From astrocytes to satellite glial cells and back: a 25 year-long journey through the purinergic modulation of glial functions in pain and more

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Abstract

Fundamental progresses have been made in pain research with a comprehensive understanding of the neuronal pathways which convey painful sensations from the periphery and viscera to the central nervous system and of the descending modulating pathways. Nevertheless, many patients still suffer from various painful conditions, which are always associated to other primary pathologies, and get no or poor relief from available painkillers. Thus, the interest of many researchers has concentrated on new and promising cellular targets and biochemical pathways. This is the case of glia cells, both in the peripheral and in the central nervous system, and of purinergic receptors. Starting from many intuitions and hypotheses raised by Prof. Geoffrey Burnstock, data have accumulated which clearly highlight the fundamental role exerted by several nucleotide and nucleoside receptors in the modulation of glial cell reaction to pain triggers and of their cross-talk with sensory neurons which significantly contributes to the transition from acute to chronic pain. The purinergic system has therefore become an appealing pharmacological target in pain research, also based on the quite unexpected discovery that purines are involved in ancient analgesic techniques such as acupuncture. A more in-depth understanding of the complex and intricated purine-orchestrated scenario in pain conditions will hopefully lead to the identification and clinical development of new and effective analgesics.

Keywords

Astrocytes, microglia, satellite glia, trigeminal pain, P2Y receptors, adenosine receptors

Abbreviations

AR-C118925: 2,8-dimethyl-5H-dibenzo[a,d]cyclohepten-5-yl}-3,4-dihydro-2-oxo-4-thioxo-1(2H)-pyrimidinyl]methyl]-N-[1H-tetrazol-5-yl]-2-furancarboxamide; AR-C69931MX:

[dichloro-[[[(2R,3S,4R,5R)-3,4-dihydroxy-5-[6-(2-methylsulfanylethylamino)-2-(3,3,3-trifluoropropylsulfanyl)purin-9-yl]oxolan-2-yl]methoxy-hydroxyphosphoryl]oxy-hydroxyphosphoryl]methyl]phosphonic acid; BK: bradykinin; CGRP: calcitonin gene-related peptide; Cl-IB-MECA: 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide; DRG: dorsal root ganglion; EAE: experimental autoimmune encephalomyelitis; FHM1: familial hemiplegic migraine type 1; GFAP: glial fibrillary acidic protein; IB-MECA: N^6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide; MRS2179: [2-[(hydroxy-oxidophosphoryl) oxymethyl]-5-(6-methylaminopurin-9-yl)oxolan-3-yl] hydrogen phosphate; MS: multiple sclerosis; PAG: periaqueductal gray; PPADS: pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; SCH58261: 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; SGC: satellite glial cell; TMJ: temporomandibular joint; TRPV1: transient receptor potential vanilloid 1.

For decades, neurons have been the center of neurobiology and neuropharmacology. Research has allowed to understand that they are excitable cells, they can communicate with one another through specific chemical neurotransmitters but also via electric signals, they are plastic and react to both external and internal signals, and their deregulation, degeneration and death are at the basis of many pathologies. All these characteristics contribute to make neurons fascinating cell types and appealing targets for drug discovery, and indeed the vast majority of currently available pharmacological approaches to nervous system disorders are directed towards a neuronal receptor, enzyme or intracellular protein.

Starting from the 1970s several revolutionary thoughts appeared in the field of neuroscience, and the two most debated have been postulated by Geoffrey Burnstock. First of all, he demonstrated the incorrectness of the dogma "one neuron, one neurotransmitter" by providing evidence of the corelease of ATP together with "classical" neurotransmitters. In parallel, he proposed a new role for ATP as a neurotransmitter itself and presented to the scientific community the so-called "purinergic" hypothesis [Burnstock, 2006]. While these ideas were fighting hard to get the appropriate recognition among scientists, another destabilizing issue was progressively taking place: other cells in the central and peripheral nervous system contribute to delivery of signals, communicate with one another and with neurons and influence their functions. These cells are collectively known as "glial" cells, and the idea that they could take the center stage in brain functions at the expenses of neurons was another bitter pill to swallow for "neuroncentric" scientists [Barron et al., 1995; Henn & Henn, 1980]. It is not difficult to imagine what did it mean to study purinergic signaling in glial cells at that time; it was exactly how my journey in science started and how I met for the first time Geoffrey Burnstock, thanks to his close friendship and collaboration with my mentor, Maria Abbracchio.

1. A tale of astrocytes and purines

At the beginning of the 90s, Geoff was fascinated by the idea to replace lost cells in neurodegenerative diseases, such as Parkinson's disease, by grafting in the damaged tissue heterologous cells previously manipulated *in vitro* by purine signaling to boost their ability to integrate and regenerate lost connections. This was one of the many pioneering idea he had in his career, and as many of them it seemed absolutely crazy. He focused his interest on astrocytes, funny star-shaped cells that were at that time mostly considered as "filler" cells in the brain to build a scaffold for neurons. Soon after my entrance in her laboratory as undergraduate student, Maria decided to join Geoff in this adventure and moved for a six-month Sabbatical leave to London. She came back with data for one of the first paper on the differential role played by ATP and adenosine in modulating astrocyte functions [Abbracchio et al., 1994], and with a laboratory book full of protocols to be implemented in our laboratory in Milan.

Ready, steady, go…in a couple of months I was observing my first GFAP-stained astrocytes under a fluorescence microscope. After getting my Master degree, I started my PhD program which was mostly focused on adenosine and astrocytes [Ceruti et al., 1997; 2000; 2003; Ceruti & Abbracchio, 2020], but in parallel, I also contributed to other research projects, including the characterization of the mechanisms of ATP-induced reactive astrogliosis [Abbracchio et al., 1995; Bolego et al., 1997; Brambilla et al., 1999; 2000]. This was a puzzling story: exposure to ATP analogues led to astrocyte activation, with the appearance of a reactive phenotype characterized by long and thin processes (Figure 1). However, the pharmacology of these responses did not correspond to any of the known G protein-coupled nucleotide P2Y receptors [Abbracchio & Burnstock, 1994; Fredholm et al., 1994; Abbracchio & Burnstock, 1998] (see below for details). Thus, long hours of discussion led to the conclusion that a new nucleotide receptor should have been involved; since at that time in Milan we were not equipped to perform molecular biology, I was selected to spend a period at University College London

in Geoff's laboratory to identify this unusual receptor. I must admit that from a scientific point of view that period was not immediately successful: several problems with astrocytic cultures and some technical issues did not allow us to come up with satisfactory results (which were several years later achieved by new members of our laboratory in Milan with the cloning and deorphanization of the new P2Y-like receptor GPR17) [Ciana et al., 2006]. On the other hand, it was a marvelous piece of my personal life: it was my first long period away from home, and I found support and friendship by Geoff, his staff including the wonderful secretary Annie, and the members of his laboratory. Geoff in particular was such a recognized scientist but I was really impressed by his protective and careful attitude towards a "simple" PhD student like me.

2. New challenges and opportunities: getting involved in pain transmission

Thanks to that experience, I deepened my knowledge on P2Y receptors. This proved fundamental some years later when our laboratory was involved in studying the role of ATP signaling in cell-to-cell communication within the trigeminal (TG) ganglion in a mouse model of genetic migraine. Before summarizing our contribution to the comprehension of the role of the purinergic system in the development of TG pain, a brief overview on how glial cells and nucleotides/nucleosides have started to be considered as key players in nociception is mandatory.

2.1 Purines and central nervous system glia: an indissoluble liaison in pain transmission

While previous sparse evidence demonstrated a pro-algogenic role of ATP in human blister base preparation, the involvement of purines in pain transmission was strengthened starting from middle 1990s following cloning and identification of ionotropic P2X3 receptors expressed by sensory neurons on pain pathways and in visceral organs [Burnstock & Wood, 1996]. In hindsight it was just logical to foresee the involvement of ATP in pain: any

inflammatory, hypoxic or traumatic event (including visceral distension due to the presence of kidney or gallstones or of excessive intestinal content during a colic) leads to a several-fold increase of the extracellular ATP concentrations which in turn can activate fast ionotropic P2X channels promoting neuronal firing. Thanks to the activity of metabolizing enzymes, i.e. ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39 or E-NTPDase1), which sequentially degrades ATP to ADP and AMP, and ecto-5'-nucleotidase (CD73) which dephosphorylates AMP [Giuliani et al., 2019], ATP is rapidly hydrolyzed within seconds to its breakdown product, adenosine, whose modulatory role on pain signaling was also recognized, with both analgesic and pro-algogenic actions depending on the expression of either the metabotropic A_1 or the A_{2A} receptor subtypes, respectively [Burnstock, 2006]. Additionally, several purinergic receptors show a peculiar localization on somata of nociceptors in sensory ganglia and on their peripheral projections, on autonomic nerves, on inflammatory cells, and on second order neurons in the spinal cord, as demonstrated in the last 25 years thus progressively reinforcing the hypothesis of a purinergic modulation of pain. Data have been later extended to different types of pain, including visceral, neuropathic and inflammatory pain, but also headache and migraine disorders [Burnstock, 2016].

At that time, whether P2Y receptors contribute to pain transmission was not known. This was mostly due to the complex pharmacology of the 8 mammals subtypes included in this receptor family [Fredholm et al., 1994]: the $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ subtypes are adenine nucleotide-sensitive receptors activated by ADP, whereas other receptor subtypes can be classified as uracil nucleotide-sensitive. UTP acts as the main agonist at the $P2Y_2$ and $P2Y_4$ subtypes, while UDP and UDP-sugars recruit $P2Y_6$ and $P2Y_{14}$ receptor subtypes, respectively [Fredholm et al., 1994]. ATP binds with different affinity to all P2Y receptor subtypes, with the exception of the $P2Y_6$, and with significant differences between mice (where it binds to $P2Y_2$ and $P2Y_4$ receptors with equimolar affinity to UTP) and humans (where it behaves as

competitive antagonist at UTP-responsive $P2Y_4$ receptors) [von Kügelgen, 2019]. A rodent homolog of the human ATP-sensitive $P2Y_{11}$ subtype has not been identified [Communi et al., 1999]. Additional complexity came from heterogeneous signaling pathways ($P2Y_{1,2,4,6,11}$ are linked to G_q/G_{11} thus recruiting PLC and eventually leading to elevation of intracellular Ca^{2+} concentrations, whereas $P2Y_{12,13,14}$ inhibit adenylyl cyclase through $G_{i/o}$) [Fredholm et al., 1\994], from their widespread expression, and from the lack of selective ligands and subtype specific antibodies [von Kügelgen, 2019]. The only available hints on the involvement of P2Y receptors in nociception derived from DRGs, where ADP inhibited neuronal N-type calcium channels through $P2Y_1$ receptors while ATP and UTP promoted calcium signaling and release of pro-algogenic CGRP likely through the $P2Y_2$ receptor subtype [Gerevich et al., 2004; Sanada et al., 2002].

A significant boost to the research on the role of purinergic receptors in general, and of P2Y receptors in particular, came from the recognition of the fundamental contribution of glial cells to pain transmission. It has, in fact, become increasingly clear that astrocytes, microglia and oligodendrocytes do not simply support neuronal activity in the central nervous system through their mechanical and structural actions; rather, they actively contribute to cell-to-cell communication through the release of multiple neurotrasmitters and the expression of their membrane receptors [Milligan & Watkins, 2009]. This is true under physiological conditions but becomes even more important in pathological settings, due to the rapid reaction of glial cells which significantly modifies their activity and functions, also through the upregulation of specific signaling systems including the purinergic one [Magni & Ceruti, 2013; 2019].

The spinal cord represents the fundamental station for the integration of painful sensations collected and transduced by nociceptors from the periphery or visceral organs, and for their eventual conveyance by second-order neurons to supra-axial centers (i.e., thalamus and cerebral cortex) for conscious perception and modulation [Yam et al., 2018]. The firing of

second-order neurons is modulated by inhibitory interneurons and by descending inhibitory pathways, but also by surrounding glial cells. This physiological cross-talk can be significantly altered by a variety of events, including drug treatments, trauma, ischemia, surgery and autoimmune disorders, which are all characterized by chronic pain [Ji et al., 2018]. It is now emerging that the development of neuroinflammation is the common feature underlying these different pain triggers. Neuroinflammation in the central nervous system (CNS) is characterized by disruption of the integrity of the blood-brain barrier, increased vascular permeability with extravasation of immune cells, and activation of microglia and astrocytes (so that chronic pain has been referred to as "gliopathy"). The latter cell types release a variety of cytokines, chemokine, neuropeptides and other substances (including ATP; see below) which auto-amplify and sustain glial activation over time and, in parallel, modulate synaptic plasticity and increase excitatory neuronal firing, driving the transition towards pathologically persistent chronic pain [Ji et al., 2018]. Neuroinflammation can occur in the peripheral nervous system as well where it involves Schwann cells and satellite glia in nerves and ganglia, respectively (see below). Thus, glial cells could represent a promising target for new non-neuronal analgesics based on their fundamental role in fostering neuronal sensitization.

As for neurons with the P2X3 receptor subtype, the most studied glial purinergic receptor involved in pain transmission is another member of the ionotropic family of receptors, the P2X4 subtype, which was found necessary and sufficient to promote microglia activation in the spinal cord as a response to nerve damage or inflammatory conditions and whose recruitment triggers a cascade of biochemical events contributing to neuronal sensitization and eventually to pain chronicization [Inoue, 2019]. Nevertheless, it soon became clear that microglia functions are also modulated by several P2Y receptors (Figure 2): the UDP-sensitive $P2Y_6$ subtype drives phagocytic activity and engulfment of debris and pathogens [Anwar et al., 2020], whereas the $P2Y_{12}$ subtype collaborates with P2X4 to promote microglia polarization,

migration, and chemotaxis toward nucleotides [von Kugelgen, 2019]. In line with this, the genetic ablation of $P2Y_{12}$ or the administration of selective antagonists (i.e., intrathecal AR-C69931MX or oral Clopidogrel) prevented the development of tactile allodynia following nerve ligation [Tozaki-Saitoh & Tsuda, 2019].

Spinal cord astrocytes react to nerve injury, traumatic and ischemic events and contribute to the modulation of neuronal firing through the release of pro- and anti-inflammatory mediators and thanks to their ability to buffer pathologically increased extracellular glutamate concentrations [Buffo et al., 2010]. Under painful conditions, a close and time-dependent connection between astrocytes and microglia reaction has been highlighted, with the latter rapidly reacting to harmful stimuli, thus directing the development of acute pain, whereas the former are recruited at later time points. Reactive astrocytes could therefore represent innovative targets for the development of cell-specific approaches to chronic pain [Nakagawa & Kaneko, 2010]. Astrocytes release ATP as "gliotransmitter" through both exocytotic and non-exocytotic pathways, and express a variety of nucleotide and nucleoside purinergic receptors; several of them are upregulated following pathological events and are directly involved in the subsequent cell reaction (Figure 2) [Rivera et al., 2016]. Despite convincing published proofs of the purinergic modulation of reactive astrogliosis, up to some years ago no definitive evidence of the involvement of specific astrocytic purinergic receptors in pain transmission has been provided. As mentioned above, the modulatory role of the A_1 and A_{2A} adenosine receptor subtypes in pain has been known for many years. It was only recently that the "youngest" member of the P1 receptor family, the A_3 adenosine receptor, which was discovered by cloning strategies at the beginning of the 90s, has been clearly identified as the major responsible for the analgesic effects exerted by adenosine. Thanks to selective agonists and antagonists, the group of Daniela Salvemini and Ken Jacobson has shown that the activation of the A_3 adenosine receptor is highly effective in reducing chronic neuropathic pain (reviewed

in [Jacobson et al., 2020]), and similar evidence has been provided in visceral pain [Antonioli et al., 2020; Lucarini et al., 2020]. Interestingly, we and others have contributed to the first demonstrations that this receptor subtype is mostly expressed by astrocytes and controls their reaction to several types of injury [Abbracchio et al., 1997]. More recently, in a rodent model of oxaliplatin-induced neurotoxicity, the astrocytic upregulation of adenosine kinase (i.e. the enzyme which phosphorylates adenosine to AMP thus reducing adenosine concentrations), accompanied by a dysregulation of A_3 adenosine receptor functions, was found responsible for the development of neuropathic pain [Wahlman et al., 2018], thus providing the first clear demonstration of the involvement of astrocytic purinergic receptors in chronic painful conditions.

2.2 A "peripheral" point of view on pain development and chronicization: purinergic receptors and sensory ganglia.

In sensory ganglia nociceptor and sensory neuron cell bodies are surrounded and wrapped by satellite glial cells (SGCs), a particular type of glial cells that share some characteristics with CNS astrocytes but have also several peculiar features which are still to be fully understood [Hanani & Spray, 2020]. Groups of SGCs are connected to one another through gap junctions and constitute a functional unit together with the specific neuron they are surrounding [Spray et al., 2019] (Figure 3). No synapses can be found within sensory ganglia (i.e. DRGs and TG ganglia), but the discovery of a somatic release of neurotransmitters and neuromodulators by sensory neurons has opened the way towards the identification of neuron-to-SGC communication and the understanding of its contribution to the modulation of neuronal firing in physiological and pathological conditions, including pain [Hanani, 2012].

As mentioned in the previous section, at the beginning of the 2000s most of available data were obtained in DRG neurons, due to their involvement in the development of pain in a

number of experimental models of neuropathic and inflammatory pain conditions which are widely utilized and studied (e.g., sciatic nerve ligation, spared nerve injury, injection of irritants in the hind paw, chemotherapy-induced pain) [Esposito et al., 2019]. It was known that DRG neurons express not only the P2X3 but also the $P2Y_{1,2,4}$ subtypes. Overall, they contribute to modulate neuronal firing through a specific P2X-P2Y but also a TRPV1-P2 cross-talk [Gerevich & Illes, 2004; Gerevich et al., 2004; Ruan & Burnstock, 2003; Sanada et al., 2002].

Conversely, much less data were available in TG ganglia, which are crucially involved in the development of orofacial pain and migraine. TG ganglia are located at the basis of the skull, and harbor the cell bodies of sensory neurons that innervate the head and face districts through their neurites which overall constitute the TG nerve, subdivided in 3 branches (V1-ophtalmic, V2-maxillary, and V3-mandibular) [Bisla & Imlach, 2019]. The TG nerve relays sensations from the periphery, including the meninges, to the brainstem and cervical spinal cord by making synapses with second order neurons in the so-called spinal-trigeminal nucleus which in turn is connected with supraspinal centers (i.e., PAG, thalamus, cortex etc.) [Bisla & Imlach, 2019]. It therefore plays a fundamental role in every type of orofacial pain and during migraine and headache attacks, and several hypotheses have been raised on whether the real trigger of migraine stands in the periphery or in the central nervous system [Haanes & Edvinsson, 2019].

A purinergic hypothesis for migraine was postulated by Geoff in 1981 and further refined in 1989, with the demonstration that both platelets and endothelial cells in the meningeal and cerebral vasculature release ATP which in turn contributes to the initial vasospasm and subsequent vasodilation, which were supposed to be at the basis of the migraine attack, together with noradrenaline and endothelial-derived factors [Burnstock, 1981; 1989]. As mentioned, much less was known on the role of purinergic system in the TG ganglion. Functional P2X3 and P2X2/3 receptors were found on sensory neurons [Burnstock, 2000], thus further confirming a role for these receptor subtypes in the modulation of neuronal function and signal

transmission from the periphery, as already shown within the CNS. Very few data were available on SGCs, with the demonstration of functional $P2Y_{1,2,4}$ subtypes in intact TG ganglia [Weick et al., 2003].

Based on our previous experience on studying the role of P2Y receptors in the modulation of reactive astrogliosis, our laboratory was included in a research project aimed at evaluating the role of purinergic receptors in a specific type of migraine with a genetic origin, the so-called Familial Hemiplegic Migraine (FHM) Type 1. This is one of the three types of migraine for which a clear correlation with specific gene mutations has been demonstrated. It is in fact well-known that migraineurs often have close relatives suffering from the same disease, but a direct correlation with alterations of specific genes is rather impossible in common migraine, due to its multifactorial nature. Conversely, FHMs are rare inherited diseases characterized by extremely severe migraine attacks which are often accompanied by hemiplegia, and specific gene mutations have been described for each of them by studying affected families [Shyti et al., 2011]. FHM1 is characterized by gain-of-function mutations of the *cacna1a* gene which encodes for the neuronal $Ca_v2.1$ calcium channel. Mutations lead to calcium channel opening at lower membrane potentials, meaning activation of neuronal firing by normally innocuous stimuli, with the generation of painful migraine attacks [Pietrobon, 2010]. In this scenario, our project coordinated by Prof. Andrea Nistri (SISSA, Trieste) was aimed at evaluating possible dysfunctions in the purinergic signaling in a mouse model of the disease, bearing the R192Q missense mutation of the human CACNA1A calcium channel [van de Ven et al., 2007]. Our collaborators focused on TG neurons and demonstrated an increased expression, higher excitability and reduced desensitization of P2X3 receptors, which overall contributed to the facilitated pain behavior observed in these animals [Hullungundi et al., 2014].

In parallel, we were involved in the evaluation of the expression and function of P2Y receptors. First of all, we performed a whole evaluation of functional P2Y receptors expressed

in WT animals and showed that both TG sensory neurons and SGCs express functional ADP-sensitive $P2Y_{1,12,13}$ and also responded to UTP, but no selective ligands were available to discriminate between the contribution of likely $P2Y_2$ or $P2Y_4$ receptors in the latter responses (see also below and Figure 3). Calcium increases as well as the percentage of responding cells in neurons were significantly lower than in SGCs, and UDP-mediated responses, likely due to $P2Y_6$ receptor recruitment, were detected in the latter cell population only [Ceruti et al., 2008]. Interestingly, exposure of TG cultures to the pro-algogenic mediator bradykinin (BK) significantly enhanced P2Y-mediated responses in SGCs, thus suggesting a potentiation of glial purinergic signaling under inflammatory painful conditions [Ceruti et al., 2008]. We next demonstrated that exposure to BK promoted the neuronal release of the migraine mediator CGRP which, in turn, acted on surrounding SGCs to boost the release of inflammatory mediators, including PGE_2, and to promote P2Y receptor functions through the recruitment of the ERK1/2 signaling pathway [Ceruti et al., 2011; Magni et al., 2015]. Of note, the cross-talk between CGRP and purinergic receptors on SGCs was significantly enhanced in cultures from R192Q CACNA1A FHM1 KI mice, thus suggesting that P2Y receptors could contribute to the increased neuronal excitability observed in these animals and that they could represent possible pharmacological targets for new anti-migraine agents to be exploited in genetic migraine as well [Ceruti et al., 2011]. In line with this, we have more recently reported basal signs of reactive microgliosis and astrogliosis in the brainstem of FHM1 KI mice [Magni et al., 2019], thus suggesting that the enhanced neuronal excitability observed as a consequence of the expression of the R192Q CACNA1A missense mutation could influence surrounding glial cells in the CNS which in turn could contribute to the pathological phenotype. Although no direct data are currently available, it is worth speculating on the possible involvement of purinergic signaling in this scenario based on the known contribution of purines to gliosis (see above).

Based on our results obtained in the mouse model of genetic migraine, we became interested in deepening our comprehension of the role of glial P2Y receptors in different and more common types of TG pain, including orofacial pain related to temporomandibular joint (TMJ) inflammation. The TMJ is innervated by the TG nerve through its V3-mandibular branch. Thus, inflammatory or traumatic events in the joint lead to retrograde neuronal sensitization in the TG ganglion with subsequent increased neuronal firing which manifests as orofacial allodynia (i.e., a painful reaction to a normally innocuous stimulus) in the vibrissae pad area where sensitized TG neurons send their projections [Villa et al., 2010a]. We set up a rat model of pain by the unilateral TMJ injection of the pro-inflammatory mixture named Complete Freund Adjuvant (CFA). In parallel with the development of orofacial sensitization, we observed a significant reaction of glial cells both in the periphery and in the CNS. In the TG ganglion, SGCs showed an activated phenotype characterized by the overexpression of the GFAP protein, the typical astrocytic protein which is a marker of cell activation in the case of SGCs, which was paralleled by an increased infiltration of macrophages with the generation of an overall pro-inflammatory environment [Villa et al., 2010a]. In the brainstem, we detected significant microgliosis with no signs of reactive astrogliosis; this is possibly due to the relatively short period of analysis (i.e., up to 72 hours) since in general astrocyte reaction to a painful stimulus manifests during pain chronicization [Villa et al., 2010a]. Concerning P2Y receptors expressed in the TG ganglion, we demonstrated that while $P2Y_1$ receptors are expressed by both neurons and SGCs, $P2Y_2$ receptors show an exclusive glial localization (Figure 3). Additionally, in line with our *in vitro* data [Ceruti et al., 2008] (see above), induction of TMJ inflammation led to a time-dependent up-regulation of both receptors, suggesting that they could play a role in the development of orofacial allodynia. Thus, we decided to treat rats from 24 hours post induction of inflammation with purinergic antagonists, namely the non-selective agent PPADS, the $P2Y_1$-selective compound MRS2179 or the $P2Y_2$-selective drug

AR-C118925. While no effect was exerted by MRS2179 and a partial inhibition was observed in the case of PPADS, a highly significant reduction in the withdrawal threshold at Von Frey test, utilized to test orofacial allodynia, was elicited by AR-C118925 which was already maximally effective at the very low dose of 1 mg/kg. This suggests that glial $P2Y_2$ receptors are crucially involved in the development of orofacial pain following TMJ inflammation [Magni et al., 2015]. Interestingly, the $P2Y_2$ antagonist was as effective as the anti-migraine agent sumatriptan and the anti-inflammatory drug acetyl salicylic acid, thus paving the way for a future clinical exploitation of agents acting on this receptor subtype in orofacial painful conditions.

The role and functions of $P2Y_2$ receptors in pain is, however, still debated, and likely depend upon the specific experimental model and pain paradigm under study. While our data on an exclusive glial localization of this receptor subtype are in agreement with published data in rat DRGs [Rozanski et al., 2013], other authors have found this receptor subtype expressed by sensory neurons in both TG ganglia and DRGs [Li et al., 2014; Malin et al., 2008]; nevertheless their data confirm an overall pro-algogenic role for this UTP-activated receptor subtype. Conversely, other data have shown an inhibitory relationship between $P2Y_2$ receptors and pro-algogenic P2X3 channels in sensory neurons [Mo et al., 2013], thus adding further complexity to these already complicated cell-to-cell communication signaling pathways.

Other members of the P2Y receptor family expressed in sensory ganglia have been involved in several types of pain, as we have recently summarized elsewhere [Magni et al., 2018; Magni & Ceruti, 2019]. For example, the $P2Y_{12}$ receptor subtype, which is known to contribute to microglia reaction to injury in the spinal cord (see above) and is not expressed by SGCs in the TG ganglion under basal conditions, is upregulated following lingual nerve crush and its blockade by selective antagonists relieves neuropathic pain in rats [Sugawara et al., 2017]. Therefore, targeting this receptor subtype might provide both a central and a peripheral

site of action for novel analgesics at least for some types of pain, e.g. neuropathic pain, with an overall reduction of side effects and a possible better control of pain.

One of the most intriguing characteristics of purinergic transmission, and of P2Y receptors in particular, that has emerged from many published papers, including ours, is a significant modulation of receptor expression and functions in the spinal-TG system and more in general in pain pathways from the periphery to the CNS. Under various pain states some receptors are upregulated, others are only expressed following nerve damage (i.e., the $P2Y_{12}$ receptor subtype in the TG; see above) (Figure 4), and all of them contribute to neuronal sensitization and to the development of pain along with its characteristic symptoms, such as allodynia and hyperalgesia. Additionally, they modulate the functions of fast-operating neuronal receptors involved in neurotransmission, including purinergic P2X3, P2X7 and vanilloid TRPV1 channels (Figure 4). Thus, although no purinergic drug has up to now reach the market as analgesic agent, P2Y receptors represent extremely interesting targets for the development of focused pharmacological strategies aimed at acting at sites where receptors are either upregulated or hyperfunctional.

3. Geoff's legacy and future directions

The overall real value of the contribution of Geoff's intuitions and visionary approach to cell biology and neuroscience cannot be quantified. His work has challenged several scientific dogma and opened up the way to many scientists worldwide who have progressively contributed to confirm and sustain his initial ideas. Accumulating evidence in the last 50 years have clearly demonstrated that the purinergic system is indeed fundamental in physiological cell-to-cell communication in all tissues and organs in the body and that dysregulations of its functioning are directly involved in several pathologies. It can be foreseen that more and more new discoveries will be made in the next years; for example, despite the clear demonstration of

specific actions mediated by guanosine and guanosine nucleotides in various organs and tissues [Mancinelli et al., 2020], including the TG ganglion [Villa et al., 2010b], the identification of a specific receptor is still lacking.

Concerning the purinergic modulation of pain pathways, the clinical exploitation of the huge amount of *in vitro* and preclinical data has been up to now slowed down by the complexity of the whole system, the long-lasting lack of really selective ligands (especially concerning P2Y receptors), and the widespread expression of both adenosine and nucleotide receptors which accounts for both their multiple functions and for possible side effects. This is, for example, the case of A_1 adenosine receptor agonists which are known to have significant cardiac activity or of the A_{2A} adenosine and $P2Y_{12}$ receptors which are crucially involved in platelet aggregation. Thanks to the fundamental support of medicinal chemists several of these issues have now started to be solved [Magni & Ceruti, 2019], and would hopefully lead to the identification of lead compounds to be proposed as purinergic analgesics. The most promising target is currently represented by the A_3 adenosine receptor, thanks to its peculiar multiple sites of actions in neuropathic and inflammatory pain and to the availability of selective ligands that are already in clinical trials for different indications (see above) [Jacobson et al., 2020].

Nevertheless, despite the current partially disappointing clinical results, research on the purinergic modulation of pain has revealed several previously unforeseen relationships among cells and has shed light on unexpected signaling pathways, and many others are yet to come. Once again, the inputs from Geoff have proved fundamental. In fact, one of his last hypotheses dealt with the role of purines in the analgesic efficacy of acupuncture. His simple and extremely logical idea was as follows: any traumatic event leads to cell damage with consequent extracellular release of ATP which is rapidly degraded to adenosine. Despite their extremely thin section, insertion of acupuncture needles in the so-called “acupoints” along meridians and their manual rotation or electrical stimulation, which are at the basis of the acupuncture practice,

are indeed traumatic events for subcutaneous tissues. Thus, local concentrations of ATP and adenosine are expected to rise several folds thus contributing to the beneficial effects of this ancient technique, including the negative modulation of painful sensation [Burnstock, 2009]. After the publication of this first hypothesis, data have progressively demonstrated that Geoff was right one more time. Increased ATP and adenosine concentrations at the site of needle insertion were detected not only in animals but also in patients [Takano et al., 2012; Tang & Illes, 2020]. Concerning the underlying mechanisms, most of available data concentrated on neuronal P2X3 receptors, with the demonstration of their desensitization or reduced expression following acupuncture analgesic treatments [He et al., 2020; Tang et al., 2019]. Nevertheless, also P2Y and adenosine receptors have been implicated [Tang & Illes, 2020]. Just as examples, A_1 adenosine receptor KO mice exposed to inflammatory pain were insensitive to acupuncture analgesia, while administration of deoxycoformicin, an inhibitor of adenosine deaminase which metabolizes adenosine to inosine, potentiated acupuncture analgesia [Goldman et al., 2010]. Additionally, in the presence of the A_{2A} selective antagonist SCH58261 the beneficial effects of electroacupuncture against autoimmune arthritis were reduced [Li et al., 2015]. The search of the biochemical pathways at the basis of acupuncture efficacy in various pathological conditions is a rapidly growing field of research, thanks to the close collaboration between Chinese researchers and colleagues from Western countries. Due to the exponential growth in the number of publications in the last 10 years, it is foreseen that new strategies to improve the efficacy of traditional treatments through the pharmacological modulation of the purinergic system may soon emerge. Interestingly, based on the known effect of methylxanthine, including caffeine, as adenosine receptor antagonists, it has been demonstrated that caffeine consumption could impair the efficacy of acupuncture in rodents [Morè et al., 2013], and a similar effect could be postulated in humans possibly depending on the caffeine-rich beverage drinking habits of each individual patient.

As I mentioned, thanks to the close collaboration of my research group with Geoff's laboratory, I started my journey in science by studying the purinergic modulation of astrocytic functions, and then moved to several different types of glial cells, including microglia, oligodendrocytes and satellite glial cells [Lecca et al., 2012]. My main focus now is on pain pathways and in the last 10 years I have been mostly working on the peripheral nervous system, but I am still fond of astrocytes. I am extremely fascinated by their peculiar characteristics and by the double-edged sword activity of reactive astrocytes [Buffo et al., 2010], and I strongly believe that the purinergic modulation of their functions contributes to pain pathways as well.

This could become especially true when dealing with chronic pain not just as a pathology itself, but as a serious secondary symptom of many diseases which increases the burden on patients and caregivers and significantly reduces their quality of life. This is the case, for example, of multiple sclerosis (MS) a progressive autoimmune degenerative disorder of the CNS characterized by massive infiltration of immune cells, progressive loss of myelin sheath, and axonal degeneration leading to gradual motor disability. Pain is a common multifaceted characteristic of MS patients, with both pain syndromes directly connected to the disease (i.e., neuromuscular and neuropathic pain, pain related to being on a wheelchair etc.) and apparently unrelated painful conditions, such as migraine and TG pain [Ceruti, 2018]. Of note, pain can appear months before the first motor or visual symptoms which lead to the diagnosis of MS, thus suggesting that there are parallel and independent mechanisms driving the different components of the disease. The role of glial cells, and particularly microglia, in the development and progression of MS has been now recognized, and the purinergic system is now of the most interesting signaling systems involved, mostly through the P2X4 and P2X7 ionotropic but also the $P2Y_{12}$ metabotropic receptor subtypes [Domerq & Matute, 2019; van Wageningen et al., 2019], but no data were available on the purinergic contribution to MS-related pain.

Based on our interest in TG painful conditions, we recently set up a rat model of relapsing-remitting MS, the most common form of MS in young adults characterized by progressive worsening of relapses and intermittent periods of remission from symptoms, called Experimental Autoimmune Encephalomyelitis (EAE) and studied the development of TG pain. We showed that these animals develop severe orofacial allodynia starting as early as 5 days after induction of the pathology, i.e. well before the appearance of the motor signs of the disease, confirming that parallel mechanisms are recruited to drive disease progression and pain development. Interestingly, we also showed the early development of reactive astrogliosis and microgliosis accompanied by increased expression of the A_3 adenosine receptor in the brainstem of EAE rats [Magni et al., 2020]. Thus, early alterations in glial cell functions which likely involve the purinergic system could be responsible for the development of TG pain and targeting the A_3 adenosine receptors could represent an innovative pharmacological approach to pain in MS patients. Based on the known role of this receptor subtype in controlling and fostering reactive astrogliosis [Abbracchio et al., 1997a; 1997b], and on the ability of astrocytes to modulate neuronal circuits through the release of mediators and neurotransmitters, including ATP [Buffo et al., 2010; Illes et al., 2019], I hypothesize that the A_3-astrocytes connection is directly involved in the early generation of EAE-associated pain. As mentioned above, the A_3AR-selective agonist IB-MECA is already in clinical trials for psoriasis and other autoimmune disorders [Magni & Ceruti, 2020], therefore its exploitation in TG pain in MS could be accelerated if our preclinical data are confirmed.

In conclusion, I can only join the sadness of many colleagues and scientists worldwide for the loss of Geoff Burnstock. We shall deeply miss his advices, his warm sympathy, and his brilliant ideas that have been such an inspiration for all of us. Personally, he significantly contributed to my career both directly and indirectly through his example and scientific activity.

We all shall do our best to continue his work and to contribute to improving knowledge in his memory.

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Conflicts of Interest

The author declares no conflict of interest.

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Figure legends

Figure 1. Scanning electron microscopy image of rat primary striatal astrocytes exposed to the ATP analog α,βmeATP. Treated cells show signs of reactive astrogliosis, with longer and thinner processes with respect to Control cultures [Brambilla et al., 2000]. This manuscript was dedicated to Geoffrey Burnstock in honor of his pioneering ideas on purinergic signaling. Reproduced with permission from Elsevier (Licence #4930081179603).

Figure 2. Expression of multiple metabotropic adenosine and P2Y receptors by neurons and glial cells involved in pain transmission both in the central and in peripheral nervous system. References are included in the reference list. Reproduced with permission from Elsevier (licence # 4930120904324) [Magni & Ceruti, 2019].

Figure 3. Differential cellular expression of the $P2Y_1$ and $P2Y_2$ receptors in the TG ganglion. Immunohistochemical analysis of rat TG ganglion sections showing co-staining of glial or neuronal markers with P2Y purinergic receptors. Satellite glial cells (SGCs) have been identified by staining with antibodies against Glutamine Synthetase (GS), while neurons express the typical markers NeuN and SNAP-25. Pictures on the left show the colocalization (yellow in Merged) of $P2Y_1$ receptor staining (green) with both glial and neuronal markers (red). Conversely, only SGCs express $P2Y_2$ receptors (green; right pictures), as shown by colocalization (yellow in Merged) with GS (red), and lack of co-staining with both NeuN and SNAP-25. Reproduced with permission from Wiley (licence #4933091176911) [Magni et al., 2015].

Figure 4. Modulation of P2Y receptor expression and function under various pain states in sensory ganglia (A) and in the spinal cord (B). Various members of the purinergic

metabotropic P2Y receptor family are expressed under physiological conditions by glial cells and neurons along pain pathways, i.e. in peripheral ganglia (DRGs and TG ganglia) and in the dorsal horn of the spinal cord. Upon induction of either inflammatory or neuropathic pain (and possibly in chronic migraine), significant plasticity of the whole system has been demonstrated. Upregulated or hypersensitive receptors are represented with thicker lines, and contribute to foster glia reaction and the cross-talk with sensory neurons, which in turn get sensitized and release cytokines and other mediators with the generation of a self-amplifying pro-algogenic circle. Some receptor subtypes are only expressed following nerve injury, i.e. the $P2Y_{12}$ subtype on SGCs (A). Additionally, P2Y receptors can modulate the functions of fast neuronal ionotropic receptors, including members of the purinergic P2X family and vanilloid TRPV1 receptors. Very little is known on the contribution to pain pathways of astrocytic P2Y receptors, while a significant role has recently emerged for various adenosine receptor subtypes (not shown; see text for details). Reproduced with permission from Elsevier (licence # 4933140768293) [Magni and Ceruti, 2013].

Figure 1

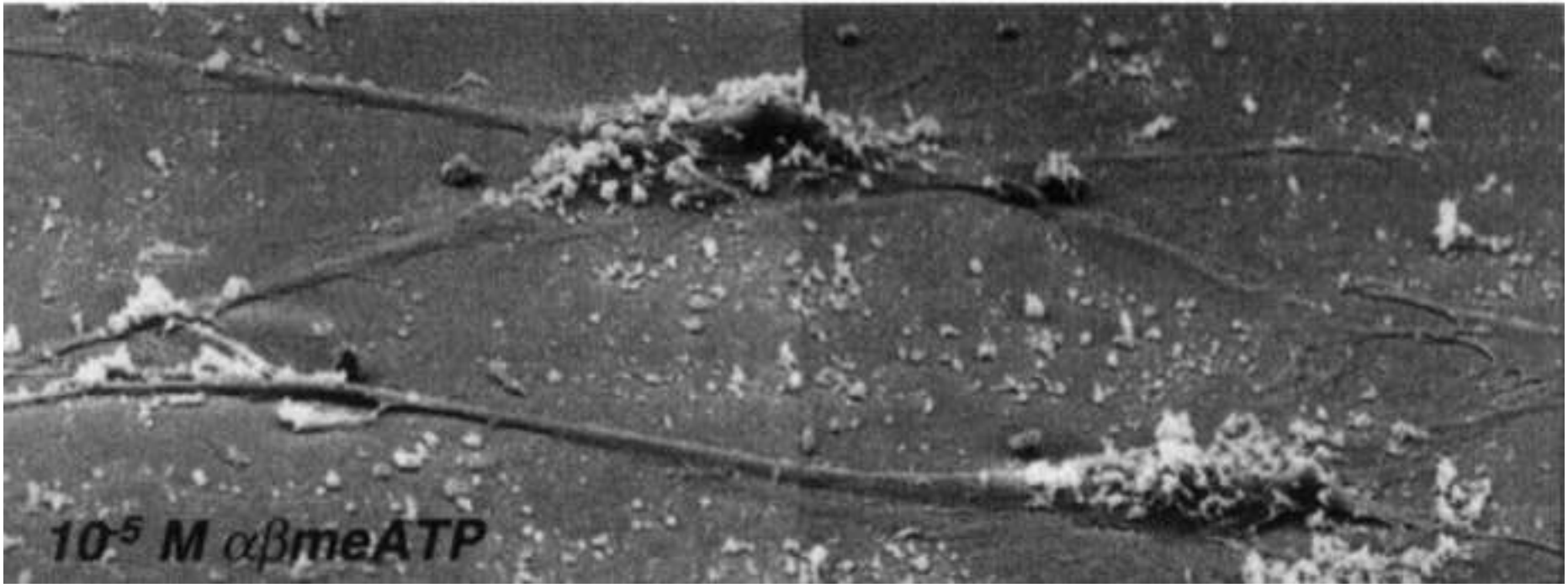

Figure 2

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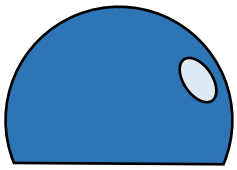
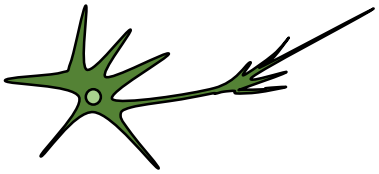
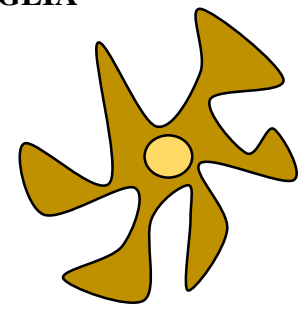
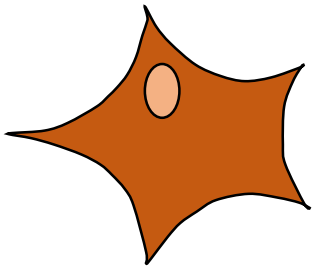
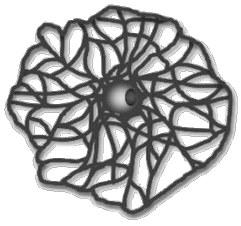
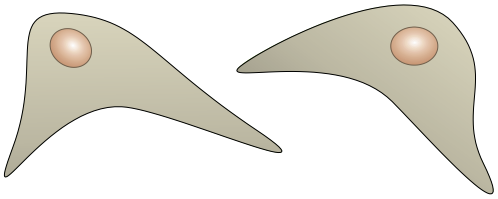
PRIMARY SENSORY NEURON 	$P2Y_1$ $P2Y_2$ $P2Y_4$	Magni et al., 2015 Molliver et al., 2002 Ruan and Burnstock, 2003
SECONDARY NEURON 	$P2Y_1$ $P2Y_4$ A_1 A_{2B}	Kobayashi et al., 2006 Kobayashi et al., 2006 Borea et al., 2018 Borea et al., 2018
MICROGLIA 	$P2Y_1$ $P2Y_2$ $P2Y_6$ $P2Y_{12}$ $P2Y_{13}$ $P2Y_{14}$ A_1 A_{2A} A_{2B} A_3	Bianco et al., 2005 Shi et al., 2017 Xu et al., 2016 Koyanagi et al., 2016 Kobayashi et al., 2012 Kobayashi et al., 2012 Luongo et al., 2014 Duarte et al., 2019 Borea et al., 2018 Borea et al., 2018
ASTROCYTES 	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$ $P2Y_{11}$ $P2Y_{14}$ A_{2A} A_{2B} A_3	Shinozaki et al., 2017 Tran, 2011 Tran, 2011 Kim et al., 2011 Barragán-Iglesias et al., 2014 Kinoshita et al., 2013 Borea et al., 2018 Borea et al., 2018 Borea et al., 2018
OLIGODENDROCYTES 	$P2Y_1$ $P2Y_{12}$ A_{2A}	Agresti et al., 2005 Amadio et al., 2014 Borea et al., 2018
SATELLITE GLIAL CELLS 	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$ $P2Y_{12}$ $P2Y_{14}$	Magni et al., 2015 Magni et al., 2015 Villa et al., 2010 Ceruti et al., 2008 Wang et al., 2018 Kobayashi et al., 2006

Figure 3

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