to increase blood cholesterol and infiltration of LDL molecules into the vascular wall, which in turn leads to endothelial damage that may affect metastasis formation. The aim of this study was to investigate the effect of high-fat dies on nucleotide alterations in the mouse model of mammary 4T1 carcinoma.
BALB/c mice were injected with 4T1 mammary carcinoma cells with with normal diet feeding (4T1) or with high-fat diet (4T1/HFD). Diet treatment was carried out for 15 weeks and 4T1 mammary carcinoma cells were injected intravenously for the last 14 days. At the end of experiment blood, serum and aortas were collected for analysis of nucleotides and nicotinamide metabolites as well as activities of extracellular adenine nucleotide catabolism enzymes. Results are presented are mean ± SEM.
4T1/HFD group had increased leukocytes and platelets number in comparison to 4T1 while activities of extracellular ATP and AMP dephosphorylation and adenosine deamination on the aortic surface were not affected. Furthermore, 4T1/HFD mice had increased plasma nicotinamide concentration (0.75 ± 0.05 vs. 0.49 ± 0.06 μmol/l), decreased nicotinamide riboside (0.38 ± 0.09 vs. 0.56 ± 0.05 μmol/l) and 1-Methylnicotinamide plasma concentrations (0.05 ± 0.01 vs. 0.20 ± 0.03 μmol/l) as well as decreased erythrocyte ATP/ADP ratio (5.4 ± 0.42 vs. 7.3 ± 0.11) in comparison to 4T1. Our earlier studies did not show such changes in mice without injection of 4T1 cancer cells.
High-fat diet in 4T1 cancer model causes substantial changes in nicotinamide metabolism that may result from combined effect of lipid abnormalities and circulating cancer cells on endothelium, where nicotinamide metabolism is especially active.
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Inhibition of CD73 aggravates rat carrageenan pleurisy

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Adenosine is a ubiquitous nucleoside whose extracellular levels dramatically increase during inflammation. The ecto-5'nucleotidase/CD73is considered the rate-limiting enzyme in the generation of extracellular adenosine [1]. Extracellular adenosine through A_{2A} receptor plays a protective role in

controlling inflammation and immune response [2]. Much evidence has been accumulated on the modulation of the inflammatory response by CD73 generating adenosine and on the modulation of the CD73 expression and activity by the inflammatory environment [3].
The aim of the present study was to investigate the role of CD73 enzyme in carrageenan-induced pleurisy in rats.
Male Wistar rats (220–260 g) were anaesthetized with enflurane; following a small skin incision at the level of the left sixth intercostal space, pleurisy was induced by injecting 200 μL of λ-carrageenan solution 1 % w/v (dissolved in distilled water) into the pleural cavity. Groups of rats were treated locally with the CD73 inhibitor (APCP, 400 μg/site), or with the respective vehicle (distilled water) immediately before carrageenan injection.
4 and 72 h following pleurisy induction, rats were sacrificed, the chest was carefully opened, and exudate was harvested by washing each pleural cavity with 2 mL of heparinized (10 U/ml) saline. The recovered exudate was quantified and analyzed for its content in cells, cytokines, and AMPase activity. On cells collected from the inflamed site and on lung tissue, CD73 expression was evaluated by ~~western~~ blotting analysis. Chemotaxis assay was performed on cells obtained from pleural exudate collected 4 h after carrageenan injection. Morphological analysis was performed on tissue samples from APCP treated and control group of rats, and from sham rats.
We found that CD73 inhibitor, APCP, significantly increased cell accumulation ($20.55 \pm 3.85 \times 10^6$/ml vs. $0.4 \pm 0.090 \times 10^6$ / ml; n = 5 $p < 0.01$) and exudate formation into the pleural cavity 4 h following carrageenan injection. APCP also increased pro-inflammatory cytokine content into the pleural cavity in the acute phase of inflammation, while only MCP-1 levels were still significantly high 72 h following carrageenan injection (TNF-α, 103.8 ± 28.28 pg/ml vs. 15.49 ± 6.34 pg/ml; n = 3, $p < 0.05$; IL-1 β, 27.60 ± 0.58 pg/ml vs. 0.82 ± 0.82 pg/ml; n = 3, $p < 0.0001$; IL-6, 122.8 ± 24.04 pg /ml vs. 51.25 ± 8.72 pg / ml; n = 3, $p < 0.05$; MCP-1 $2554 \times 10^3 \pm 298.1$ pg/ml vs $1885 \times 10^3 \pm 90.28$ pg/ml; n = 3, $p < 0.05$).
Interestingly we found that cells collected from APCP treated rats 4 h following carrageenan-induced pleurisy showed increased ability to migrate both spontaneously and under a chemotactic stimulus. In parallel, these cells showed a reduced CD73 expression and activity compared to cells collected from control group. In contrast, cells from control groups did not migrate in the absence of a chemotactic stimulus but their ability to migrate was strongly increased following in vitro treatment with APCP. Morphological analysis of lung sections of carrageenan-treated rats showed cell infiltration of the bronchial and perivascular space as well as lung injury. No histological alterations were found in sham-rats. Local treatment with APCP increased both lung cell infiltration and lung injury 4 h following carrageenan-induced pleurisy.
Taken together, our results show that CD73 plays a major role in the early phase of acute inflammation, especially by controlling cell migration. Our ~~datastrengthenthe~~ important role of CD73/adenosine signalling as endogenous modulator of inflammation and further highlight the importance of adenosine signalling pathway as a therapeutic target that could be successfully used to treat inflammatory diseases.

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P2X7 receptor modulates extracellular ATP levels and immune system during oncogenesis

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Extracellular ATP (eATP) is a key mediator of the immune response and it is one of the components of tumor microenvironment, where it affects cancer growth, immune cell functions and tumor-host interaction [1-2]. The ATP gated ion channel P2X7 is a receptor widely expressed by cancer cells, which has a pivotal role in host-tumor crosstalk, stimulating cancer cell proliferation and immune cell activation [1]. Our group recently demonstrated that pharmacological blockade of P2X7 has a strong *in vivo* anti-tumor effect but also that lack of P2X7 in tumor host favors oncogenesis [1]. The mechanisms underlying this different behavior were not clarified, thus in an effort to understand whether the levels of eATP in tumor microenvironment could be involved in this phenomenon, we investigated the role of P2X7 on eATP and immune response modulation during oncogenesis. Thanks to a luciferase derived eATP probe (PmeLUC) we measured the levels of the nucleotide in the growing tumors obtained either in P2X7 null or in antagonist treated mice.
Our data show that tumoral levels of eATP can be reduced by the absence ~~of host~~ P2X7. Moreover, the inhibition of the receptor with A740003, while not affecting eATP, alters the quantity and nature of the immune infiltrate. Taken together our data suggest that P2X7 receptor can affect the tumor microenvironment by eATP and immune system modulation.

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Investigation of the P2X7R localization and function in colonic myenteric plexus preparations using a BAC transgenic P2X7-EGFP mouse model

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The purinergic P2X7 receptor (P2X7R) is expressed in immune cells and plays a role in cytokine release and programmed cell death. Its upregulation has been described in inflammatory bowel disease (IBD) and its blockade or deletion has been shown to ameliorate symptoms in disease models and patients [1]. Recent data suggest that P2X7Rs on enteric neurons and glia mediate neuronal death during IBD [2]. However, its precise cellular localization as well as molecular and physiological functions are still unclear which has been attributed to the complex pharmacology in native tissues and a lack of

specific antibodies. To resolve this issue, we made use of a BAC transgenic mouse model in which an EGFP-tagged P2X7R is overexpressed under the control of its endogenous promoter. This model allows the determination of P2X7R localization and possible effects of its overexpression. Immunofluorescence studies in myenteric plexus (MP) preparations from P2X7-EGFP transgenic mice demonstrate an overlap of P2X7-EGFP transgene expression with endogenous P2X7 expression and marker proteins for macrophages (Iba1) and interstitial cells of Cajal (ANO1) but not with neuronal (HuC/D) or glial (GFAP) marker proteins. P2X7-EGFP overexpressing mice appear healthy but show increased macrophage numbers in MP and increased RNA expression of interleukin-1β, a key mediator of inflammation. In addition, increased fecal water content and IgA levels were observed. ATP application on MP preparations from transgenic mice causes significantly increased neuronal cell death that is only moderate in wt mice and not seen in $P2X7^{-/-}$ mice.
In conclusion, our data support a role for P2X7R in enteric neuron death but suggest an indirect effect via activation of macrophage P2X7 receptors.

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The selective block of adenosine A_{2B} receptors prevents neuronal death in CA1 hippocampus after oxygen glucose deprivation

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Cerebral ischemia results from severe reduction in cerebral blood flow after cardiac arrest, occlusion of vessels supplying nervous tissues, or prolonged systemic hypotension. Each year about 700,000 people suffer new or recurrent stroke, a major cause of long-term disability, and the third most common cause of death in Western countries. The availability of drugs able to counteract stroke induced neurodegeneration is still an unmet need.
Extracellular concentrations of adenosine significantly increase during cerebral ischemia [1] reaching the μmolar range, thus activating all four adenosine receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3. The role of adenosine A_1, A_{2A} and A_3 receptors was already investigated in the in vitro model of "severe ischemia" induced by 7 min of oxygen and glucose deprivation (OGD) in the CA1 region of rat hippocampal slices [2]. No data on the involvement of A_{2B} receptor in this model of cerebral ischemia are so far available.
In this work we investigated the role of the adenosine A_{2B} receptor during OGD by the use of a selective antagonist ofthe A_{2B} receptor, PSB 603. The OGD-induced cell injury and PSB 603 effect on the extent of CA1 pyramidal neuron injury was assessedby immunohistochemistry for NeuN (a typical neuronal cell body marker), in hippocampal slices fixed in paraformaldehyde 1 h after the end of OGD.
The severe ischemic insult caused substantial damage to CA1 pyramidal neurons, as determined by the reduction in NeuN fluorescence that is indicative of a decrease in the number of neurons. In addition, a significant increase of pyknotic nuclei (+696 %, n = 7; $p = 0.0010$) and of degenerating, a-nucleated neurons (+1400 %, n = 7; $p = 0.0009$) were found in OGD-untreated slices in comparison to control slices. These effects were completely prevented by the antagonist PSB 603 (50 nM, n = 7). OGD did not affect astrocytes or microglia, at least at 1 h after the end of OGD.
Results show for the first time that selective antagonism of adenosine A_{2B}receptor improves neuronal survival following a severe OGD period in the CA1 hippocampus.

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P2X7 receptor involvement in the pathogenesis of Hidradenitis Suppurativa

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The P2X7 ~~receptor(P2X7R)~~ is an ATP-gated plasma membrane ion channel widely distributed in human tissues, the highest expression being in cells of the immune and inflammatory systems, especially of the myeloid lineage [1]. The P2X7R plays different functions depending on the cell type and agonist concentration. One of the most relevant P2X7R activities consists in the regulation of inflammatory responses mainly through NLRP3 inflammasome activation and IL-1β processing and release [2].
Hidradenitis suppurativa (HS) is a chronic skin disease characterized by recurrent, painful nodules and abscesses of apocrine glands. Follicular occlusion and subsequent rupture are central events in HS. HS pathogenesis is not yet understood and many researches have recently focused on possible involvement of components of the immune system, mainly cytokines. Indeed, dysregulated cytokine expression, e.g. IL-1β, TNF-α, IL-6, was found in skin, plasma and immune cells of HS patients [3-6].
The aim of this study was to investigate a possible role of P2X7R in the pathogenesis of HS, also in view of a possible use of P2X7R antagonists in the treatment of this pathology with few options for effective therapy.
For this 30 HS patients compared to 30 matched healthy control subjects, have been studied as regard skin biopsies, plasma and peripheral blood mononuclear cells (PBMC). Results show increased P2X7R expression in skin biopsies of HS patients respect to healthy controls (Fig. 1). Higher P2X7R immunostaining was shown by cheratinocytes as well as by some inflammatory cells in the derma, mainly macrophages and plasma cells. Plasma IL-1β levels were increased in HS patients respect to healthy controls suggesting a

role for this cytokine in disease development. In apparent contrast with the last finding, PBMC from HS patients appeared defective in IL-1β release upon P2X7R stimulation. This is in agreement with deregulated and compartmentalized cytokine responses found by other authors [3,6]. Further research is necessary to identify the main source of circulating IL-1β as well as the expression of inflammasome components in HS lesional and possibly perilesional skin.

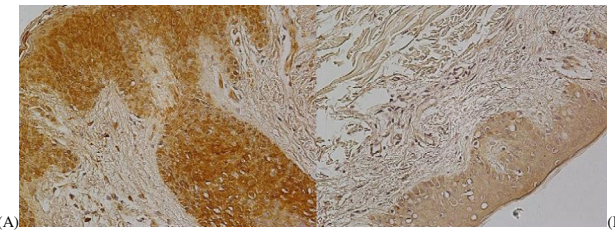
(A)  (B)

Figure 1. Increased P2X7 immunostaining of skin section from HS patient (A) compared to control subject (B).

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Role of CD73 in organ remodeling and wound healing after acute kidney injury

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Despite preventive strategies and treatment, acute kidney injury (AKI) often progresses to chronic kidney disease and continues to be associated with high morbidity and mortality [1]. Novel therapeutic strategies depend upon a critical understanding of the molecular mechanisms that occur after injury and contribute to the development of fibrosis. After ischemia-reperfusion injury (IRI) ATP is released into the kidney microenvironment by ~~apoptotic/ necrotic~~ cells and by activated immune cells and functions as a danger signal promoting pro-inflammatory responses [2]. Extracellular ATP is rapidly metabolized by CD39 to AMP and further by CD73 to adenosine, which signals via its specific adenosine receptors to regulate immune responses and healing processes [3]. Loss of CD73 increases kidney injury after IRI, but its contribution to mechanisms of kidney fibrosis has not been examined [4]. We hypothesize that CD73 mediates tissue recovery after kidney injury by blocking fibroblast-myofibroblast transformation and resolution of inflammation.

24 hrs after IRI, the initial injury was similar between ~~fibroblast/ pericyte~~ specific CD73-/- mice compared to littermate control mice as assessed by plasma creatinine, kidney tissue KIM1 and NGAL mRNA. Interestingly, plasma creatinine levels were higher in ~~fibroblast/ pericyte~~ specific CD73-/- mice compared to littermate control mice 14 days after subjecting kidneys to 20' unilateral IRI. Collagen formation and matrix deposition were increased in mice lacking CD73 on ~~fibroblasts/ pericytes~~. Moreover there was an increase in the area occupied by PDGFR-β-immunoreactive cells and in expression of markers for myofibroblasts in the injured kidney of fibroblast/ pericyte specific CD73-/- mice after IRI, suggesting a role of CD73 in regulating the phenotype switch of fibroblasts to myofibroblasts. We also found that inflammation was increased in mice lacking CD73 on ~~fibroblasts/ pericytes~~ after injury resulting in impaired resolution of immune cell infiltration. Interestingly, fibroblast/ pericyte specific CD73-/- mice could be rescued by administration of soluble 5'NT (CD73) starting on day 2 after IRI. Lastly, fibroblasts isolated from CD73-/- mouse kidneys and cultured *in vitro* displayed a hyperproliferative phenotype compared to fibroblasts from WT kidneys.

These results demonstrate the contribution of fibroblast/ pericyte CD73 on kidney fibrosis after IRI. We show that a lack of CD73 on ~~fibroblasts/ pericytes~~ resulted in an increase of fibrosis after kidney injury and displayed a hyperproliferative phenotype. An understanding of the molecular mechanisms by which ~~fibroblast/ pericyte~~ CD73 regulates fibroblast-myofibroblast transformation could provide a new therapeutic approach to treat progressive kidney fibrosis.

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Adenosine A_{2B} receptors block adenosine A_{2A} receptor signaling

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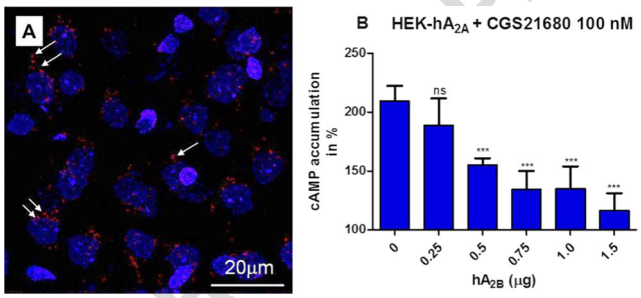
The adenosine receptor (AR) subtypes A_{2A} and A_{2B} are G protein-coupled receptors whose expression is highly regulated under pathological, e.g. hypoxic or inflammatory conditions. They are co-expressed on many different cell types and tissues e.g. on T-cells [1], in neuroendocrine tumors [2] and prostate cancer cells [3]. Thus, both receptors play important roles in inflammatory diseases and have become major drug targets in immunooncology [4]. It is well accepted that G protein-coupled receptors are able to form homomers, heteromers or oligomers with unique pharmacological properties and therefore they represent interesting novel targets for drug development [5]. In the present study we examined possible heteromeric interactions between the adenosine A_{2A} and A_{2B} receptor subtypes. We used bioluminescence resonance energy transfer (BRET), bimolecular fluorescence complementation (BiFC) and proximity ligation assays (PLA) to identify these complexes in co-transfected CHO cells and in the *dorsal hippocampus* of the rat. The functional impact was examined by measuring A_{2A}- and A_{2B}-specific agonist-induced activation of cAMP accumulation in co-transfected HEK293T cells. The results suggest formation of A_{2A}-A_{2B}AR heteromeric complexes in co-transfected CHO cells and in native tissues. Moreover we observed an altered pharmacology of the A_{2A}AR when co-expressed with the A_{2B}AR: the A_{2A}-selective agonist CGS21680 lost its affinity for the A_{2A}/A_{2B} heteromer and receptor activation was abolished as shown in cAMP accumulation assays suggesting an inhibition of A_{2A}ARs through heteromerization with A_{2B}ARs.



A. A_{2A}-A_{2B} specific proximity ligation assay (PLA) clusters in the *CA3* region of the rat *dorsal hippocampus.*
B. Stimulation of cAMP accumulation in human A_{2A}AR-expressing HEK cells transiently transfected with increasing DNA amounts of A_{2B}AR.

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The molecular basis of PPADS antagonism at hP2X1 receptors

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P2X receptors (P2X1-P2X7) activated by ATP are widely expressed throughout the human body and mediate various physiological and pathological processes. For example, P2X1 receptors underlie a component of arterial contraction and contribute to thromboembolism. Antagonists of P2X1 receptors are targets for novel treatment of cardiovascular diseases. Structural information on zebrafish P2X4 and human P2X3 and P2X7 receptors provided a major advance in understanding agonist and subtype selective antagonist actions. But we know little about the molecular basis of antagonism of general antagonists. PPADS is a nonselective antagonist but less effective at P2X4 receptors. Previous studies showed mutation of glutamate (E) to lysine (K) at position 249 in P2X4 receptors recovered their sensitivity to PPADS [1]. However, the contribution of residue 249 and other residues involved in PPADS binding at P2X1 receptors are unclear.

In this study, human P2X1 receptors have been used as a model to map the binding site of the antagonist PPADS. We hypothesized residues involved in PPADS binding would be in a ring centred on 249 with a radius of the length of PPADS. Individual residues in the ring were investigated by cysteine mutagenesis, accessibility and sensitivity tests. The cysteine accessibility method has been successfully used to map ATP induced changes at human P2X1 receptors [2-4]. Individual residues that showed decrease in accessibility were subsequently tested to see if the mutation had an effect on PPADS sensitivity. PPADS sensitivity decreased for several mutants (for example K249C), indicating they are directly involved in PPADS binding. However there was no change in the antagonist action for some mutants (for example T75C), suggesting that the reduction in accessibility at these sites results from a PPADS induced conformational change. Mapping these data allows us to provide a model of the antagonist PPADS binding sites at P2X receptors.

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Vascular adenosine deaminase as an early marker of endothelial activation and vascular inflammation

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Extracellular nucleotides and adenosine may affect vascular pathologies by controlling inflammation, lipid accumulation and thrombosis. Membrane-bound ecto-enzymes effectively control local concentrations of these metabolites. Our recent studies highlighted that changes in activities of these ecto-enzymes may be ~~relate~~ with valve dysfunction [1] and atherosclerosis [2]. Vascular activity of ecto adenosine deaminase (eADA) consistently increased in all these pathologies. This work aimed to investigate cellular sources of eADA in pathological human vessels and to correlate this activity with the markers of endothelial activation and inflammation. Furthermore, the effects of inflammatory factors on eADA activity were tested.
In human fragment of atherosclerotic aorta immunofluorescence showed that at early stage of pathological process eADA originated from endothelial cells, not from migrating vascular smooth muscle cells (Figure 1a-b). Moreover, co-localization between ADA and CD26 protein was observed, which proves that CD26 could bind eADA on the cell surface. Further studies exhibited that activity of eADA positively correlated with vascular expression of adhesion molecules (ICAM-1, VCAM-1) (Figure 2a-b). In later stages of pathological process, eADA also originated from immune infiltrate and correlated with plasma concentration of IL-6 (Figure 2c). In LPS-treated rats, activity of vascular eADA was increased (control rats: 0.98 ± 0.09; LPS-treated rats: 2.00 ± 0.52 nmol/min/cm^2) and positively correlated with plasma IL-6 concentration ($r = 0.79$, $p < 0.01$), while activity of e5'NT correlated negatively with this parameter ($r = -0.76$, $p < 0.05$). In IL-6-/- mice, we observed a decreased activity of vascular eADA (WT: 1.75 ± 0.45; IL6-/-: 0.99 ± 0.23 nmol/min/cm^2;) that was reflected by higher concentration of peripheral blood adenosine (WT: 75 ± 18.5 nM; IL6-/-: 92 ± 21 nM).
This study highlights the role of extracellular adenosine catabolism in endothelial activation and vascular inflammation. Activity of vascular eADA could be considered as a potential marker of early stages vascular pathologies.

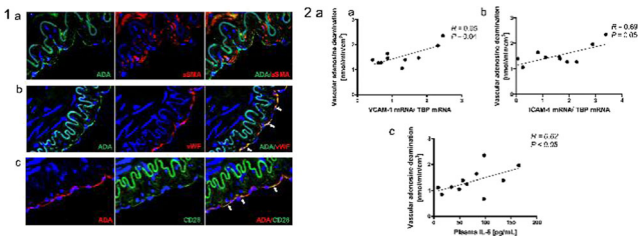

Figure 1. Immunofluorescence co-localization of adenosine deaminase (ADA) with alpha smooth muscle actin (aSMA) (a), von Willebrand factor (vWF) (b) and CD26 protein (c) in human IVA artery during neointima formation.
Figure 2. Correlation of eADA on the surface of human ascending aorta with the vascular expression of VCAM-1 (a), ICAM-1 (b) and plasma IL-6 concentration (c). Results are shown as plots of Spearman correlation.
This study was supported by National Science Centre of Poland (2014/13/N/NZ4/04471).

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Purinergic signaling in calcific aortic valve disease

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Calcific aortic valve disease is characterized by the deformation of valve leaflets through the modification of extracellular structures, modification of cell pattern and finally formation of calcium deposits. Extracellular nucleotides in blood and their catabolites may modify valve pathology by the activation of purinergic receptors distributed on the aortic valve cells. The balance between extracellular nucleotides and nucleosides in the aortic valve microenvironment is controlled by ecto-enzymes anchored to the surface of valve cells or inflammatory infiltrate. This study aimed to investigate disturbances of processes related to purinergic signaling during calcific aortic valve disease including enzyme activities, regulatory metabolite concentrations and receptor expression.

After incubation of the non-stenotic aortic valves in Hanks solution (30 min), concentration of purines (adenosine, inosine and hypoxanthine) in medium increased to 2.17 ± 0.31 μM, while uridine increased to 0.58 ± 0.06 μM. In pathologically changed aortic valves purines increased to 1.69 ± 0.24 μM and uridine increased to 1.34 ± 0.25 μM. Both adenine and uracil nucleotides were hydrolyzed on the stenotic and non-stenotic valve surfaces. The rates of adenine nucleotide and adenosine hydrolysis on the fibrosa surface of non-stenotic valve were as followed: 2.52 ± 0.29 nmol/min/cm^2 (ATP hydrolysis), 1.95 ± 0.21 nmol/min/cm^2 (AMP hydrolysis) and 0.54 ± 0.08 nmol/min/cm^2 (adenosine deamination). On the stenotic valve, the hydrolysis of ATP (1.80 ± 0.09) and AMP (1.23 ± 0.07) were lower, while adenosine deamination was higher (0.95 ± 0.08) than in non-stenotic valve. No differences between stenotic and non-stenotic valves were observed on the ventricular side. The rates of UTP hydrolysis did not differ significantly in comparison to ATP hydrolysis. Similarly, degradation rates of extracellular UMP to uridine and AMP to adenosine were at comparable levels. Using specific inhibitors for particular ecto-enzymes, we demonstrated that ecto-nucleoside triphosphate diphosphohydrolase 1 (eNTPD1, CD39) is responsible for ATP and UTP hydrolysis, ecto-5'nucleotidase (e5NT, CD73) for AMP and UMP hydrolysis and ecto-adenosine deaminase (eADA, ADA1) for adenosine deamination. Molecular studies showed reduced expression of mRNA levels for genes encoded CD39 and CD73 in stenotic valves, while immunofluorescence and flow cytometry pointed cellular sources of these proteins. Valvular endothelial and interstitial cells expressed predominantly CD39 and CD73, while immune infiltrate in stenotic valves ~~mostly expressed~~ ADA1 and ADA1-anchoring protein - CD26. In both non-stenotic and stenotic aortic valves, all genes encoding adenosine receptors were expressed (ADORA1, ADORA2a, ADORA2b and ADORA3), while only the expression of ADORA2a and ADORA2b were reduced in stenotic valves as compared to non-stenotic.

Our results indicate that many aspects of purinergic signalling are adversely modified in calcific aortic valve disease (Fig. 1), including the activities of extracellular nucleotide metabolism and expression of adenosine receptors. Reduction in CD39 and CD73 activity with the increase of eADA activity on the fibrosa surface of stenotic valve could affect extracellular nucleotide and adenosine concentrations in the way that favors valve inflammation and calcification. Moreover, the presence of all types of adenosine receptors in aortic valves and the reduction of ADORA2a and ADORA2b expression in stenotic valves underlines the role of adenosine signaling in aortic valve pathology.

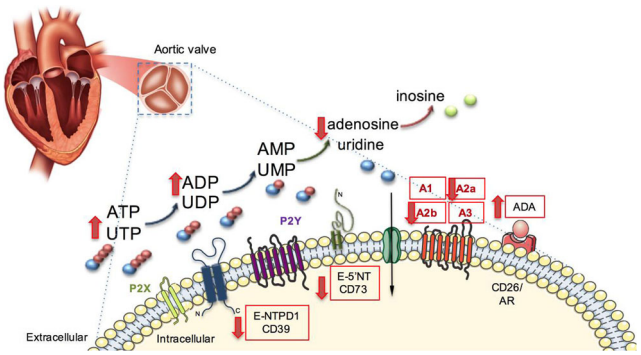

Figure 1. Purinergic signaling in calcific aortic valve disease. Red arrows point increase/decrease in the activities of ecto-nucleoside triphosphate diphospho-hydrolase 1 (eNTPD1), ecto5'nucleotidase(e5NT) and adenosine deaminase (ADA) on the stenotic valve surface and predicted changes in the concentration of nucleotides and their catabolites in aortic valve microenvironment as well as changes in the expression of adenosine receptors.

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Adenosine A_{2A} receptor as a new therapeutic target in Fragile X Syndrome

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Fragile X Syndrome (FXS) is an autism spectrum disorder in which the lack of the protein FMRP leads to an exacerbated signaling at metabotropic glutamate receptors 5 (mGlu5Rs). Converging literature and our own data indicate that adenosine A2A receptors (A2ARs), some effective modulators of neuronal damage [1], may represent a suitable therapeutic target for FXS. Indeed, a synaptic co-localization and a strong permissive interaction between A2A and mGlu5 receptors in the hippocampus have been reported by our group [2], suggesting that blocking A2ARs might normalize the mGlu5R-mediated effects of FXS. Furthermore, the reduction of the phosphatase STEP is beneficial in FXS (Fmr1-KO) mice, and the pharmacological or genetic blockade of A2ARs inhibit the activity of STEP [3]. Very importantly, as a proof of the involvement of A2ARs in FXS, we found that immunoprecipitated FMRP binds A2ARs' RNA in mouse total cortex extracts. To verify if A2AR blockade could be beneficial in FXS, we performed extracellular electrophysiology experiments in hippocampal slices. In Fmr1-KO mice, the selective mGlu5R agonist CHPG (300 μM over 10 min) induced a depression of fEPSPs' slope (74,39 ± 5,26 % of basal, N = 7; $*p < 0.05$ vs wild-type, Mann-Whitney U-test) that was completely blocked by the selective A2AR antagonist ZM241385 (100nM; 109,4 ± 10,13 % of basal, N = 4; $°p < 0.05$ vs CHPG).
Moreover, in Fmr1-KO mice treated for 4 weeks with the A2AR antagonist KW6002 (istradefylline, 3 mg kg–1 per day, PO), we found that DHPG-induced LTD (100 μM over 5 min) was fully abolished, since the fEPSPs' slope completely recovered (110,1 ± 10,99 % of basal, N = 4; $°p < 0.05$ vs vehicle). Finally, the application of ZM241385 (500nM over 3 h) to FXS patients' fibroblasts fully prevented the hyperphosphorylation of Erk1/2 signaling. According to all these preclinical evidence, antagonists of A2ARs are strongly supported as a new therapeutic strategy for FXS.

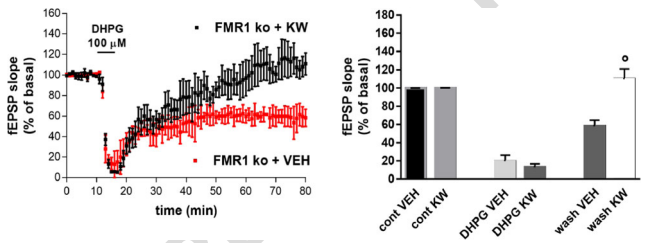

Figure. In vivo oral administration of the selective A2AR antagonist KW6002 abolished the exacerbated mGlu5Rdependent LTD in Fmr1 KO mice.

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PKC-regulated membrane resealing underlies a major mechanically-evoked ATP release pathway in murine osteoblasts

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ATP release is one of the first events to occur in response to mechanical stimulation of mammalian cells. However, there is ongoing debate about the dominant pathways involved in the release of this ubiquitous signalling molecule. We mechanically-stimulated compact bone-derived osteoblasts through direct membrane deformation or turbulent fluid shear. Using an ATP bioluminescence assay, we demonstrated that ATP was released in response to mechanical stimuli. We next examined the contribution of vesicular exocytosis to mechanically-induced ATP release [1]. Using confocal microscopy, we confirmed that acidophilic dye, quinacrine, and fluorescent ATP analog, MANT-ATP, co-localize within the same vesicles (Fig. 1A). Mechanical stimulation of single osteoblast evoked rapid exocytosis of quinacrine-positive vesicles. Activation of protein kinase C (PKC) with phorbol-12-myristate 13-acetate (PMA) significantly potentiated vesicular exocytosis, while inhibition of PKC with a broad-spectrum PKC inhibitor, bisindolylmaleimide II (BIS), significantly reduced exocytosis. Unexpectedly, increase in vesicular release coincided with a significant decrease in ATP released, and vice versa. Since PKC-mediated vesicular release has been implicated in membrane resealing [2], we examined membrane integrity in mechanically stimulated cells. By applying a membrane impermeable dye, Trypan blue (TB), before and 5 min after the mechanical stimulation, we have found that the membrane integrity was compromised in 26.6 ± 7.0 % of cells immediately upon mechanical stimulation, however this effect was reversible and within 5 min only 1.5 ± 1.2 % of cells remained TB-permeable, indicating active membrane resealing. PMA pre-treatment significantly reduced immediate TB uptake,

while in cells treated with BIS, 30.4 ± 5.4 % of cells remained TB-permeable 5 min after mechanical stimulation. Next, we treated samples with vehicle, PMA, BIS as well as other modulators of PKC and vesicular homeostasis including incubation in calcium free media, PLC inhibitor (U73122), conventional PKC inhibitor (Go6976), N-ethylmaleimide (NEM) sensitive factor inhibitor (NEM), and hemi-channel blockers (carbenoxolone, flufenamic acid and octanol), and examined single cell vesicular release, bulk ATP release and degree of membrane integrity. We have found that ATP release strongly and negatively correlates with vesicular release (Fig. 1B), and positively correlates with membrane disruption (Fig. 1C). We conclude that (i) mechanical stimuli compromise the integrity of the cellular membrane, allowing ATP release into the extracellular space, (ii) PKC-dependent vesicular exocytosis is critical for membrane resealing and (iii) cytosolic ATP, rather then vesicular ATP, is the primary source of ATP released in response to mechanical stimulation.

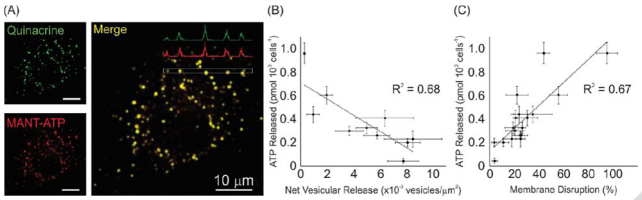

Figure 1. Vesicular ATP is not primary source of mechanically evoked ATP release. (A) Quinacrine, and MANT-ATP co-localize in granular compartments in primary murine osteoblasts. (B) Mechanically evoked ATP and vesicular exocytosis are negatively correlated. (C) Mechanically evoked ATP and membrane disruption are positively correlated.

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Protective effect of A_{2A}-receptor overexpression in mouse atria

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In the mammalian heart the actions of adenosine can be mediated by A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Previously, we studied mice with overexpression of A_1 or A_3 receptors. Both of which have been claimed to be involved in protecting the heart against ischemia and reperfusion injury. In the present work, we tested the hypothesis that cardiac protection might also be mediated via A_{2A} receptors. In order to amplify putative beneficial effects of A_{2A} receptor activation, we generated transgenic mice which overexpress the A_{2A} receptor in a cardiac specific way by using the alpha myosin promoter: TG). Left atria from these mice were mounted in the organ bath, electrically stimulated at 1 Hz and isometrically measured force was recorded. After stabilization (normoxic conditions), perfusion buffer was switched from 95 % oxygen and 5 % carbon dioxide to 95 % nitrogen and 5 % carbon dioxide, to simulate hypoxia in vivo. After 30 min, re-oxygenation was achieved by switching back the gas supply and 30 min thereafter a second round of ischemia and re-oxygenation was performed. In atria of WT (=littermate wild type mice), force of contraction was 56 ± 10 % of the normoxic value, in TG this value amounted to 98 ± 13 (N = 8-11, $p < 0.05$: TG vs WT). After the second ischemia these values amounted to 107 ± 13 % and 104 ± 20 % respectively (n = 8-11, $p > 0.05$ TG vs. WT). The time to 50 % reduction in force after hypoxia was not different between TG and WT, neither during the first nor the second hypoxia. Furthermore, in hypoxia cessation of contraction in isolated spontaneously beating right atria occurred later in TG vs WT.
The present results might be interpreted that A_{2A}-receptors protect against the initial (first) hypoxia by being constitutively active. The second hypoxia might be sustained better in WT because the first hypoxia led to preconditioning of the WT atria, therefore no difference between WT and TG is measurable any more. It is tempting to speculate that agonists at A_{2A} receptors might exert protective function against hypoxia in patient with ischemic heart disease.

Role of P2X7 polymorphisms in idiopathic infertility and HHV-6A infection

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P2X7 receptor is an ATP gated ion channel well known for its pro-inflammatory, anti-infectious activity. The human gene for P2X7 receptor is highly polymorphic accounting for more than 1500 SNPs, among which ten loss and three gain of function variants. P2X7 SNPs have been the focus of numerous studies trying to exploit them as biomarkers in pathologies as different as infectious diseases, oncological conditions, mental illnesses and many others [1]. Nevertheless, the relation between P2X7 ~~gene variants~~ and fertility was never investigated. Here we analysed the expression of gain (489C > T) and loss (1513A > C) of function P2X7 SNP in 103 patients subdivided into three cohorts: 50 fertile women, 30 idiopathic primary (unsuccessful pregnancies) and 23 secondary (previous pregnancy) infertile women. P2X7 genotyping was also correlated with cytokine levels in uterine flushing and positivity for HHV-6A infection, a condition previously associated with primary infertility [2].

We observed no differences in 1513 AC genotype, while we found a decrease in 489 TT genotype in primary infertile women (3 %) in comparison with secondary infertile (25 %) and fertile (24 %) women ($p < 0.0001$; Fisher exact test). Interestingly, VEGF levels were lower in secondary infertile (15.7 ± 5.5 pg/ml) and fertile (14.8 ± 2.3 pg/ml) women, in comparison with primary infertile women (19.5 ± 5.8 pg/ml) ($p = 0.02$; Mann Whitney U test), independently from P2X7 489CT genotype. When we looked at HHV-6A infection, we found positive samples only in primary infertile women (10/30; 33 %)[2]. When we subdivided the subjects according with P2X7 489 CT genotype and HHV-6A infection, we found HHV-6A positivity only in 489CC subjects. IL1α and IL1β levels were higher in HHV-6A infected primary infertile women (54.2 ± 20.1; 49.2 ± 17.3 pg/ml, respectively) in comparison with uninfected primary infertile women (29.3 ± 12.3; 3.4 ± 4.5 pg/ml, respectively) ($p < 0.001$, Mann Whitney U test). Similarly, VEGF levels were higher in HHV-6A infected primary infertile women (19.7 ± 6.8 pg/ml) in comparison with uninfected primary infertile women (8.9 ± 2.6 pg/ml) ($p < 0.001$; Mann Whitney U test). These data suggest that the 489 TT genotype, responsible for P2X7 gain of function, might have a protective role against HHV-6A infection onset. On the contrary women with P2X7 489 CC genotype present altered cytokines/growth factors profile in the uterine environment and show an increased susceptibility to HHV-6A infection and infertile condition. In conclusion, our preliminary data support the hypothesis that P2X7 could be exploited as predictive biomarker and potential therapeutic target in primary idiopathic infertility associated with HHV-6A infection.

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Metabolism of 4-pyridone-3-carboxamide ribonucleoside (4PYR) in endothelial and cancer cells and its effect on cellular energy metabolism

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Endothelium plays a vital role in the regulation of physiological processes including immune response, inflammation and is also critical for cancer metastasis. Healthy endothelial cells facilitates vascular repair and attenuate metastasis. Dysfunction in endothelial cells caused by toxins could impair these functions. Accumulation of unusual nucleosides in blood is a common scenario in different types of cancer. One such nucleoside: 4-pirydone-3-carboxamide-1-b-D-ribonucleoside (4PYR) could be metabolised in the cells giving rise to mono-, di- and triphosphates (4PYMP, 4PYDP and 4PYTP) and analogue of ~~NAD- 4PYRAD~~. Previous studies demonstrated that 4PYR disrupts cellular energy balance. The aim of this study was to investigate the metabolism of the 4PYR in cancer cells and to analyse effect of this nucleoside on cellular energetics.
Human neuroblastoma cells (SH-SY5Y) and human malignant melanoma cells (A375) were plated at density of 4x10^4/well in 24-well plates. Next day, medium was changed and cells were incubated for 0, 24, 48 and 72 h with addition of 100 μM 4PYR. After incubation cell extracts were analyzed on cellular ATP, NAD, 4PYMP and 4PYRAD concentration. Glycolysis was analysed in human dermal microvascular endothelial cells (HMEC-1) using a Seahorse Agilent XFp metabolic flux analyzer. Cells were plated in XFp and the next day culture medium was changed and cells were incubated for 0, 24, 48 and 72 h with addition of 100 μM 4PYR. Analysis has been performed according to manufacture protocol. Both SH-SY5Y and A375 cells were capable to metabolize 4PYR to 4PYMP and 4PYRAD. 4PYR administration caused significant reduction of ATP and NAD concentration in A375 cells. 4PYMP concentration in A375 cells reached about 30 nmol/mg protein which was similar to accumulation in HMEC-1 cells. In SH-SY5Y cells 4PYR administration did not affect ATP and NAD concentration. Glycolytic function measured as extracellular acidification rate (ECAR) decreased in HMEC-1 cells after 72 h of treatment with 100 μM 4PYR. Significant differences were observed in parameters such as Glycolytic Capacity, Glycolytic Reserve and Non-glycolytic acidification that were deteriorated in 4PYR treated cells. Both types of cancer cells were capable to form nucleotides (4PYMP, 4PYTP, 4PYRAD) from 4PYR but the effect on ATP and NAD concentration was diverse. 4PYR by formation of its intracellular derivatives was found to interfere with cellular energetics by disruption of glycolysis.
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Neuroprotective effect of nucleotide and growth factor receptors by regulating dual phosphatases activity

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Extracellular nucleotides behave as important trophic signals in neural models. They can regulate multiple functions, as proliferation, differentiation and survival, through the activation of both P2Y metabotropic and P2X ionotropic receptors. In cerebellar cultures of neurons and astrocytes, nucleotide receptors work together with growth factor receptors and share the same intracellular signalling cascades. For $P2Y_{13}$ receptors, PI3K/Akt/GSK3 signalling allowed them to couple to survival pathways, such as the antioxidant axis Nrf2/heme oxigenase-1. As well, trophic functions of P2X7 receptors were dependent on the activation of PKD and GSK3 signalling proteins, in the same way than neurotrophin BDNF [1-4].
One of the signalling mechanisms most recently investigated cover the regulation of MAPK activation and deactivation. In both neurons and astrocytes, EGF receptors elicited robust and transient ERK1,2 phosphorylation, followed by signalling termination through the activation of protein phosphatases. Indeed, EGF was able to regulate expression of dual specificity phosphatase DUSP6, which selectively dephosphorylated cytosolic ERK1,2 proteins. P2X7 nucleotide receptors behaved similarly to EGF receptors and regulated DUSP6 activity, through a common biphasic mechanism. In the first phase, both receptors triggered proteasome-dependent degradation of DUSP6 protein, to prolong ERK signalling. This was followed by a second delay phase of DUSP6 protein recovery that is due to *Dusp6* gene induced expression. Both DUSP6 degradation and expression were dependent on ERK1,2-mediated events, indicating that EGF and P2X7 receptors converge on negative feedback regulation of ERKs signalling. Additionally, $P2Y_{13}$ receptors regulated the activity of another member of DUSP family, the nuclear inducible phosphatase DUSP2. $P2Y_{13}$ receptor stimulation induced DUSP2 expression in an ERK-dependent way, and prevented the accumulation of nuclear phosphorylated form of p38-MAPK occurring after cytotoxic stimuli. Overall, nucleotide receptors contribute to cell survival in both neurons and astrocytes through the regulation of MAPK signalling by targeting DUSP phosphatases.

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Visualising allosteric modulation of adenosine A_1 receptors in real-time

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Allosteric binding sites are less conserved across receptor subtypes or species, therefore allosteric modulation is a promising tool to increase target specificity. In the case of Adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR, A_3AR) highly distributed throughout the human body, this principle will reduce off-target side effects in a treatment with subtype-unspecific agonists. In this study we investigated the adenosine receptor subtype A1AR with a subtype-specific positive allosteric modulator (PAM) PD 81,723. Standard methods like radio-ligand binding approaches fail to investigate any effects of endogenous adenosine. Our approach allows us to follow conformational changes caused by allosteric modulators in living cells. We used our recently developed fluorescence resonance energy transfer (FRET) approach and designed human and rat A1 receptor based FRET-sensors. These sensors are modified with a cyan fluorescent protein at the C-terminus and a six amino acid FlAsH-binding motif within the third intracellular loop as previously described. These sensors were well expressed at the cell surface and retained their functional properties- [1] With both sensors we determined the affinity of PD 81,723 in combination with adenosine as well as the influence of PD 81,723 on the concentration response curve of adenosine for the human A1 receptor. Furthermore, we evaluated the probe dependency of PD 81,723 using the non-selective agonist NECA. Using a recently developed Gi-protein based FRET sensor [2] we were able to visualise the allosteric modulation even at the G-protein level of the human A1 receptor. Taken together, this study shows that our designed human and rat A1 FRET-sensors are highly suitable to monitor real-time allosteric modulation in living cells and provide unique insights into allosteric modulation in combination with the endogenous ligand adenosine at receptor and G-protein level.

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The role of the extracellular loop 2 in A_{2A} and A_{2B} adenosine receptors

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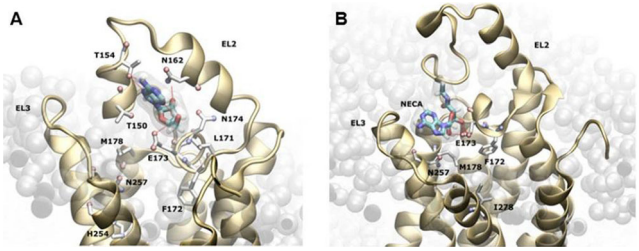
It is well established that the extracellular loop 2 (ECL2) of G protein-coupled receptors (GPCRs) participates in ligand recognition and may contribute to receptor selectivity [1-3]. While human adenosine A_{2A} and A_{2B} receptor (AR) subtypes show a high overall identity, the ECL2 is much less conserved. The A_{2B}AR possesses the longest ECL2 of all four adenosine receptor subtypes and contains four cysteine residues of which three are homologous to the ones found in the A_{2A}AR. Despite the high overall sequence conservation, the endogenous ligand adenosine and its derivatives show considerably higher affinity for the A_{2A} than for the A_{2B}AR subtype. Using mutagenesis studies and homology modeling, we previously showed that the ECL2 contributes to subtype-selectivity of A_{2A} and A_{2B} ARs [3].
We have now generated and characterized a chimera of the human A_{2A}AR, in which its ECL2 was exchanged for that of the A_{2B}AR (A_{2A}(ECL2-A_{2B})). The A_{2A}(ECL2-A_{2B}) chimera showed slightly reduced affinities and similar efficacies for NECA and the A_{2A}-selective agonist CGS21680 as compared to the wt A_{2A}AR, but a decrease in affinity and efficacy for a larger 2-substituted adenosine derivative (PSB-826). The most dramatic effect was seen with the endogenous ligand adenosine which displayed a 60-fold reduced potency at theA_{2A}(ECL2-A_{2B}) mutant receptor in comparison with the wt hA_{2A}AR; its potency was similar to that of the wt A_{2B}AR. Radioligand binding studies provided further evidence for the involvement of the ECL2 of A_{2A} and A_{2B}ARs in ligand binding and receptor subtype selectivity. In order to better understand the reason of

this dramatic difference between the structurally similar agonists adenosine and NECA, we generated a homology model of the loop mutant receptor and performed supervised molecular dynamics simulations (SuMD). The simulations suggested that adenosine can form a stable intermediate complex with the ECL2 of the A_{2B}AR with an energetic stabilization which is twice as low as compared to the energy obtained when bound to the orthosteric site, while NECA was not able to form an intermediate complex as stable as that of adenosine (see Figure). On the contrary, the major energetic stabilization appeared between NECA and the orthosteric binding site.



Meta stable complexes of the human A_{2A}(ECL2-hA_{2B}) mutant receptor with (**A**) adenosine, and (**B**) NECA, obtained with Supervised Molecular Dynamics simulation (SuMD).

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Mouse airway smooth muscle cells express P2X receptors

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Asthma is an inflammatory disease that results in airway obstruction, remodelling and airway smooth muscle (ASM) hyperresponsiveness. The triggers responsible for airway smooth muscle hyperresponsiveness remain unclear. Under both physiological and pathophysiological conditions cells release mediators, including nucleotides. Interestingly, the concentration of the extracellular nucleotide ATP is increased in the lung fluid of chronic ~~smokersand~~ patients with chronic obstructive pulmonary disease [1], cystic fibrosis [2], idiopathic pulmonary fibrosis [3] and asthma [4]. Extracellular nucleotides such as ATP can activate P2 receptors, which are known to regulate a range of physiological functions including contraction of smooth muscles from vas deferens [5], bladder [6] and ileum [7]. The ligand-gated P2X receptors play a major role in the modulation of smooth muscle function. We hypothesise that functional P2X receptors are expressed in ASM and contribute to the contraction of ~~airwaysunder~~ healthy and asthmatic conditions. Therefore, in the first instance, we investigated the expression of P2X receptors in ASM. Whole trachea and lungs were isolated from C57BL6/J mice after culling by schedule 1 (exposure to isofluorane followed by increasing concentrations of CO_2). The tracheas were dissected further to isolate the ASM which was used with the whole lung for qPCR and western blot experiments, while both whole lung and whole trachea were used for immunohistochemistry (DAB) experiments. P2X1-7 receptor subtype transcript expression levels were measured by qPCR. The expression of P2X1, P2X4 and P2X7 receptor proteins in tracheal smooth muscle and lung was assessed by western blot and by immunohistochemistry to further investigate their specific tissue and cellular localisation. qPCR experiments gave the following P2X receptor transcript expression profile for tracheal ASM and lung P2X4 > P2X6 ≈ P2X7 > P2X5 > P2X1 ≈ P2X2 ≈ P2X3. In addition, P2X1, P2X4 and P2X7 receptor proteins were also detected by ~~western~~ blot in tracheal ASM and lungs. Immunohistochemistry experiments showed that P2X1, P2X4 and P2X7 receptor proteins co-localise with the smooth muscle in trachea and lung, whilst P2X4 and P2X7 receptor proteins also co-localise with the epithelium.
In conclusion, ASM from mouse trachea and lungs express P2X receptor transcripts and P2X1, P2X4 and P2X7 receptor proteins.

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Extracellular ATP as *energy reservoir* in cancer progression

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Extracellular ATP is a signalling molecule implicated in chemotaxis, cytokine release, neurosecretion and cell growth. Release of ATP into extracellular space occurs in response to a multiplicity of physiological and phatological stimuli. Several plasma membrane ATP-releasing pathways have been suggested such as vesicular exocytosis, membrane anion carriers and non selective channels [1]. In healthy tissues the extracellular space is very low in ATP at variance with the tumor microenvironment (TME) where ATP can reach the hundred micromolar level [2]. Tumor cells have in general higher intracellular ATP content than healty cells due to overactive glycolysis and oxidative phosphorylation [3]. The higher intracellular ATP content provides the increased energy stores to power enhanced growth and mobility typical of cancer cells as well the source to feed the increased extracellular ATP levels. In order to understand the mechanism of regulation of the extracellular ATP and the relationship between intracellular and extracellular ATP levels, we measured extracellular ATP in B16F10 mouse melanoma cells cultured in presence and absence of serum. Serum deprivation severely blunted cell growth, which in serum starved B16F10 cells was reduced to about 20 % of controls. Extracellular ATP levels were much higher in serum starved than in control cells, while intracellular ATP was much higher in control cells. Moreover cytosolic calcium was also lower in serum starved versus control B16F10 cells, in contrast glycolysis was potentiated leading to a strong acidification of the extracellular medium.
Extracellular ATP might be a source of energy that cancer cells exploit under stress or limiting growing conditions. It has been shown that cancer cells may convert the high extracellular ATP content typical of the TME into creatin-phosphate which is then taken up fuel the intracellular energy stores [4]. This process is centered on the ability of cancer cells to release creatine kinase B (CKB) into extracellular space. B16F10 melanoma cells express high CKB levels which are unaffected during serum starvation. B16F10 cells release CKB via plasma membrane-derived microvesicles, thus the ATP rich microenvironment of serum-starved cells is also enriched in CKB. These findings suggest that accumulation of ATP within the extracellular space can serve multiple tasks, among which an energy store.

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The effect of 4-pyridone-3-carboxamide ribonucleoside (4PYR) in the mouse model of mammary 4T1 carcinoma

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Our earlier studies identified novel aspect of nicotinamide metabolism that involve 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (4PYR). This nicotinamide derivative could be metabolized in all cell types resulting in formation of nucleotide derivatives (4PYMP, 4PYTP, 4PYRAD). The accumulation of these compounds induces cytotoxic effect through the disruption of energy balance, but its exact mechanism is not known. Elevated blood concentration of 4PYR was observed in patients with cancer. The aim of this study was to investigate the effect of 4PYR and its metabolites in the mouse model of mammary 4T1 carcinoma.
BALB/c mice were divided into four groups: 1) bearing 4T1 mammary carcinoma as a control, 2) treated with 4PYR before 4T1 cells injection, 3) treated with 4PYR after 4PYR injection and 4) treated with 4PYR before and after 4T1 injection. 4T1 cells were injected intravenously and animals were maintained for 28 days. 4PYR (100 mg/kg/24 h) was administrated subcutaneously every 12 h in the last week. At the end of experiment blood and serum samples, lungs and aortas were collected for analysis of nucleotides, nicotinamide metabolites and 4PYR with its metabolites, as well as metastases number.
Metabolic effects of 4PYR administration include elevation of blood 4PYR and accumulation of erythrocyte 4PYMP and 4PYRAD as compared to control. 1-Methylnicotinamide (MetNA) and N-methyl-4-pyridone-3-carboxamide (Met4PY) concentration decreased while N-methyl-2-pyridone-5-

carboxamide (Met2PY) concentration increased in plasma of 4PYR treated groups in comparison to control. Furthermore, 4PYR treatment caused decrease of erythrocyte ATP/ADP and NAD/NADH ratio compared to control. 4PYR administration did not affect ATP and AMP hydrolysis, but cause increase in vascular adenosine deaminase activity on the aortic surface. 4PYR treatment led to decreased number of lung metastases.
4PYR treatment in 4T1 cancer model induces profound alterations in nicotinamide metabolism that may affect cancer progression and especially metastasis number.

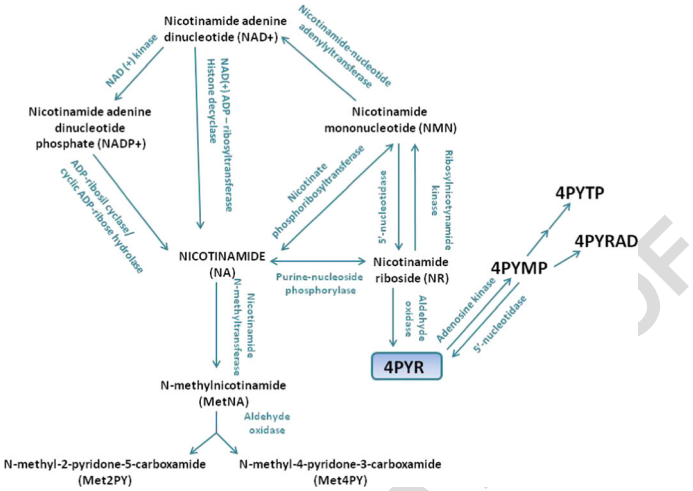

Figure 1. Formation and metabolism of 4-pyridone 3-carboxyamide-1-B-D-ribonucleoside (4PYR).

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