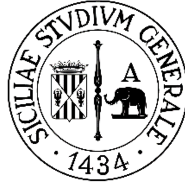

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IONOTROPIC PURINERGIC RECEPTORS AND THEIR ROLE IN MICROGLIA-MEDIATED NEUROINFLAMMATION

Michele Barraco

Tutor: Prof. Claudio Bucolo

Co-tutor: Prof. Mariangela Chisari

University of Catania
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Abstract

Neuroinflammation is the most common phenomenon involved in the progression of neurodegenerative diseases, mainly mediated by microglia. It is known that purinergic receptors are involved in such conditions, observations also supported by increased ATP extracellular levels. Microglia express both purinergic ionotropic P2X4 and P2X7 receptors. The latter has been extensively studied as main player in inflammatory response mediated by microglia. However, since glutamate extracellular levels are similarly increased in neuroinflammation and metabotropic glutamate $mGlu_5$ receptor is expressed in these cells, an interaction between these two receptor subtypes has been studied in the first part of this project. Moreover, since during neuroinflammation neuroactive steroids are known to play a role, effects in the modulation of both P2X4 and P2X7 receptors has been investigated in the second part of this study. Several cholesterol metabolites, such as testosterone butyrate, allopregnanolone, 17β-estradiol and 25-hydroxycholesterol, were tested. Taken together, data reported here provide new insights in purinergic current modulation in microglia cells, suggesting new interactions in signal transduction pathways and structure-activity relationship of molecules that together will help to develop new drugs for neuroinflammation treatment.

Keywords

Purinergic ionotropic P2X4 and P2X7 receptors; metabotropic glutamate $mGlu_5$ receptor; neuroactive steroids; oxysterols; neuroinflammation; microglia

General introduction

Microglia and functions in the central nervous system

Microglia are resident macrophages in the central nervous system (CNS) (Norris and Kipnis, 2019), making the innate immune cells in this tissue. Among other cells present in this area, they are less numerous than other cell types (~ 10% of the total amount) (Frost and Schafer, 2016). They originate from myeloid progenitors in the yolk-sac, migrating to the brain and spinal cord during early stages of embryonic development (Ginhoux et al., 2013). In adulthood, microglia cells change morphology based on their functions (Olah et al., 2011). They show a ramified phenotype with several processes protruding from the main body cell during the resting state, also known as patrolling microglia, carrying out a surveillance role in the CNS (Verdonk et al., 2016) (Fig. 1). Beside this role, resting microglia exert a control in both neuronal proliferation and differentiation (Deierborg et al., 2010). In the brain, especially during development, the number of synapses might be higher than those required, so the unused ones can be removed (Kierdorf and Prinz, 2017). This process, also known as synaptic pruning, is mediated by microglia following neuronal secretion of signaling molecules, such as chemokine fracktalkine/CX3CL1, acting on microglial receptors (CX3CR1 in this case), as chemoattractant signal that help microglia to identify synapses (Paolicelli et al., 2011; Pawelec et al., 2020). Moreover, microglia, as well as astrocytes, contact endothelial cells participating to the integrity of blood-brain barrier (BBB) (Dudvarski Stankovic et al., 2016; Ronaldson and Davis, 2020).

The major role of microglia is observed during the inflammatory response, where these cells switch to an activated state and modify their morphology, loosing membrane processes and gaining an amoeboid phenotype (Salvi et al., 2017). Activated microglia states can be distinguished in M1 and M2 phenotypes (Fig. 1), based on mediators released in the brain.

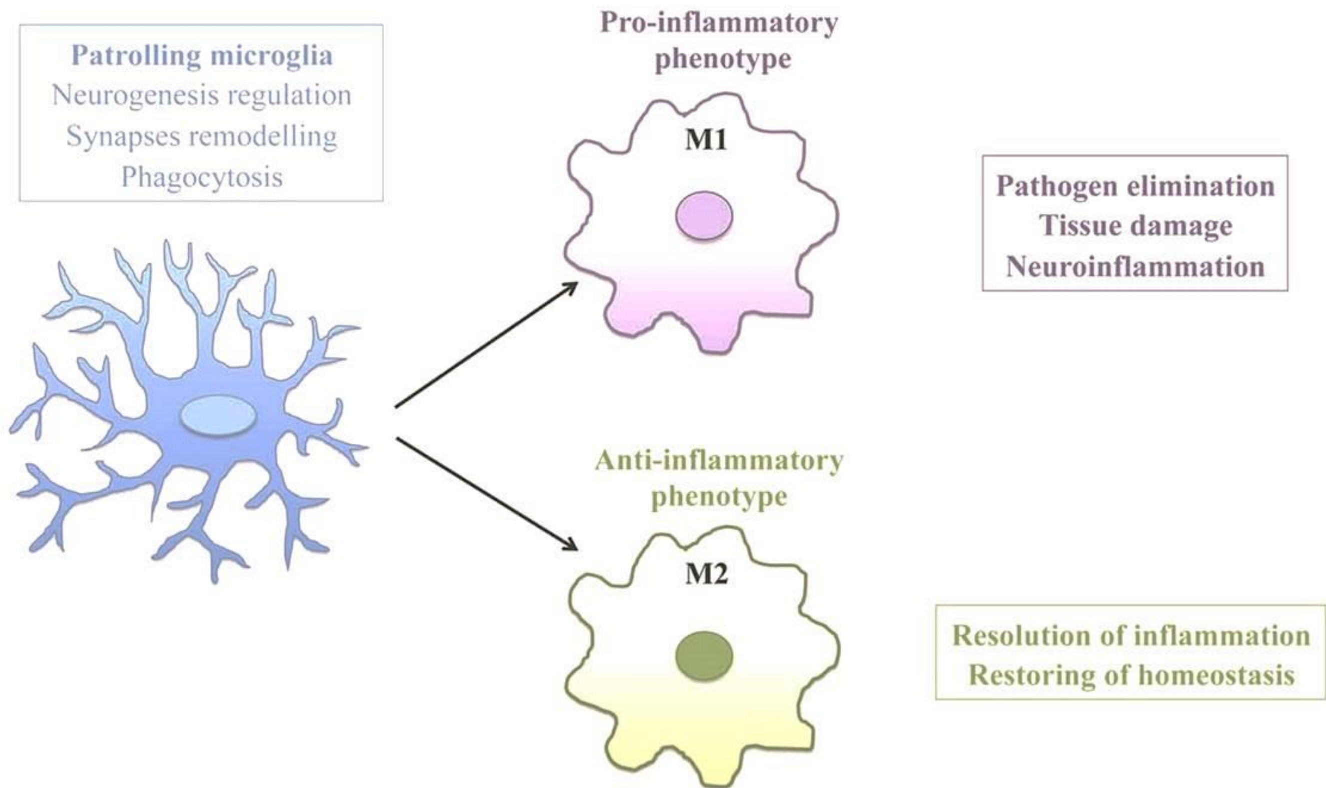

Figure 1. *Overview of microglia morphology changes from the resting state (patrolling microglia) to activated M1 and M2 phenotypes, pro and anti-inflammatory, respectively (modified from Salvi et al., 2017).*

M1 phenotype is promoted by different toxic insults in the brain, such as damaged or dying neurons that release soluble mediators belonging to the complement factor family (McGeer et al., 2005), promoting microglia migration to the site of such release (Suzumura, 2013) and afterwards facilitating phagocytosis (Colonna and Butovsky, 2017). Other signals that activate microglia are components of bacterial cell wall or toxins, such as peptidoglycans and lipopolysaccharide (LPS), recognized from microglia by membrane-bound Toll-like receptors (TLRs) (Cunha et al., 2016). Microglia M2 phenotype is mainly facilitated during release of anti-inflammatory mediators by other cells and promote brain homeostasis by resolving inflammation. Moreover, microglia in the activated state

synthetize and release pro-inflammatory mediators such as reactive oxygen species (ROS), tumor necrosis factor α (TNFα), interferon γ (IFNγ) and interleukin (IL)-1 β (Hanisch, 2002).

To date, it is well-established that the inflammatory response mediated by microglia is a hallmark of several neurodegenerative diseases, such as Alzheimer's (Selkoe and Hardy, 2016), Parkinson's (Qian et al., 2010) and Huntington's (Crotti et al., 2014). As consequence, all these agents released by persistent microglial activation lead towards a detrimental microenvironment, that might affect even the viability of healthy cells (Chew et al., 2006). Several neurotransmitters are present in the CNS extracellular space in low concentrations but during the neuroinflammation these levels increase (Idzko et al., 2014; Haroon et al., 2017). This is the case of glutamate and adenosine triphosphate (ATP) (Vesce et al., 2007; Fiebich et al., 2014). Microglia cells express both glutamatergic and purinergic receptors that are shown to have several implications in microglia functions (Jesudasan et al., 2021), including the inflammatory response.

Purinergic transmission

The nucleotide ATP is the most important molecule involved in cell metabolism (Erecinska and Silver, 1989). Despite this function, Burnstock's pioneering work provided evidences about the possibility that ATP can activate receptors expressed by neurons and glial cells (Burnstock, 1972), suggesting a role as neurotransmitter. The extensive research over the past decades (Edwards and Gibb, 1993; Wieraszko, 1996; Smith et al., 2001; Vultaggio-Poma et al., 2022), confirmed Burnstock's idea. ATP can be released from several cell types, including neurons, astrocytes and microglia (Butt, 2011), by many different mechanisms (Giuliani et al., 2019). As other neurotransmitters, ATP molecules are accumulated in vesicles at high concentrations by the action of vesicular nucleotide transporter (VNUT) (Miras-Portugal et al., 2019) and they are released in the synaptic cleft in a Ca^{2+}-dependent manner (Praetorius and Leipziger, 2009). In astrocytes and microglia, ATP can be released through

channels permeable to either cations or anions (Taruno, 2018), connexins (Lohman and Isakson, 2014), lysosomal exocytosis (Imura et al., 2013), specific ATP binding cassette (ABC) transporters (Gibson et al., 2012), and purinergic ionotropic receptor subtype 7 (Brandao-Burch et al., 2012) (Fig. 2).

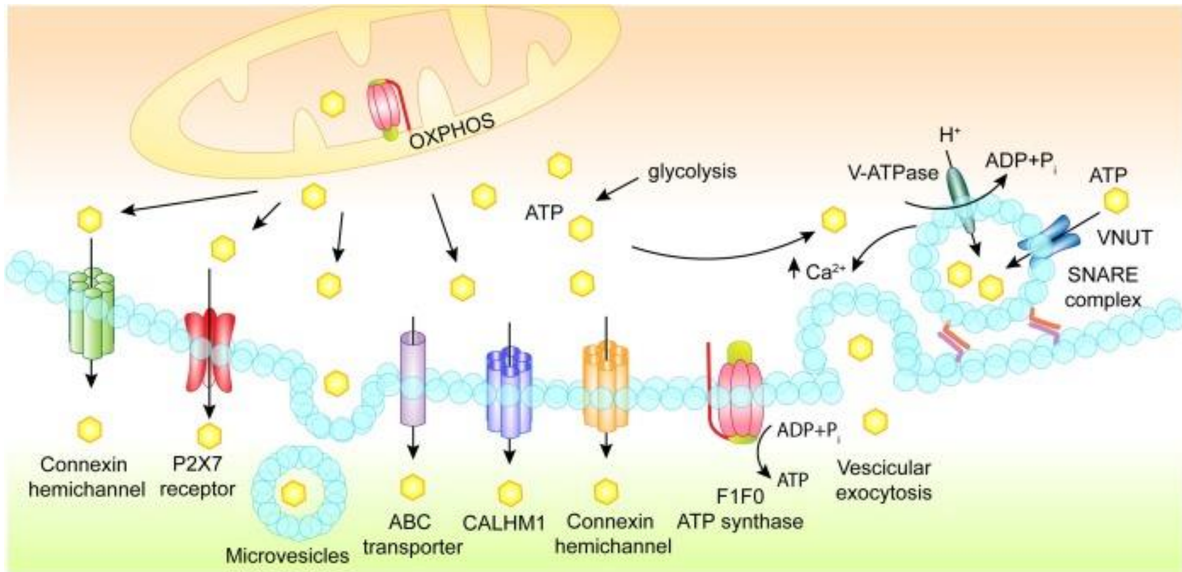

Figure 2. *Overview of different mechanisms involved in ATP release, as described in the text (Giuliani et al., 2019).*

In the extracellular space, ATP is hydrolyzed by ectonucleotidase CD39 in ADP and AMP, which is transformed in adenosine by the ectonucleotidase CD73 (Yegutkin, 2008). Adenosine and ATP can act on specific receptors belonging to the superfamily of purinergic receptors, divided in P1 and P2 receptors (Fredholm et al., 1997). Metabotropic P1 receptors are activated by adenosine and they are classified in several subtypes (A1, A2a, A2b and A3) expressed in both neurons and glial cells (Borea et al., 2018). On the other hand, ATP acts on P2 receptors, divided in G-protein coupled receptor (P2Y) and ligand-gated ion channels (P2X) receptors. Among metabotropic P2Y receptor, several subtypes are Gq-coupled such as P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11, whereas other subtypes are Gi-coupled,

such as P2Y12, P2Y13 and P2Y14 (Abbracchio et al., 2008). Purinergic receptors are expressed in several tissues and cell types in the human body. Microglia express both metabotropic and ionotropic purinergic receptors that are involved in several actions. Metabotropic P2Y6 receptor mediates the activation of phagocytosis (Inoue et al., 2006), while the P2Y12 receptor in response to ATP stimulates the chemotaxis of microglia (Honda et al., 2001). Furthermore, metabotropic P2Y12 and P2Y13 receptor subtypes seem to be involved in the microglia activation state, promoting the release of pro-inflammatory mediators, such as IL-1β, IL-6 and TNF-α (Liu et al., 2017). On the other side, P2X receptors are classified in 7 subtypes (P2X1-7), formed by three subunits that surround a non-selective cation channel, permeable to Na^+, K^+, and Ca^{2+} (Peverini et al., 2018) (Fig. 3).

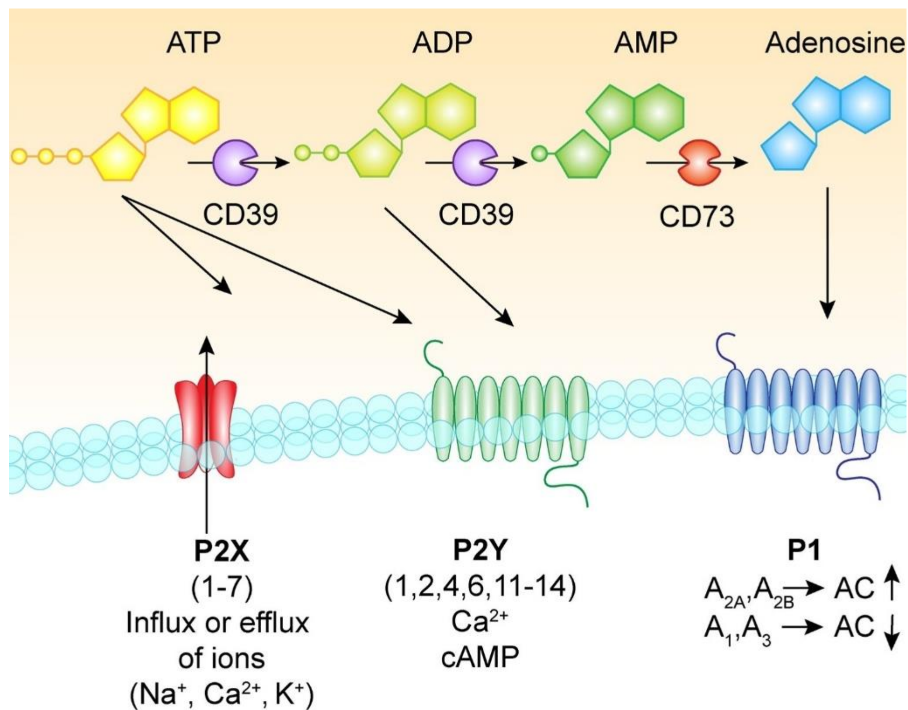

Figure 3. *ATP degradation by ectonucleotidase (as indicated, upper panel) and purinergic receptor subtypes with indication of selective endogenous agonists (bottom panel, modified from Giuliani et al., 2019).*

All P2X receptors are assembled as homotrimers but some of them can also form heteroreceptors, such as P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, P2X4/6, P2X4/7 (Burnstock, 2007; Saul et al., 2013). After resolving the crystal structure of the zebrafish P2X4 receptor (Kawate et al., 2009), it was possible to identify the ATP binding site in each subunit in the extracellular receptor portion (Hattori and Gouaux, 2012). Moreover, both N- and C-terminus are expressed intracellularly, and they modulate signal transduction pathways (Kaczmarek-Hájek et al., 2012) and help stabilizing the receptor in the plasma membrane (Chaumont et al., 2004), respectively. P2X receptor activation in the CNS elicits several outcomes, such as neurotransmitter release from neurons (Gu et al., 1997), vasoconstriction of cerebral vessels elicited by endothelial cells (Lewis et al., 2000), inflammatory response by astrocytes (Franke et al., 2001) and microglia (Verkhratsky et al., 2012). Among P2X receptors, the P2X7 subtype plays a key role in microglia-mediated neuroinflammation (Adinolfi et al., 2018; Sophocleous et al., 2022).

Ionotropic P2X7 receptor, a well-known player in neuroinflammation

Ionotropic P2X7 receptor is the only among this family that shows a low affinity for ATP, and high concentrations (> 100 μM) are needed to activate it (Roberts et al., 2006). This receptor has a peculiar feature about its activation. It has been shown that upon sustained P2X7 receptor stimulation with the endogenous agonist, the pore of this receptor enlarges, becoming permeable also to big inorganic molecules, such as the cation N-methyl-D-glutamine (NMDG) and the fluorescent dye YO-PRO-1 (Pelegrín, 2011). Several studies highlighted the P2X7 receptor contribution in microglia function. TNF-α release by these cells has been associated to P2X7 receptor activation and, despite the pro-inflammatory nature of this cytokine, a neuroprotective effect has been shown in glutamate-induced toxicity (Suzuki et al., 2004). However, to date, it is well-known the role of P2X7 receptor in both activation and microglia-mediated neuroinflammation (Sanz et al., 2009; Monif et al., 2010;

Burnstock, 2016), therefore associating this receptor to several brain diseases. For instance, overexpression of P2X7 receptor is found in activated microglia of multiple sclerosis and amyotrophic lateral sclerosis spinal cord (Yiangou et al., 2006), and this is thought to promote oligodendrocytes death. P2X7 receptor activation promotes proliferation and chemiotaxis in several tumors (Adinolfi et al., 2019), such as human glioma (Ji et al., 2018), esophageal squamous cell carcinoma (Santos et al., 2017), pancreatic ductal adenocarcinoma (Giannuzzo et al., 2015), and human breast cancer cells (Jelassi et al., 2013). Moreover, P2X7 receptor is also involved in retinal degeneration such as age-related macular degeneration, optic neuropathy, and diabetic retinopathy (Platania et al., 2022). The neuroinflammatory role of this receptor has been shown through several mechanisms. High ATP concentrations activate the P2X7 receptor which in turn triggers the multimerization of elements forming the inflammasome (caspase-1, ASC and NLRP3), within the cytosol of microglia cells. The mature inflammasome induces the transition of pro-IL-1β to its mature form, which can either freely permeate the plasma membrane or being accumulated in lysosomes and then released (Dubyak, 2012). Also, it has been shown that P2X7 receptor activation induces the formation and subsequent release of microvesicles (MVs) (Bianco et al., 2005). Their formation is induced upon P2X7 receptor activation and the involvement of p38 and mitogen-activated protein kinase (MAPK) cascade, which in turn activate the acid sphingomyelinase enzyme (A-SMase) (Bianco et al. 2009). It has also been shown that MVs contain mature IL-1β and this additional secretory pathway represents an extra way to the inflammasome release of this pro-inflammatory cytokine, that exacerbate neuroinflammation (Turola et al., 2012). The IL-1β exerts its detrimental role increasing glutaminase levels in neurons, inducing a greater glutamate synthesis, responsible for cytotoxicity and neuronal death (Ye et al., 2013). Is important to note that in IL-1β knock-out animals the rate of neuronal death was similar to control animals, suggesting that this cytokine is not required in the neurotoxic effect mediated upon P2X7 activation in microglia (Brough et al., 2002). Another well-established role of this receptor in the

inflammatory response mediated by microglia is due to production and release of radical species, such as nitric oxide (NO) by the activation of inducible nitric oxide synthase (Gendron at al., 2003), and ROS through activation of NADPH oxidase (Parvathenani et al., 2003; Bartlett et al., 2013).

Ionotropic P2X4 receptor and its debating role in neuroinflammation

The P2X4 receptor has a high affinity for ATP and, in contrast to P2X7 receptor, the concentration range for its activation by the endogenous agonist is lower (1-100 μM). Moreover, P2X4 receptor shows a weak current desensitization during prolonged ATP application (Coddou et al., 2011). In the CNS, P2X4 receptor is involved in physiological and pathological phenomena. In hippocampal neurons, activation of P2X4 receptor enhances synaptic strength and influences the long-term potentiation (LTP) (Baxter et al., 2011). On the other hand, an increased P2X4 receptor expression and functions are responsible for neuronal death mediated by exposure to the β-amyloid peptide (Varma et al., 2009). Interestingly, it has been shown that functional P2X4 receptors are expressed in the plasma membrane, where they can be activated by ATP, but also in intracellular organelles, such as lysosomes (Cao et al., 2015). The receptor trafficking to plasma membrane, favored by an increase in lysosomal pH (Murrell-Lagnado and Frick, 2019), increases the number of receptors available for ATP activation (Qureshi et al., 2007). The trafficking rate varies among different cell types in the CNS and it is more prominent in microglia in the activated state (Boumechache et al., 2009). Therefore, the increased expression of P2X4 receptors in the plasma membrane suggests an important feature in pathological conditions affecting the CNS (Domercq et al., 2013). In contrast, upon P2X4 activation in microglia, the exocytotic release of BDNF is allowed by p38-MAPK and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Trang et al., 2009). Upon BDNF release, TrkB receptor activation induces phosphorylation of several intracellular proteins, such as the cAMP responsive element binding protein (CREB). This nuclear transcription factor promotes the activation of genes

involved in the formation of new synapses (Yoshii and Constantine-Paton, 2010). This pathway has beneficial effects in the brain since it supports neuronal viability (Oliveira et al., 2012). Consistently, the beneficial activity by BDNF released upon P2X4 activation in microglia consists also in the remyelination of damaged axons and the differentiation of oligodendrocytes (Di Virgilio and Sarti, 2018; Domercq and Matute, 2019; Su et al., 2019).

In neuropathic pain, many studies suggested a detrimental outcome mediated by microglia and the BDNF release upon P2X4 receptor activation (Tsuda et al., 2003; Ulmann et al., 2008; Lalisse et al., 2018). P2X4 receptor expression increases in activated spinal microglia (Tsuda et al., 2005; Tsuda et al., 2013) and the activation of TrkB receptor expressed in sensory neurons by BDNF determines an increase in intracellular Cl^- concentration, mediated by the downregulation of K^+-Cl^- cotransporter (KCC2) (Rivera et al., 2002). Such condition is responsible for a depolarizing shift in the anion reversal potential in lamina I neurons in spinal cord, converting the $GABA_A$ and glycine transmissions of these cells from inhibitory to excitatory (Coull et al., 2005). Therefore, P2X4 receptor antagonists have been proposed as a therapeutic treatment for neuropathic pain (Beggs et al., 2012), but the complexity of effects mediated by this receptor require a deeper knowledge on its involvement in CNS diseases.

Among other receptors expressed in microglia, glutamate receptors might be involved in neuroinflammation, as extracellular concentrations of glutamate are increased during this condition.

Metabotropic mGlu$_5$ receptor, beneficial or detrimental actions in neuroinflammation?

Glutamate is the main excitatory neurotransmitter in the CNS. Neurons release glutamate in the synaptic cleft (reaching 1 mM in concentration) with a Ca^{2+}-dependent mechanism that increases the fusion of the glutamate-containing vesicles in the cytoplasm with the plasma membrane (Hackett and Ueda, 2015). Afterwards, extracellular glutamate levels are reduced (< 20 nM) (Moussawi et al., 2011) by specific excitatory amino acid transporters (EAATs) expressed in both glial and neuronal cells

(Malik and Willnow, 2019). Upon release, glutamate activates different receptor subtypes, classified as ionotropic receptors (amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA; N-methyl-D-aspartate, NMDA; kainic acid, KA) and metabotropic (mGlu) receptors (Bleich et al., 2003). The structure of the first group of receptors consists in: N-terminus domain and a ligand binding domain for the agonist, in the extracellular side; four transmembrane subunits forming the channel permeable to cations and a C-terminus domain within the cell (Twomey et al., 2018). In neurons, ionotropic receptors elicit excitatory postsynaptic currents (EPSCs), plasma membrane depolarizing events that leads to the action potential onset (Nicoll and Malenka, 1999). In the hippocampus, ionotropic glutamate receptor subtypes are involved in synaptic plasticity, supporting new synapse formation, as well as the LTP, a process promoting learning and memory (Bliss and Collingridge, 2013). Metabotropic glutamate receptors are divided in three classes: group I ($mGlu_1$ and $mGlu_5$) includes receptors coupled to $G_{q/11}$; group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) receptors coupled to $G_{i/o}$ (Conn, 2006). Functional mGlu receptors exist as dimers and group I receptors can form homodimers (e.g., $mGlu_5$-$mGlu_5$) or heterodimers (e.g., $mGlu_1$-$mGlu_5$) within the same group subtypes, whereas receptors belonging to group II and III can be found as homodimers or intergroup heterodimers (e.g., $mGlu_2$-$mGlu_4$) (Doumazane et al., 2011). In general, a mGlu receptor shows a venus fly trap domain (VFTD) in the extracellular side, consisting in two lobes forming a cleft, where glutamate binds. The VFTD is linked to the seven transmembrane domains by a cysteine-reach domain (CRD) and the C-terminus is located intracellularly (Nicoletti et al., 2011) (Fig. 4).

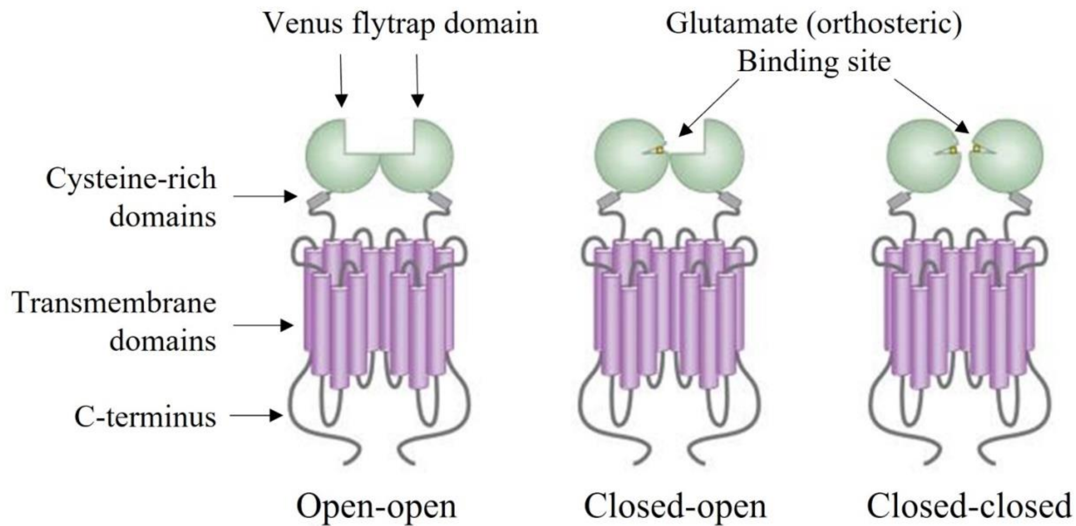

Figure 4. *Schematic representation of a metabotropic glutamate receptor (modified from Colleen et al., 2010).*

Metabotropic glutamate receptors are expressed in microglia, and they have roles in both physiological and pathological conditions (Liu et al., 2016). It has been shown that microglial $mGlu_2$ and $mGlu_3$ receptors have an opposite role in neuronal viability. Activation of $mGlu_2$ receptors has been suggested as a signal promoting the microglial release of pro-inflammatory TNF-α (Taylor et al., 2005). In a multiple sclerosis model, antagonizing this receptor subtype or activating $mGlu_3$ receptors, both expressed in microglia, have implications in preventing neurotoxicity (Pinteaux-Jones et al., 2008). Moreover, activation of group III receptors ($mGlu_4$, $mGlu_6$ and $mGlu_8$) it has been shown to have neuroprotective effects during the inflammatory state of microglia (Taylor et al., 2003). Furthermore, it has been shown that glutamate activation of both ionotropic and metabotropic receptor subtypes in microglia induces the production of neurotrophic factors, such as BDNF, glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF), highlighting the pivotal role of these cells in neuronal survival and growth (Liang et al., 2010; Tremblay et al., 2010).

About $mGlu_5$ receptor, it is known to be expressed in several cells in the CNS, including microglia (Drouin-Ouellet et al., 2011), but its role in the modulation of the inflammatory response is not clearly defined. Upon its stimulation, the Gq-mediated mechanism, with production of inositol triphosphate (IP3) and diacylglycerol (DAG) induced by phospholipase C (PLC) on phosphatidylinositol 4,5-bisphosphate, is activated (Niswender and Conn, 2010). However, other signal transduction pathways of its activation have been reported, such as an increase in AKT phosphorylation (Bhat et al., 2021) and the interaction with scaffold proteins belonging to Homer family (Fagni et al., 2002). Moreover, different studies highlighted the possibility that $mGlu_5$ receptors coupled to other receptors activated by glutamate (NMDA) or adenosine (A2A), expanding chances of signal transduction mechanisms (Bird and Lawrence, 2009).

It has been shown that in primary culture of microglia, the treatment with LPS induced the activation of these cells and such activation was reduced by stimulation with selective agonist of group I receptors (Farso et al., 2009). Also, the selective stimulation of $mGlu_5$ receptor reduced levels of pro-inflammatory mediators, such as NO, ROS and TNF-α, in microglia cells pre-exposed to LPS (Byrnes et al., 2009). Therefore, these evidences support a positive effect of this receptor in the modulation of neuroinflammatory response of microglia. In contrast, further evidences demonstrate the pro-inflammatory role of $mGlu_5$ receptor in microglia. Using immunocytochemistry and an antibody-based fluorescence resonance energy transfer (FRET) technology, it has been shown the co-localization of LPS and $mGlu_5$ receptors in microglia, suggesting that this receptor is a target for microglial activation by the bacterial toxin (Liu et al., 2014). Furthermore, it has been shown that $mGlu_5$ activation increase the P2X7-dependent release of microvesicles containing pro-inflammatory microRNAs (Beneventano et al., 2017).

Beside neurotransmitters, other endogenous compounds such as neuroactive steroids (NSs), produced by cholesterol metabolism, are involved in neuroinflammation (Yilmaz et al., 2019).

Cholesterol metabolism and steroids in the brain

Cholesterol is the major lipid of cell membranes that regulates membrane fluidity, ion permeability, synaptogenesis and myelin formation (Mauch et al., 2001; Saher et al., 2011; Ho et al., 2022). Approximately the 23% of the whole body's cholesterol is synthesized in the CNS (Dietschy and Turley, 2004). Cholesterol chemical structure consists in four rings, indicated from A to D, with a hydroxyl group in C-3 position and a long 6-methylheptan-2yl side chain at C-17 position (Nes, 2011) (Fig. 5). Several cell types, including neurons and astrocytes, express enzymes acting on cholesterol to obtain different metabolites (Pfrieger and Ungerer, 2011). The enzyme CYP46A, is an isoform of cytochrome P450 mainly expressed in neurons, which transforms cholesterol in one of the most abundant brain oxysterols, known as 24-(S)-hydroxycholesterol (24-(S)-HC) (Ramirez et al., 2008). On the other hand, CYP27A1 is found in neurons, astrocytes and microglia cells and it catalyzes the synthesis of 27-hydroxycholesterol (27-HC), another oxysterol (Gilardi et al., 2009). Cholesterol-25-hydroxylase (CH25H), CYP46A, CYP27A1 and CYP3A4 are enzymes involved in the synthesis of 25-hydroxycholesterol (25-HC) (Mutemberezi et al., 2016) (Fig. 5). Moreover, cholesterol is the substrate of the cytochrome P450 side-chain cleavage (P450scc) and this enzyme mediates the first step in the synthesis of both NSs and steroid hormones. The first metabolite produced by P450scc is pregnenolone that is transformed in allopregnanolone, one of the most studied NSs, by 3β-hydroxysteroid dehydrogenase (3β-HSD), 5α-reductase and 3α-hydroxysteroid oxidoreductase (3α-HSOR). On the other hand, pregnenolone can be the substrate of other enzymes, such as P450c17, 3β-HSD, 17β-HSOR, leading to the synthesis of the steroid hormone testosterone, precursor of 17β-estradiol obtained after aromatase activation (Schumacher et al., 2000) (Fig. 5).

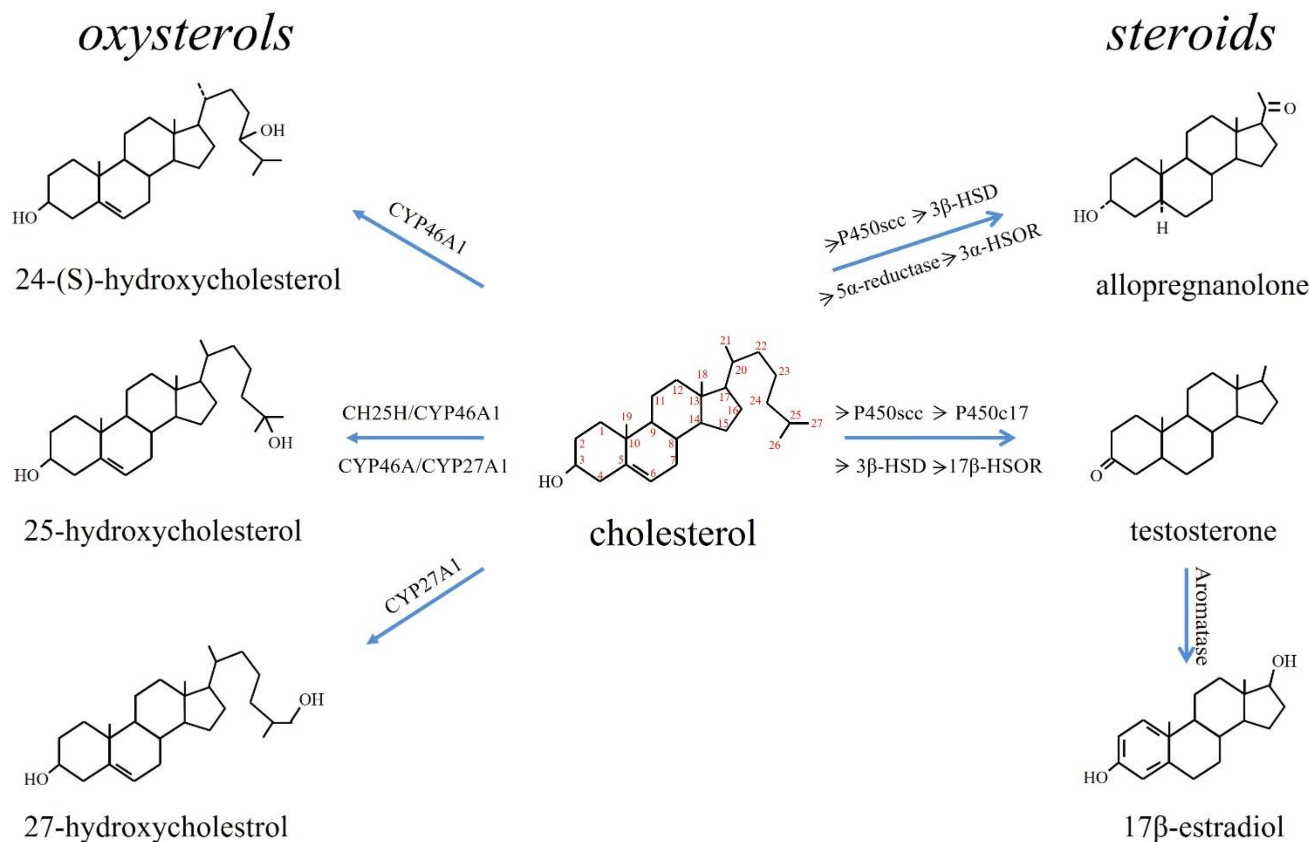

Figure 5. *Cholesterol metabolism in the brain. Cholesterol chemical structure, enzymes and metabolites are indicated.*

Evidences accumulated over the last decades indicate that cholesterol metabolites can affect brain functions acting on different receptor classes via genomic and non-genomic pathways. Oxysterol 24-(S)-HC, in the brain, activates the nuclear hormone liver X receptors (LXRs) (Peet et al., 1998), inducing gene regulation of protein involved in cholesterol homeostasis (Wang et al., 2002). Oxysterols and NSs are able to affect the excitability of the CNS, acting on $GABA_A$ (Lambert et al., 2003; Fodor et al., 2005) and NMDA receptors (Korinek et al., 2011; Paul et al., 2013). $GABA_A$ receptor potentiation suggests that these compounds might be useful in the treatment of brain pathologies with a hypofunction of the GABAergic system, such as depression (Zorumski et al., 2019), anxiety (Longone et al., 2011) and epilepsy (Lévesque et al., 2017). As evidence of such clinical use, the

allopregnanolone-based treatment brexanolone has been approved in 2019 by the Food and Drug Administration (FDA) for postpartum depression (PPD) treatment (Scott, 2019).

On the other hand, NMDA receptor modulation suggests that cholesterol metabolites might be a good candidate useful to compensate dysfunctions of glutamatergic system in neuropsychiatric disorders (Tang et al., 2020). Additional roles for cholesterol metabolites are relevant in neurogenesis and inflammation. The NS dehydroepiandrosterone (DHEA) increases the birth of new granule neurons in the rat dentate gyrus of the hippocampus (Karishma and Herbert, 2002), but the mechanism is not yet fully elucidated. This finding potentially extends NS functions to learning and memory processes. About steroid hormones, it has been shown that 17β-estradiol inhibits the Aβ-induced neuroinflammation in microglia, probably due to the inhibition of nuclear factor kappa-B (NF-κB) (Yun et al., 2018). Moreover, this steroid hormone is shown to promote the phagocytosis of Aβ plaques activating the estrogen receptor β expressed in microglia (Li et al., 2002). Taken together, these observations support the important role of cholesterol metabolites in brain homeostasis and pathological conditions.

Aim of the project

In the last decades, many studies have been performed to better understand mechanisms underlying brain neuroinflammation and the role of microglia (Liu et al., 2003; Perry et al., 2010; Woodburn et al., 2021). All these efforts aimed to find new strategies for the treatment of neurodegenerative diseases, that share inflammation as a hallmark (Fu et al., 2018; Lee et al., 2019). During neuroinflammation, many mediators are involved (Yang and Zhou, 2018) and among them, ATP and glutamate extracellular levels are found increased compared to physiological conditions (Byrnes et al., 2009; Di Virgilio et al., 2017). The main interest during this project was to elucidate the function of P2X7 receptor in microglia, a well-known player in neuroinflammation. To date, the separate contribution of ATP and glutamate in the modulation of microglia function has been highlighted (Czapski and Strosznajder, 2021; Engel et al., 2021), but it cannot be excluded an interaction among purinergic and glutamatergic receptors during microglia-mediate inflammation.

In order to explore a link between purinergic and glutamatergic transmissions in microglia, part of the project aimed to evaluate the potential crosstalk between ionotropic purinergic P2X7 receptor and metabotropic glutamate $mGlu_5$ receptor, using a microglia murine cell line, BV2 cells, as experimental model.

Several endogenous molecules such as cholesterol metabolites are shown to act on ionotropic receptors ($GABA_A$ and NMDA) (Ziolkowski et al., 2021), modulating current elicited upon selective activation of these ligand-gated ion channels. Recent evidences show that cholesterol metabolites affect the activity of P2X receptor, such as neuronal P2X2 receptor (De Roo et al., 2003) and P2X4 receptor in a heterologous system (Codocedo et al., 2009; Sivcev et al., 2019).

Therefore, the second part of this project aimed to investigate how native P2X4 and P2X7 receptors and current elicited upon their activation in BV2 cells can be affected by different cholesterol metabolites, such as testosterone butyrate, allopregnanolone, 25-HC and 17β-estradiol. Results shown

in the following chapters highlight new mechanisms of ionotropic purinergic current modulation in microglia, useful to identify new targets to treat neuroinflammation.

Chapter I

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Purinergic ionotropic P2X7 and metabotropic glutamate mGlu$_5$ receptors crosstalk influences pro-inflammatory conditions in microglia

Mariangela Chisari[a,*], Michele Barraco[b], Claudio Bucolo[a], Lucia Ciranna[c], Maria Angela Sortino[a]

Dept. of Biomedical and Biotechnological Sciences, [a]Section of Pharmacology, and [c]Physiology, University of Catania, Italy

[b]PhD program in Neuroscience, University of Catania, Italy

*Corresponding author. Dept. of Biomedical and Biotechnological Sciences, Section of Pharmacology, University of Catania, via S. Sofia 97, 95123, Catania, Italy; email address: chisarim@unict.it

Abstract

Microglia represent the resident immune system in the brain. They mediate neuroinflammatory processes and have been described as important regulators of homeostasis in the central nervous system (CNS). Among several players and mechanisms contributing to microglial function in inflammation, ATP and glutamate have been shown to be involved in microgliosis. In this study, we focused on receptor subtypes that respond to these neurotransmitters, purinergic ionotropic P2X7 receptor and metabotropic glutamate mGlu$_5$ receptor. We found that both receptors are functionally expressed in a murine microglia cell line, BV2 cells, and we performed patch-clamp experiments to measure purinergic ionotropic P2X7 receptor ion flux in control condition and after metabotropic glutamate mGlu$_5$ receptor activation. The selective purinergic ionotropic P2X7 receptor agonist, 2′(3′)-O-(4-

benzoylbenzoyl)adenosine-5′-triphosphate (BzATP, 100 μM), elicited a robust current that was prevented by the selective purinergic ionotropic P2X7 receptor antagonist A438079 (10 μM). When BV2 cells were acutely stimulated with the selective metabotropic glutamate $mGlu_5$ agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG, 200 μM), purinergic ionotropic P2X7 receptor current was increased. This positive modulation was prevented by the selective metabotropic glutamate $mGlu_5$ receptor antagonist 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP, 1 μM). Moreover, nitric oxide synthesis elicited by purinergic ionotropic P2X7 receptor activation was enhanced by metabotropic glutamate $mGlu_5$ receptor co-stimulation. Taken together, our results suggest an important crosstalk between ATP and glutamate in inflammation. Pro-inflammatory effects mediated by purinergic ionotropic P2X7 receptor might be exacerbated by simultaneous exposure of microglia to ATP and glutamate, suggesting new pharmacological targets to modulate neuroinflammation.

Keywords

Purinergic ionotropic P2X7 receptor; metabotropic glutamate $mGlu_5$ receptor; nitric oxide; patch-clamp; inflammation; microglia

Introduction

Microglia are important regulators for brain homeostasis (Hanisch and Kettenmann, 2007), since they are responsible for synapse formation, elimination and modulation (Chung et al., 2015). However, their main function is in inflammation, when concentrations of two major neurotransmitters, ATP and glutamate, are increased in the central nervous system (CNS) extracellular space (Verkhratsky et al., 2014). Among several targets, ATP binds to ionotropic purinergic receptor (P2X), such as purinergic P2X7 receptor. This is a ligand operated ion channel formed by three subunits that, upon activation, allows non-selective cation flux (Sluyter, 2017). Compared to other ionotropic purinergic P2X

receptors, ATP affinity for purinergic P2X7 receptor is very low (Coddou et al., 2011), suggesting that this receptor recruitment is possible only in conditions where ATP extracellular levels are very high, mainly during neuroinflammation (di Virgilio et al., 2017; Oliveira-Giacomelli et al., 2021). Beside ion flux, purinergic P2X7 receptor activation leads to an increase in its own pore size (Martínez-cuesta et al., 2020), providing an extra source responsible for high extracellular ATP levels (Suadicani et al., 2006; le Feuvre et al., 2002). Moreover, in microglia, purinergic P2X7 receptor has been associated with release of interleukin (IL)-1β (Brough et al., 2002), an inflammation hallmark, and nitric oxide (NO) (Gendron et al., 2003), a molecule involved in microglia activation (Boje and Arora, 1992). Such phenomena have been implicated in several CNS disorders including epilepsy (Bhattacharya, 2018), Alzheimer's and Parkinson's diseases (Bhattacharya and Biber, 2016). On the other side, glutamate can activate several receptors either in neurons and glia. Microglia express metabotropic glutamate 5 receptor ($mGlu_5$) during neuroinflammation, but its role is still controversial in such conditions. It has been shown that NO is reduced during glutamate $mGlu_5$ receptor activation in microglia (Byrnes et al., 2009), but this receptor has also been identified as a target for lipopolysaccharide (LPS), suggesting a role in the inflammatory mechanism activated by this agent (Liu et al., 2014). Moreover, very recently, it has been shown that reducing metabotropic glutamate $mGlu_5$ receptor activation in microglia cells restores the synaptic density and prevents cognitive impairment in a mouse model for Alzheimer's disease (Spurrier et al., 2022).

In this study we explored the interaction between these two receptor subtypes in microglia-mediated neuroinflammatory processes.

In our previous work (Beneventano et al., 2017), we observed that, in microglia BV2 cell line, long-term exposure (24 h) to the selective glutamate $mGlu_5$ receptor agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG, 200 μM), positively modulates purinergic P2X7 receptor currents elicited by selective activation with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP,

100 μM). Since such effects were not present in BV2 cells exposed to LPS, we hypothesized that crosstalk between these receptors is important in a milieu promoting inflammation, but not in a well-established inflammatory condition. Therefore, in this study, we reduced the exposure time to CHPG (10 s), exploring early responses and mechanisms involved. Crosstalk between ATP and glutamate through purinergic P2X7 and glutamate $mGlu_5$ receptors, respectively, might contribute to elucidate the role of these two players in neuroinflammation.

Materials and Methods

2.1 Cell cultures: the murine BV2 cell line was cultured in 75 mm flasks in Dulbecco's Modified Eagle's medium (DMEM) with high glucose (4500 mg/l) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml-100 μg/ml) (all from Thermo Fisher Scientific, Italy). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Since this cell line undergoes rapid changes during culturing, experiments were performed in a limited number of passages in trypsin (7-12). For following experiments, cells were plated in 35 mm dishes and maintained with supplemented DMEM medium. Glass bottom dishes (20 mm insert, 1.5 thickness; In Vitro Scientific, IBL Baustoff + Labor GmbH, Austria) were used for live cell imaging experiments.

2.2 Cell transfection: BV2 cells were transiently transfected with muscarinic acetylcholine M_3 receptor, green fluorescent protein (GFP) or pleckstrin homology domain of phospholipase δ tagged with mCherry (PHmCh) fluorescent protein. Constructs, expressed in pcDNA3.1 vector, were kindly provided by N. Gautam (Washington University in St Louis, MO, USA). Transfections were performed with Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's instructions. Experiments were performed within 24h after removing Lipofectamine 2000.

2.3 Live cell imaging: transfected cells were transferred from culture medium to a saline solution containing (in mM): 138 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES at pH 7.25.

Muscarinic acetylcholine M_3 receptor agonist, carbachol 100 μM, was applied manually after acquiring 6 images (5 s interval) to determine baseline fluorescence. Cells were imaged with Axio Observer Z1 microscope, EC Plan Neofluar 40X/0.75 objective, Axiocam MRM and appropriate filter sets (Carl Zeiss). For clarity, brightness and contrast were adjusted with software ImageJ (Rasband, W.S., ImageJ, U.S. NIH).

To test metabotropic glutamate $mGlu_5$ receptor function in BV2 cells, Fluo-4 dye was added in cultured cells according to manufacturer's instructions. After washing the dye, cells were transferred to saline solution already described in this section and selective metabotropic glutamate $mGlu_5$ receptor agonist, CHPG 200 μM, was added manually. The same equipment was used for these experiments and, also in this case, 6 images (5 s interval) were acquired to determine baseline Fluo-4 fluorescence. Fluorescence analysis was again performed with the software ImageJ.

2.4 Electrophysiology: patch-clamp recordings were performed in whole-cell configuration at room temperature (RT). Cells were transferred from culture medium to an extracellular recording solution as mentioned in imaging section. Purinergic ionotropic P2X7 receptor current was recorded in a low divalent solution (0.1 mM $CaCl_2$ and no $MgCl_2$). Patch pipettes (4–5.2 MΩ) were filled with a solution containing (in mM): 115 K gluconate, 20 KCl, 2 EGTA, 10 HEPES at pH 7.25. Cells were clamped at -80 mV. Saline solutions and drugs (BzATP 100 μM, A438079 10 μM, CHPG 200 μM, 3-((2-Methyl-4-Thiazolyl)ethynyl)pyridine – MTEP- 1 μM, as indicated in corresponding figures) were applied with a computer-controlled multibarrel, gravity-driven local superfusion system (Automate Scientific, USA).

Measuring the junction current changes of an open tip pipette, the estimation of the superfusion system exchange times was 126.2 ± 9 ms. An EPC7 Plus amplifier (HEKA Elektronik, Germany) was used for patch-clamp experiments in voltage-clamp configuration. Data acquisition was performed at 5 kHz, filtered at 1 kHz using a 7-pole Bessel filter and digitized with Digidata 1440A (Molecular Devices, United States).

2.5 Nitrite determination by the Griess assay: cells plated in 24-well plate were placed in the saline external solution used for patch-clamp experiments. Treatments with BzATP 100 μM and CHPG 200 μM lasted for 30 min. For extracellular nitrite quantification, 100 μL of supernatant was taken from each condition and added to 100 μL of Griess reagent in a 96-well plate. After 15 min at RT in the dark, the absorbance was measured at 540 nm using a plate reader (Varioskan Flash, ThermoFisher Scientific). A nitrite standard calibration curve was also prepared using nitrite standards from 1 to 100 μM.

2.6 Data analysis and statistical procedures: images of living cells were analyzed using ImageJ software. Images were acquired over time and then stacked together. Cells in movies, obtained by such method, were analyzed by line scanning method. Lines were positioned in areas not including the nucleus to show the fluorescence changes in plasma membrane and cytosol before and after receptor stimulation. Fluorescence profiles of cells expressing PHmCh in presence or absence of muscarinic acetylcholine M_3 receptors, were normalized to the minimum value observed before carbachol stimulation (basal conditions). The same method was used to evaluate fluorescence in Fluo-4 experiments. Electrophysiology data acquisition and analysis were performed using pCLAMP 10 software (Molecular Devices, Crisel Instruments, Italy). Peak current (I) was measured for each trace obtained by the recording protocol and the ratio with the corresponding cell membrane capacitance (C_m) was evaluated to obtain normalized current (current density, pA/pF), thus taking in consideration cell size variability. Data from different experiments were initially processed with Microsoft Excel and presented as means ± S.E.M. Statistical significance was determined using a Student's two-tailed t-test or ANOVA analysis followed by the appropriate multiple comparisons test, as indicated in corresponding figure legends. Plots, bar diagrams, statistical analysis, and figure preparations were finalized with GraphPad Prism (GraphPad Software, USA).

2.7 Drugs: drugs were from Sigma-Aldrich (Italy) except for Fluo-4 obtained from Life Technologies (Italy), A438079, CHPG and MTEP obtained from Tocris (Bio-Techne, Italy).

Results

In order to test our hypothesis about purinergic ionotropic P2X7 receptor current modulation by metabotropic glutamate $mGlu_5$ receptor activation, we first assessed functional expression of both receptor subtypes in our experimental model, BV2 microglia cells. Metabotropic glutamate $mGlu_5$ receptor has been reported to be expressed in BV2 cells (Loane et al., 2009). In our model, we confirmed that this receptor is functional using live cell imaging experiments. Cells were loaded with Fluo-4 calcium chelator, an indicator that increases in fluorescence in presence of cytosolic Ca^{2+}. Fluorescence changes were evaluated before and after metabotropic glutamate $mGlu_5$ receptor activation using the selective agonist, CHPG 200 μM. The Fluo-4 fluorescence increase (ΔF) was measured at the peak fluorescence (during receptor activation) normalized to basal fluorescence (before receptor activation). Images were acquired with 5 s interval. Normalized basal fluorescence was set as 1, while increased fluorescence detected after agonist administration was 1.63 ± 0.18 ($n = 6$), $p < 0.01$ by Student's t-test. To evaluate the functional expression of purinergic ionotropic P2X7 receptor, we performed patch-clamp experiments in whole-cell configuration. Cells were exposed to BzATP 100 μM, and corresponding current was observed in patch-clamp recordings (Fig. 1A, grey trace). Since it is known that purinergic ionotropic P2X7 receptor undergoes pore enlargement (Martínez-cuesta et al., 2020), we applied the selective agonist every 30 s, up to three stimulations.

As expected, second and third stimulations with BzATP elicited an increased current compared to the first stimulation (Fig. 1A, black and red traces).

To ensure that this largest current was due only to purinergic ionotropic P2X7 receptor activation and not to the contribution of other channels, we co-applied BzATP with the selective purinergic ionotropic

P2X7 receptor antagonist, A438079 10 μM. Current was inhibited in presence of the antagonist, even the larger current elicited by the third BzATP stimulation (Fig. 1A and B).

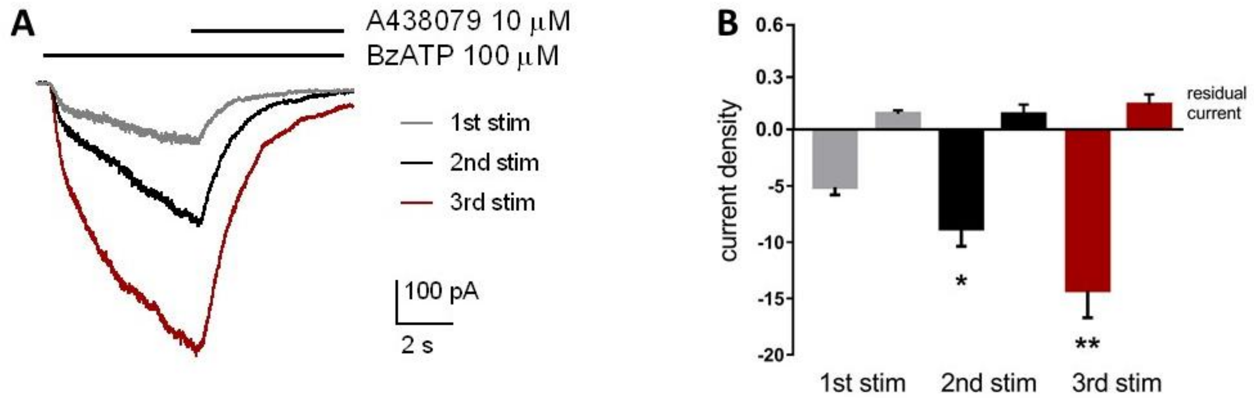

Figure 1. *Multiple stimulations of purinergic ionotropic P2X7 channel determine receptor sensitization. A. Representative traces of current elicited after multiple stimulation of purinergic ionotropic P2X7 receptor. Receptors were activated with the same concentration of the selective agonist, BzATP 100 μM, followed by the application of the selective antagonist, A438079 10 μM. BzATP was applied every 30 s. B. Bars show quantified peak current of purinergic ionotropic P2X7 receptor (negative values) as elicited in A, and the residual current (positive values) at the end of both agonist and antagonist application. Current for each cell was normalized to corresponding* C_m. *Residual current was normalized to the corresponding peak current. Bars are average ± SEM, n = 9, * $p < 0.05$, ** $p < 0.01$ according to one-way ANOVA for repeated measurements followed by Tukey's multiple comparisons test (vs 1st stimulation bar).*

We performed patch-clamp experiments to record purinergic ionotropic P2X7 receptor current in presence and absence of metabotropic glutamate $mGlu_5$ receptor activation. Based on the previous observation of increased purinergic ionotropic P2X7 receptor current after multiple agonist stimulation, experiments were carried out in different groups of BV2 cells, exposed to BzATP 100 μM alone as control group or to both agonists, BzATP and CHPG 200 μM. Cells were exposed to CHPG for 10 s to

allow metabotropic glutamate $mGlu_5$ receptor activation, followed by co-stimulation with both selective agonists. A strong current increase was observed in these cells compared to control group cells (Fig. 2A) and purinergic current quantification revealed a significant effect (Fig. 2B; $p < 0.05$ by unpaired t-test). Quantification of the C_m suggested a comparable size among cells (Fig. 2C).

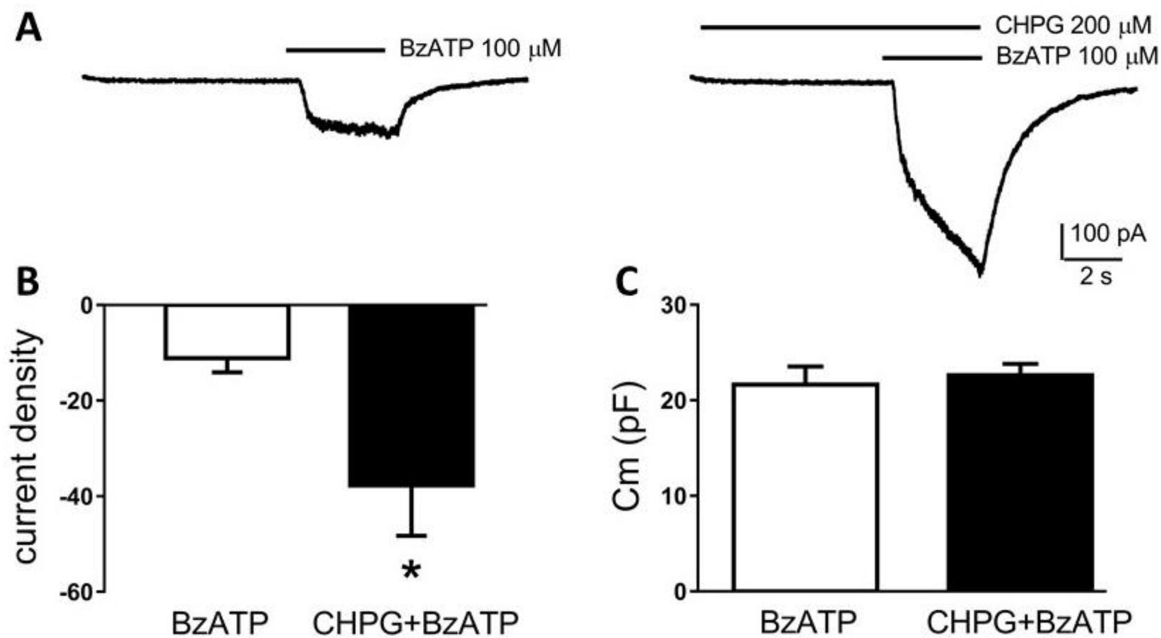

Figure 2. *Acute stimulation of metabotropic glutamate* $mGlu_5$ *receptor increases purinergic ionotropic P2X7 receptor current. A. Representative traces of current elicited in BV2 cells upon purinergic ionotropic P2X7 receptor activation with BzATP 100 µM (left panel) and pre-exposed to CHPG 200 µM for 10 s before co-application of both CHPG and BzATP (right panel). B. Bar diagram shows corresponding quantification of purinergic ionotropic P2X7 receptor current in control conditions (left bar) or after pre-application/co-application of CHPG (right bar). Current for each cell was normalized to corresponding* C_m. *C. Comparison of cell* C_m *from experiments in A and B. Bars are average ± SEM, n = 8, * p < 0.05 (unpaired t test).*

A different set of cells was used to evaluate CHPG effect on sensitized current of purinergic ionotropic P2X7 receptor (Fig. 3) using the same protocol stimulation as described in Fig. 2. Cells were stimulated up to three times by BzATP (control group) and normalized current values were compared to data obtained in cells pre-treated/co-stimulated with CHPG. Each activation in presence of CHPG showed significantly higher purinergic ionotropic P2X7 receptor current compared to control group in patched cell (Fig. 3A and B). Again, C_m did not show any differences in cells size (Fig. 3C).

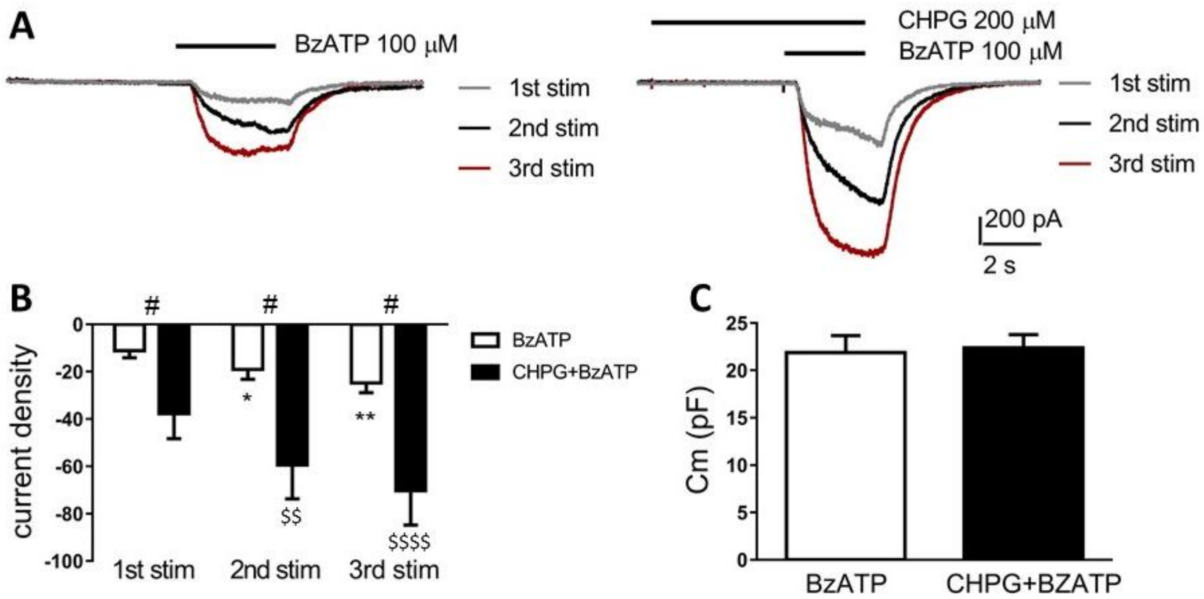

Figure 3. *CHPG enhances the sensitized current of purinergic ionotropic P2X7 receptor. A. Representative traces of purinergic ionotropic P2X7 receptor current after multiple stimulation by BzATP 100 µM (left panel) and exposed to CHPG 200 µM pre- and co-BzATP stimulation (right panel). B. Bars show quantified current of purinergic ionotropic P2X7 receptor in cells treated as in A. Current for each cell was normalized to corresponding C_m. C. Comparison of cell C_m from experiments in A and B. Bars are average ± SEM, n = 7 for BzATP treated cells, n = 6 for CHPG+BzATP treated cells; * and # $p < 0.05$; **, §§ and ## $p < 0.01$; §§§§ $p < 0.0001$ (two-way ANOVA followed by Sidak's multiple comparison test; symbols indicate: * significance referred to BzATP in first*

stimulations, § significance referred to CHPG + BzATP in first stimulations, # significance in each stimulation +/- CHPG treatment).

We next tested the effect of a metabotropic glutamate $mGlu_5$ receptor antagonist, MTEP 1 μM, to determine whether receptor blockade prevented the potentiation of CHPG-activated purinergic ionotropic P2X7 receptor current. Cells were treated in three different conditions (separate groups): exposed to BzATP, exposed to CHPG and BzATP and, finally, exposed for 15 s to MTEP before applying agonists (Fig. 4A). Current density for each cell in the specific group was evaluated and our results show that cells pre-exposed to MTEP did not show an increase in current compared to cells co-stimulated with CHPG and BzATP (Fig. 4B). C_m analysis did not show any differences in size for these three experimental groups (Fig. 4C).

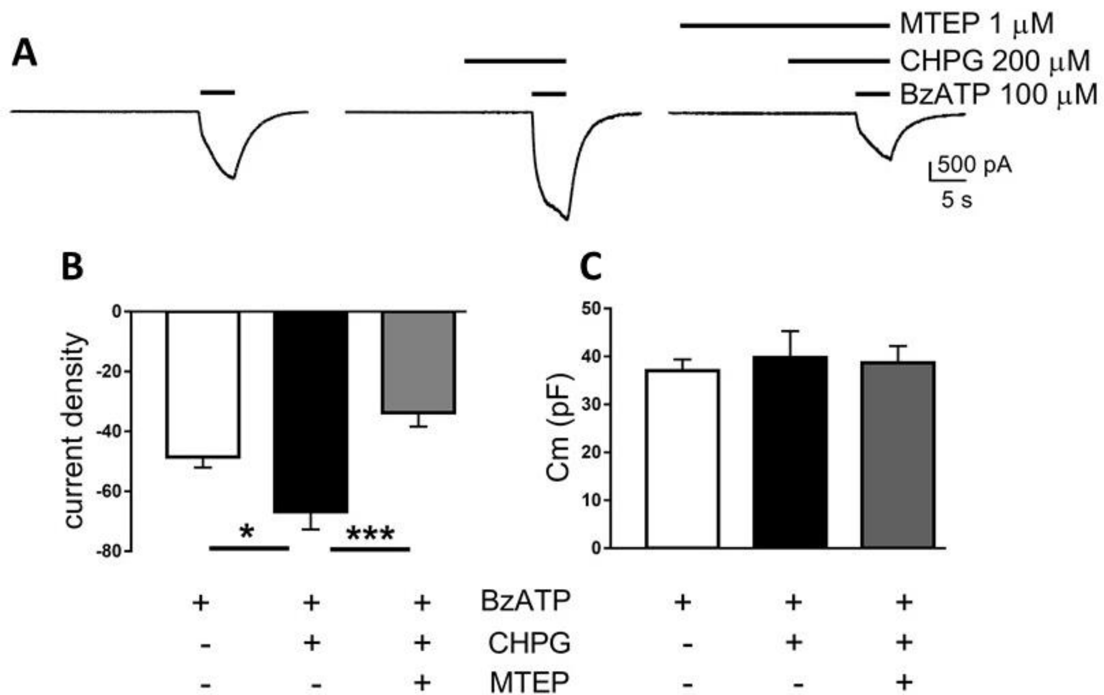

Figure 4. *MTEP prevents the increase in purinergic ionotropic P2X7 receptor current elicited by CHPG. A. Representative traces of purinergic ionotropic P2X7 receptor current elicited in three different cell groups: by BzATP 100 µM (left panel), by pre-/co-stimulation with CHPG 200 µM (middle panel), and by 15 s MTEP 1 µM incubation before CHPG and BzATP stimulation (right*

*panel). B. Bars show quantification of purinergic ionotropic P2X7 receptor current in all three groups in A. C. Comparison of cell C_m from experiments in A and B. Bars are average ± SEM ($n = 8$ for both BzATP and CHPG + BzATP groups, $n = 9$ for MTEP pre-treated group); * $p < 0.05$ (BzATP vs CHPG + BzATP bars), *** $p < 0.001$ (CHPG + BzATP vs MTEP bars) according to one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

To evaluate whether the increase in purinergic ionotropic P2X7 receptor current was specifically determined by metabotropic glutamate $mGlu_5$ receptor activation and not elicited by any Gq-coupled receptors, we examined stimulation of muscarinic acetylcholine M_3 receptor. We transfected cells with the PHmCh construct, used as reporter of muscarinic acetylcholine M_3 receptor activity. Upon Gq-activation, PHmCh translocates from plasma membrane to cytosol
(Chisari et al., 2009; Várnai and Balla, 1998). To test endogenous muscarinic acetylcholine M_3 receptor expression in BV2, PHmCh transfected cells were analyzed in live cell imaging experiments upon muscarinic acetylcholine M_3 receptor stimulation by the selective agonist carbachol 100 μM. Since fluorescence changes were not observed (Fig. 5A and B, top panels), we concluded that in our experimental model muscarinic acetylcholine M_3 receptors were not expressed.

Therefore, we transfected the muscarinic acetylcholine M_3 receptor along with PHmCh that, after carbachol stimulation, translocated to the cytosolic compartment (Fig. 5A and B, bottom panels).

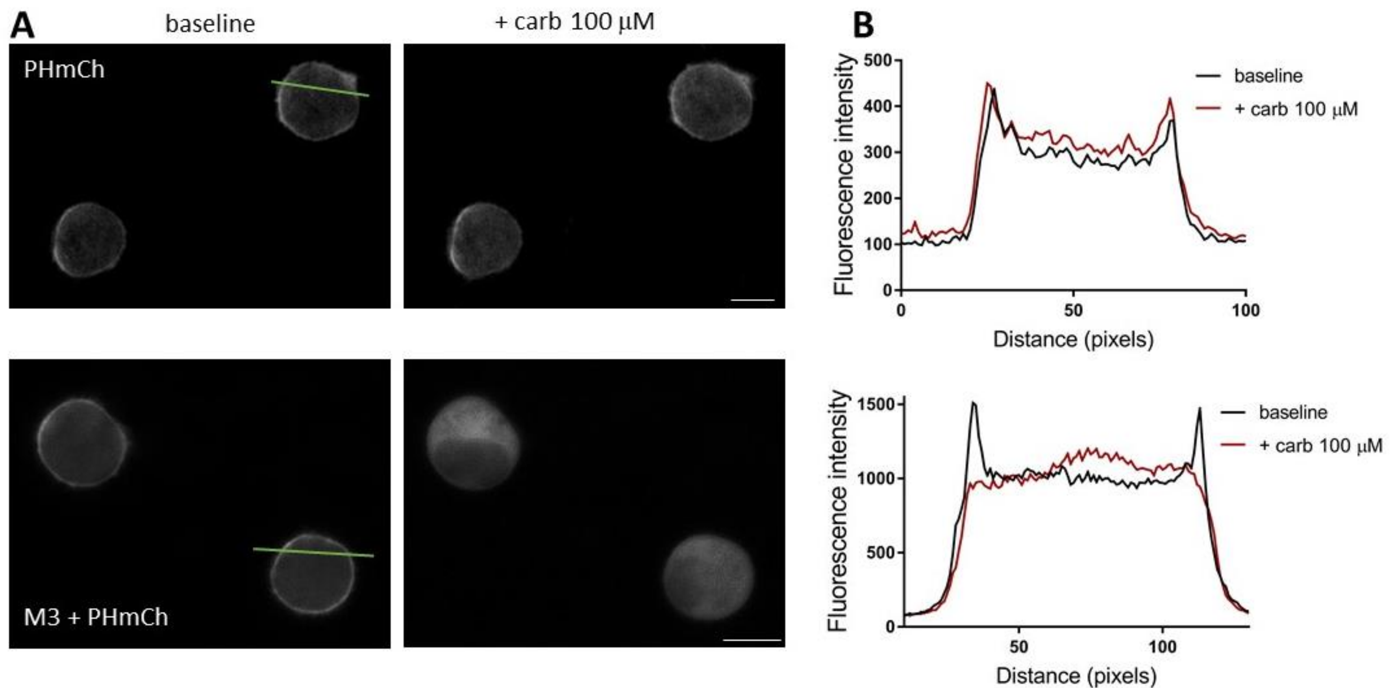

Figure 5. *BV2 cells do not express endogenous muscarinic acetylcholine M_3 receptors. A. Representative images of BV2 cells transfected with pleckstrin homology domain of PLC δ tagged with mCherry (PHmCh). In bottom panels, muscarinic acetylcholine M_3 receptor was transfected. Images were acquired every 5 s before and after agonist stimulation (carbachol, carb, 100 µM) (as indicated, bar = 10 µm). Activation of muscarinic acetylcholine M_3 receptor showed PHmCh translocation from plasma membrane to cytosol. B. Representative fluorescence quantifications by line scanning method (green line) in conditions shown in A. Changes in fluorescence distribution in bottom panels reflect PHmCh translocation.*

Based on these observations, we transfected BV2 cells with muscarinic acetylcholine M_3 receptor along with GFP (to identify transfected cells). Carbachol 100 µM was pre-applied for 10 s to activate muscarinic acetylcholine M_3 receptor and then co-applied with BzATP 100 µM (Fig. 6A, right panel). Purinergic ionotropic P2X7 receptor current recorded from these cells was normalized to corresponding C_m and compared to current density obtained from cells transfected only with GFP (Fig. 6A, middle panel), to test any influences on recordings by the transfection protocol. All data were compared to

current recorded in naïve cells (Fig. 6A, left panel). BV2 cells were stimulated twice and, in all groups, an increase in current by the second BzATP application was observed, but carbachol pre-/co-application with BzATP did not show any further increase (Fig. 6A-C) in either condition. Evaluation of C_m did not show any statistical differences in cells size (Fig. 6D).

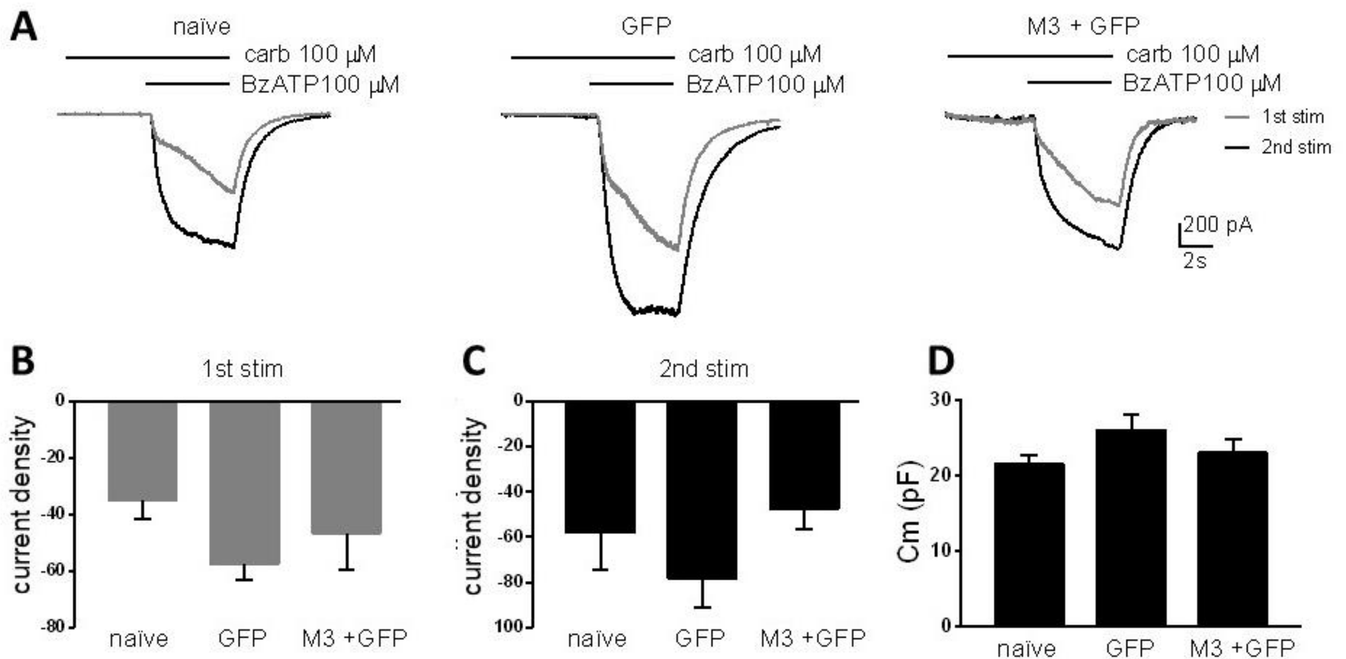

Figure 6. *Activation of muscarinic acetylcholine M_3 receptor does not affect purinergic ionotropic P2X7 receptor current. A. Representative traces of current elicited in BV2 cells after multiple stimulations of purinergic ionotropic P2X7 receptors by BzATP 100 μM in: non-transfected cells (naïve, right panel), in GFP (middle panel) and M_3 + GFP transfected cells (right panel). All cells were pre-exposed for 10 s to muscarinic acetylcholine M_3 receptor agonist, carb 100 μM, and stimulated simultaneously with both carb and BzATP. First 5 s carb application are not shown in traces. B and C. Bars indicate quantification of purinergic ionotropic P2X7 receptor current in all conditions showed in A. Current for each cell was normalized to corresponding C_m. D. Comparison of cell C_m from experiments in A and B. Bars in B, C and D are average ± SEM, n = 7 for both naïve and*

GFP groups, n = 8 for M_3 + GFP group. One-way ANOVA for repeated measurements and Tukey's multiple comparisons test showed no statistical differences.

Since purinergic ionotropic P2X7 receptor activation in microglia leads to several outcomes of an inflammatory response, including increase in NO release (Gendron et al., 2003), we tested the hypothesis that combination of BzATP and CHPG might influence such production. To test this, we performed a Griess assay to evaluate nitrite concentration as a measurement of NO levels in BV2 cells. We performed this experiment in four different conditions: control cells to determine basal nitrite production, cells treated for 30 min with CHPG 200 μM to identify a potential contribution on nitrite production by metabotropic glutamate $mGlu_5$ receptor, cells stimulated for 30 min with BzATP 100 μM to assess the amount of nitrite produced by purinergic ionotropic P2X7 receptor stimulation, and a fourth group of cells treated with both agonists (mix). Our data showed that nitrite concentration was comparable in control and CHPG groups, but was significantly increased by BzATP and this increase was even higher in combination with CHPG (Fig. 7, as indicated).

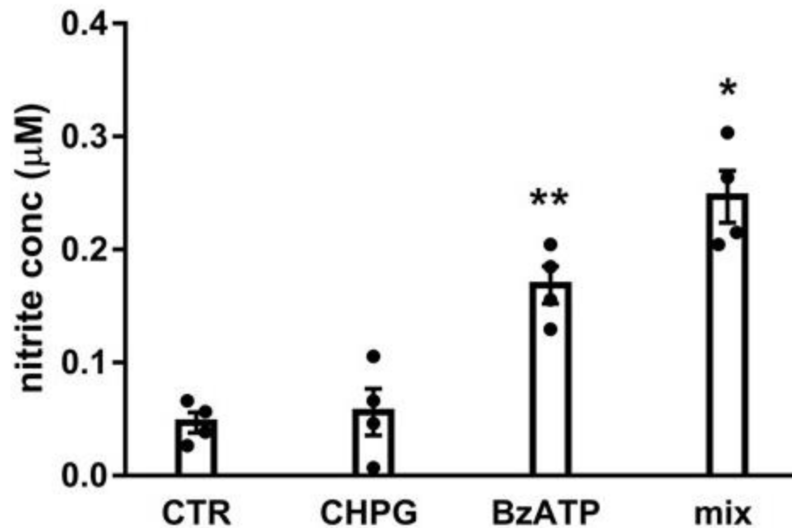

Figure 7. *Nitrite production by purinergic ionotropic P2X7 receptor is enhanced by metabotropic glutamate mGlu$_5$ receptor activation. Bars indicate extracellular nitrite concentration in BV2 cells in basal conditions, upon stimulation with CHPG 200 μM, by BzATP 100 μM treatment and by simultaneous exposure to both drugs (mix). Treatments lasted for 30 min. Bars are average ± SEM, n = 4 independent experiments; * $p < 0.05$ (mix vs BzATP), ** $p < 0.01$ (BzATP vs both CTR and CHPG) according to one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

Discussion

The purinergic ionotropic P2X7 receptor is a well-known player in inflammation in different organs and tissues, including the CNS (Calovi et al., 2019). Both its expression and function in microglia havebeen well characterized, suggesting an important role to promote activation of these cells (Monif et al., 2010), a hallmark for microgliosis or neuroinflammation. Compared to other purinergic receptors, purinergic ionotropic P2X7 receptor activation requires high ATP concentrations (> 100 μM) (North, 2002). Since in physiological conditions extracellular ATP levels are in the nanomolar range (Pegg et al., 2010), this feature suggests that purinergic ionotropic P2X7 receptor is a silent receptor that becomes involved during inflammation (Savio et al., 2018). Purinergic ionotropic P2X7 receptor

activation in microglia leads to neuroinflammation (Fiebich et al., 2014), characterized also by enhanced production and release of IL-1β and NO (Ferrari et al., 2006; Gendron et al., 2003). Several groups, including us, also observed that, upon prolonged activation, the pore of purinergic ionotropic P2X7 receptor enlarges (Yan et al., 2008) to allow organic ions and ATP to permeate it (Martínez-cuesta et al., 2020; Suadicani et al., 2006). This receptor is endogenously expressed in our model, BV2 cells, a murine microglia cell line frequently used to study microglia functions avoiding limitations of primary cultures (Henn et al., 2009).

Among metabotropic glutamate receptors, $mGlu_5$ receptor is expressed in microglia where its role has not been clearly elucidated. Activation of metabotropic glutamate $mGlu_5$ receptor has been shown to reduce the release of inflammatory molecules by microglia (Byrnes et al., 2009; Hsieh et al., 2012), thus, suggesting a potential neuroprotective role for this receptor. Accordingly, evidence showed that metabotropic glutamate $mGlu_5$ receptor blockade by selective antagonists caused an increase in reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) expression (Chantong et al., 2014). On the other hand, microglia activation mediated by LPS is thought to involve the metabotropic glutamate $mGlu_5$ receptor (Liu et al., 2014), indicating a pro-inflammatory effect mediated by this target.

To date, scientists have described the contribution that each individual receptor subtype has in neuroinflammatory events, focusing on these targets to find a treatment for neuroinflammatory diseases. Data in this study broaden this knowledge as we identified new features in neuroinflammation persistence. The presence of multiple transmitters in the extracellular space in the CNS certainly regulates the activity of cells involved in damage repair, primarily astrocytes and microglia. To our knowledge, no consolidated evidence exists on the concurrent activation of multiple receptors that respond to different transmitters in microglia. This study highlights this possibility since it investigates how microglia function can be modulated when purinergic ionotropic P2X7 and metabotropic

glutamate $mGlu_5$ receptors are activated simultaneously. Experiments shown here confirmed the ability of the purinergic ionotropic P2X7 receptor to undergo a sensitization process with repeated stimulations by the selective ATP analogue, BzATP. Since an increase in purinergic ionotropic P2X7 receptor current might determine a higher activation of signal transduction pathways, this result allows us to speculate, in accordance with evidences known so far, that the role of purinergic ionotropic P2X7 receptor favors the pro-inflammatory process in microglia (Santana et al., 2015; Monif et al., 2010). The concomitant metabotropic glutamate $mGlu_5$ receptor activation results in a notable increase in purinergic ionotropic P2X7 receptor current, as shown by quantitation of current elicited by co-stimulation with both agonists compared to BzATP alone. Moreover, the sensitization process of purinergic ionotropic P2X7 receptor is amplified by metabotropic glutamate $mGlu_5$ receptor activation. Since purinergic ionotropic P2X7 receptor current increase after repeated stimulation could be explained by a higher open channel probability (Dunning et al., 2021), we can speculate that metabotropic glutamate $mGlu_5$ receptor activation promotes such phenomenon. However, it has been shown that group I mGlu receptors promote NMDA current potentiation by increasing receptor trafficking on the plasma membrane (Lan et al., 2001). It has been shown that purinergic ionotropic P2X7 receptors are highly coupled with adenosine A2A receptors in microglia, and the inhibition of either receptor subtype reduces microglia reactivity, leading to neuroprotection (Dias et al., 2021). Adenosine A2A receptors are also found to couple to metabotropic glutamate $mGlu_5$ receptors in neurons (Borroto-Escuela et al., 2018). Such interaction might play a role also in microglia activation, and could be the bridge that regulates effects of glutamatergic transmission on the purinergic system. Therefore, more studies are required to better define the potential interaction between metabotropic glutamate $mGlu_5$ and purinergic ionotropic P2X7 receptors.

Since nitrite is obtained by oxidation of NO, a neurotoxic agent produced also by activated microglia (Boje and Arora, 1992), we speculate that an increase in nitrite levels elicited by the combination of

BzATP and CHPG supports a neuroinflammatory condition mediated by microglia. It is important to note that, in our experiments, CHPG alone does not change nitrite levels compared to control conditions, thereby excluding a role for metabotropic glutamate $mGlu_5$ receptor, *per se,* in promoting inflammation in microglia cells. Also, during pre-exposure to CHPG, baseline current in BV2 cells is not affected, suggesting an inability of CHPG to directly bind and open purinergic ionotropic P2X7 receptors, at least in our experimental protocol. However, we cannot exclude that CHPG binds other proteins expressed in BV2 cells. The role of metabotropic glutamate $mGlu_5$ receptor in enhancing of purinergic ionotropic P2X7 receptor current is also supported by the use of the metabotropic glutamate $mGlu_5$ receptor antagonist, MTEP (Lea IV and Faden, 2006).

Pre-treatment with this antagonist decreased the ability of CHPG to bind its receptor, and consequently, purinergic ionotropic P2X7 receptor current was not potentiated. We also tried to identify mechanisms underlying such communication. Our data suggest that cellular machinery specifically activated by metabotropic glutamate $mGlu_5$ receptor is responsible for such response.

The lack of effect mediated by muscarinic acetylcholine M_3 receptor is a good indicator that not all Gq-coupled receptors can mediate an increase in purinergic ionotropic P2X7 receptor current. This suggests, as well, that our observations are not mediated by Ca^{2+} increase in the cytosol, an effect determined by inositol 1,4,5-trisphosphate produced by Gq-mediated phospholipase C activation. Purinergic ionotropic P2X7 and metabotropic glutamate $mGlu_5$ receptors were both linked to the phosphorylation state of AKT. While the activation of P2X7 current by BzATP leads to AKT dephosphorylation (He et al., 2017), metabotropic glutamate $mGlu_5$ receptors lead to an increase in AKT phosphorylation when a positive allosteric modulator is present and not when CHPG is used as an orthosteric agonist (Bhat et al., 2021), suggesting the complexity of metabotropic glutamate $mGlu_5$ receptor signaling. Based on this evidence, future experiments are required to identify other effectors involved in the signal transduction pathway of metabotropic glutamate $mGlu_5$ receptor in microglia and

to relate them to modulation of purinergic ionotropic P2X7 receptor current. It is important to note that the enhancement of purinergic ionotropic P2X7 receptor current following metabotropic glutamate $mGlu_5$ receptor activation is very rapid, since only a 10 s exposure to CHPG was required. In our experimental model, removing or shortening such pre-exposure was not helpful because we observed variability in the increase in purinergic ionotropic P2X7 receptor current after co-stimulation with CHPG and BzATP. Moreover, we observed previously that a long incubation with CHPG for 20-24h at 37°C still shows an increase in purinergic ionotropic P2X7 receptor current (Beneventano et al., 2017). Therefore, it appears that the signal transduction pathway activated by metabotropic glutamate $mGlu_5$ receptor involves a rapid activation and long-lasting cascade of events.

In conclusion, the potentiation of purinergic ionotropic P2X7 receptor current mediated by metabotropic glutamate $mGlu_5$ receptor in microglia is a potentially important phenomenon that, based on our results, might promote an inflammatory environment in the CNS. The identification of signal transduction pathways underlying this modulation will be useful to identify new targets and possibly new drugs for pathologies with neuroinflammation as a hallmark.

CRediT authorship contribution statement

Mariangela Chisari: Conceptualization, Methodology, Supervision, Validation, Investigation, Formal analysis, Visualization, Data curation, Resources, Funding acquisition, Writing – Original draft, Writing - Review & Editing; **Michele Barraco:** Investigation, Formal analysis, Visualization; **Claudio Bucolo:** Resources, Writing - Review & Editing; **Lucia Ciranna:** Resources, Writing - Review & Editing; **Maria Angela Sortino:** Resources, Funding acquisition, Writing - Review & Editing

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Chapter II

Article in preparation

Cholesterol metabolites modulate ionotropic P2X4 and P2X7 receptor current in microglia cells.

Michele Barraco[1-2], Eva Kudova[3], Claudio Bucolo[2], Lucia Ciranna[4], Maria Angela Sortino[2], Mariangela Chisari[2,*]

[1]PhD program in Neuroscience, University of Catania, Italy;

Dept. of Biomedical and Biotechnological Sciences, [2]Section of Pharmacology, and [4]Physiology, University of Catania, Italy;

[2]Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Flemingovo namesti 2, 16610 Prague 6, Czech Republic;

*Corresponding author. Dept. of Biomedical and Biotechnological Sciences, Section of Pharmacology, University of Catania, via S. Sofia 97, 95123, Catania, Italy; email address: chisarim@unict.it

Abstract

The central nervous system is a well-known steroidogenic tissue producing, among others, cholesterol metabolites such as neuroactive steroids, oxysterols and steroid hormones. It is well known that these classes of endogenous molecules affect several receptor classes, including ionotropic GABAergic and NMDA glutamatergic receptors in neurons. It has been shown that also ionotropic purinergic (P2X) receptors are cholesterol metabolites' targets. Among P2X receptors, purinergic P2X4 and P2X7 receptors are expressed in microglia, the innate immune cells involved in the brain inflammatory response. In this study, we explore the ionotropic purinergic receptors modulation by cholesterol metabolites in microglia. Patch-clamp experiments were performed in BV2 cells, a microglia cell line, to evaluate effects of cholesterol metabolites using micro- and nanomolar concentrations. About P2X4

receptor, we found that testosterone butyrate (20 μM and 200 nM) and allopregnanolone (10 μM and 100 nM) both potentiated its current, while neither 25-hydroxycholesterol (10 μM and 100 nM) nor 17β-estradiol (1 μM) showed any effects. On the other hand, P2X7 receptor current was potentiated by allopregnanolone (10 μM) and 25-hydroxycholesterol (10 μM and 100 nM). Taken together, our data show that modulation of either P2X4 and P2X7 current is affected differently by cholesterol metabolites, suggesting a structure-activity relationship among these players. Identifying the possible link between purinergic transmission, microglia and cholesterol metabolites will allow to define new targets for drug development to treat neuroinflammation.

Keywords

Purinergic ionotropic P2X4 and P2X7 receptors; neuroactive steroids; oxysterols; microglia; neuroinflammation

Introduction

Microglia are the innate immune cells in the central nervous system (Norris and Kipnis, 2019). They interact with neurons and astrocytes, participating in the maintenance of brain homeostasis and inflammatory response (Hansson and Rönnbäck, 2003). In response to harmful or inflammatory stimuli, microglia pass from a resting to an activate state, which allow them to regulate the inflammatory response (Luo and Chen, 2012). It has been reported that during neuroinflammation levels of ATP are elevated compared to physiological conditions (Idzko et al., 2014). Among others, microglia express purinergic receptors activated by ATP, both metabotropic (P2Y) and ionotropic (P2X) (Calovi et al., 2019). P2X receptors are formed by three subunits delimitating the channel that,

upon ATP activation, allows a non-selective flow of cations (North, 2002), Na^{+}, K^{+} and Ca^{2+} (Samways et al., 2014). Microglia express mainly P2X4 and P2X7 receptors (Raouf et al., 2007). P2X7 receptor function in microglia is widely confirmed to lead to pro-inflammatory conditions (Deng et al., 2022). This receptor subtype has a low affinity to ATP, thus high ATP concentrations (> 100 μM) are needed to activate it (Duan and Neary, 2006). The signal transduction pathway activated by P2X7 receptor leads to the release of reactive oxygen species (ROS) (Bartlett et al., 2013), interleukin (IL)-1β (Mingam et al., 2008), as well as the inflammasome assembly (Franceschini et al., 2015). On the other hand, P2X4 receptors are activated by lower ATP concentrations (≤ 100 μM), based on the high affinity of this neurotransmitter for this receptor subtype (Coddou et al., 2011). P2X4 receptor is expressed in microglia, where promotes myelin debris phagocytosis (Zabala et al., 2018) and the release of brain derived neurotrophic factor (BDNF) (Trang et al., 2009), involved in the formation of new synapses (Parkhurst al., 2013). It is also known that in the spinal cord the up-regulation of P2X4 receptors in microglia favors the pathogenesis of neuropathic pain (Ulmann et al., 2008), suggesting a detrimental role linked to this receptor subtype.

Recently, brain cholesterol metabolism impairment has been linked to neuroinflammation (Vance, 2012; González-Guevara al., 2020). Among several molecules derived from cholesterol, neuroactive steroids (NSs), synthetized by the activity of cytochrome P450 side-chain cleavage (P450scc) on cholesterol (Schumacher et al., 2000), are well known to modulate neuronal NMDA and $GABA_A$ receptors (Korinek et al., 2011; Zorumski et al., 2013). It has been shown that some NSs affect purinergic transmission, acting as positive allosteric modulators of P2X2 and P2X4 receptors (De Roo et al., 2003, Codocedo et al., 2009, Sivcev et al., 2019). Along with NSs, cholesterol can be oxidized by other enzymes such as microsomal cytochrome P450 family enzymes, producing oxysterols (Mutemberezi et al., 2016). These compounds, such as 24(S)-hydroxycholesterol and 25-hydroxycholesterol (25-HC), are also known to positively modulate NDMA receptor current

(Linsenbardt et al., 2014). Other cholesterol metabolites are steroid hormones, such as testosterone and 17β-estradiol (Kawato et al., 2003). The variety of metabolites obtained by cholesterol allows to compare their chemical structures where many features are retained but different groups are present at C-3 and C-17.

In this study we tested the possibility that cholesterol metabolites with different chemical features, such as testosterone butyrate (TB-T), allopregnanolone (alloP), 25-HC and 17β-estradiol, might modulate P2X4 and P2X7 receptors endogenously expressed in BV2 microglia cells. Comparing current modulation effects highlights structure-activity features important to design and develop new drugs to treat neuroinflammatory conditions.

Materials and methods

Cell cultures: BV2 cells (murine cell line) were grown in flasks (25 cm^2 and 75 cm^2, Falcon, Italy) in Dulbecco's Modified Eagle medium with high glucose (4.5 g/l) enriched with 10% heat-inactivated fetal bovine serum and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml) (all from Thermo Fisher Scientific, Italy). Flasks were maintained in a humidified incubator at 37°C and 5% CO_2. For further experiments, cells were seeded on 35 mm dishes (Falcon, Italy), after exposing cell in flasks to 0.05% trypsin in Hank's balanced salt solution (Thermo Fisher Scientific, Italy). An important feature of BV2 cells is that long culturing affects their viability and functions, therefore cells in a narrow number of passages in trypsin (7-12) were used for experiments in this study.

Electrophysiology: to record purinergic current, cells were transferred from culture medium to a bath solution containing in mM: 138 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES at pH 7.25. During patch-clamp experiments in whole-cell configuration, cells were exposed to a solution where divalent cation concentrations were changed (no $MgCl_2$ and 0.1 mM $CaCl_2$) to allow any blocks by

these ions in purinergic current. Borosilicate patch electrodes, obtained using P-1000 puller (Sutter Instrument, USA), were filled with an internal solution containing in mM: 115 K-gluconate, 20 KCl, 2 mM EGTA and 10 HEPES at pH 7.35. Using this internal solution, pipette tip resistance was 4-5 MΩ. Recordings were performed at room temperature (RT), in voltage-clamp configuration (holding potential -80 mV), using an EPC7 Plus amplifier (HEKA Electronik, Germany). Data were acquired at 5 kHz, filtered at 1 kHz using a 7-pole Bessel filter and digitized using Digidata 1440A (Molecular Devices, USA). A computer-controlled multibarrel, gravity-driven local superfusion system (Automate Scientific, USA), was used to apply solutions. The flow exchange time measured by the junction current changes of an open tip pipette was 126.2 ± 9 ms.

Data analysis and statistical procedures: P2X4 and P2X7 currents were analyzed with pClamp 10 (Molecular Devices, Crisel Instrument, Italy), processed initially with Microsoft Excel and then transferred to GraphPad Prism (GraphPad Software, La Jolla, CA) for statistical analysis and figure finalization. Data shown in graphs are the mean ± SEM. The concentration-response curve fitting for averaged points was obtained with a non-linear four-parameter logistic function. Values of basal P2X4 peak current were set to 1 (first bar in graphs, Fig. 3 and 5) and P2X4 responses with tested drugs were normalized to basal ATP-evoked current. About P2X7 current, values of peak current were measured at first and second stimulations in both control and treated groups, as indicated in the Results section. Their ratio ($1^{st}/1^{st}$ stimulation, and $2^{nd}/1^{st}$ stimulation) was used for current normalization. Student's two-tailed t-test and one-way ANOVA followed by Tukey's multiple comparisons test were used to determine statistical significance, as indicated in figure legends.

Drugs: testosterone butyrate (TB-T) was synthesized in Dr. Eva Kudova's laboratory at Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic. ATP disodium salt and 17β-estradiol were purchased from Sigma-Aldrich (Italy), whereas A438079

hydrochloride, 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD), allopregnanolone (alloP), and 25-hydroxycholesterol (25-HC) were obtained from Tocris.

Results

BV2 cells are known to express different receptors (Atwood et al., 2011), including ionotropic receptors activated by purinergic transmission (Brautigam et al., 2005). We first tested the possibility to identify both P2X4 and P2X7 receptors in this cell line, exploiting the different ATP sensitivity to activate them separately. We already shown that P2X7 receptor is functionally expressed in BV2 cells and its activation by the selective agonist BzATP, 100 μM, leads to a strong current increase (Chisari et al., 2023). Moreover, repeated stimulations with BzATP 100 μM show an increase in current, suggesting a receptor sensitization due to a higher pore size (Martínez-Cuesta et al., 2020). Here, we used the endogenous agonist ATP and we observed a sensitization in P2X7 current during the second stimulation with ATP 300 μM for 5 s (Fig. 1A, red vs black trace). To verify the P2X7 receptor selective activation, at the end of each ATP stimulation we co-applied the selective P2X7 receptor antagonist, A438079 10 μM, for 10 s and current was strongly reduced (Fig. 1, A and B).

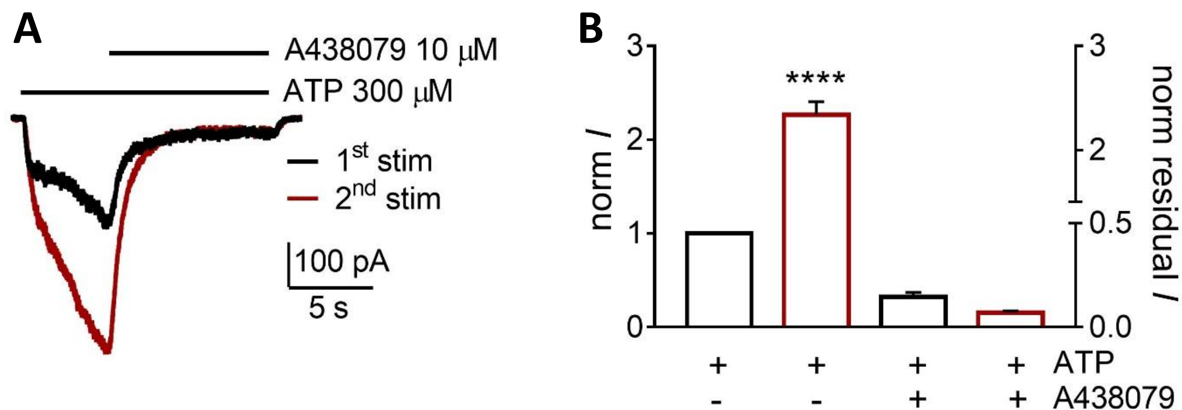

Figure 1. *Identification of P2X7 current in BV2 cells.* **A.** *Representative traces of P2X7 current elicited by multiple ATP 300 μM stimulations for 5 s (as indicated), followed by co-application with selective*

antagonist A438079 10 μM for 10 s. **B.** *Quantification of P2X7 current from traces in A. Peak current in each trace was normalized to the first ATP application (indicated as 1). The residual current at the end of the antagonist application was normalized to the peak current of the corresponding trace. Bars are average ± SEM, n = 8,* **** *p < 0.0001 (vs first ATP application, paired t test).*

Afterwards, to identify P2X4 receptor, cells were stimulated with increasing ATP concentrations, from 0.3 to 300 μM (Fig. 2A). Further concentrations were not tested to avoid P2X7 receptor recruitment. Peak current from each trace was plotted to obtain a concentration-response curve, and each point was fitted by Hill equation (Fig. 2B). Such fitting showed an EC_{50} = 3.99 μM and Hill slope = 1. For practical reasons, we decided to use ATP 5 μM for 5 s to test selective P2X4 activation (Fig. 2C). The concomitant application of ATP and the selective P2X4 antagonist, 5-BDBD 10 μM, for 15 s (Fig. 2C, left trace) showed an inhibition of P2X4 current. To quantify the extent of it, the residual current at the end of the co-application was normalized to the steady state of current elicited with ATP alone (Fig. 2D). Also, in order to facilitate this comparison, steady state ATP current was plotted as 1 (Fig. 2D). 5-BDBD was inhibiting current elicited by ATP 5 μM but such inhibition was not full, as shown by a reduction in current ~ 60%. We hypothesized that an increase in 5-BDBD concentration might be helpful to obtain a higher current inhibition, therefore, we increased the 5-BDBD concentration to 30 μM (Fig. 2C, right trace). Even with this higher concentration, the residual current was not statistically different from current obtained with previous concentration (Fig. 2D), but the high percentage of inhibition suggested that ATP 5 μM primarily activated P2X4 receptors.

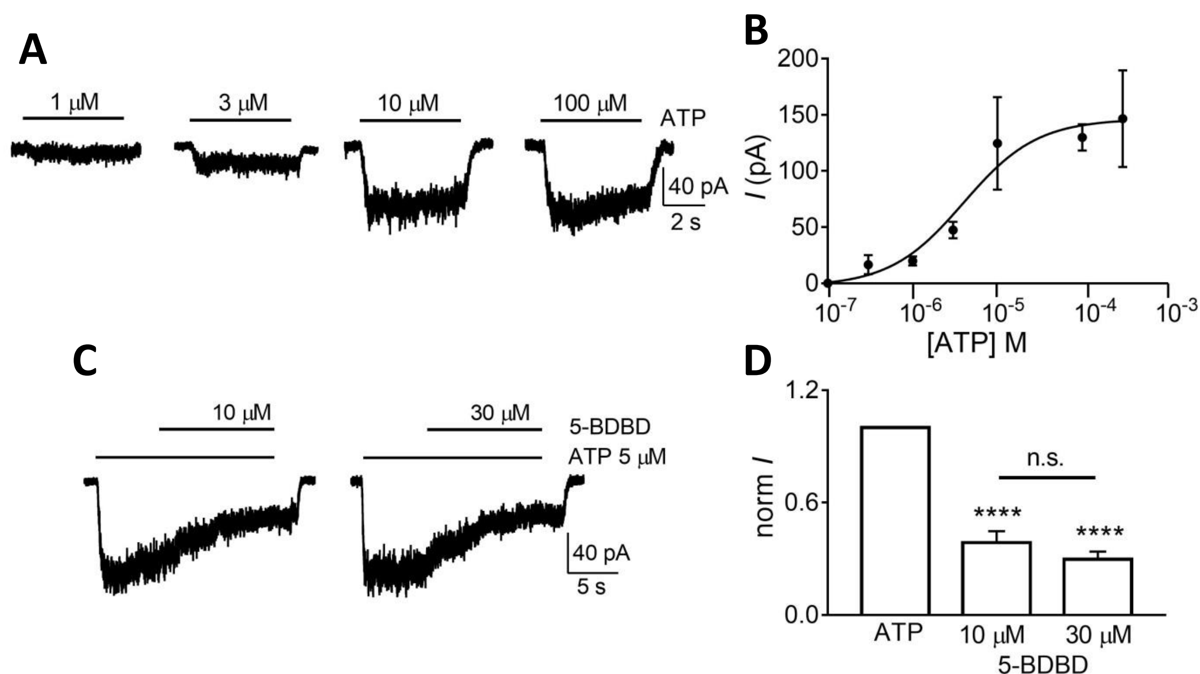

Figure 2. *Identification of P2X4 current in BV2 cells.* **A**. *Representative traces of P2X4 current elicited by increasing ATP concentrations (as indicated).* **B.** *ATP concentration-response curve obtained by recordings in A. Points are average ± SEM, n = 4-5 cells for each concentration. For display purposes, lines represent fits of the Hill equation to the averaged data points, the summary fits indicate EC_{50} = 3.99 μM and Hill slope = 1.* **C.** *Representative traces of P2X4 current elicited by ATP 5 μM, followed by co-application with selective antagonist 5-BDBD, 10 and 30 μM.* **D.** *Quantification of P2X4 current elicited in experiments shown in C. Current obtained at the end of ATP application (indicated as 1) was used to normalize the residual current at the end of the antagonist co-application. Bars are average ± SEM, n = 7,* **** *$p < 0.0001$ (vs ATP bar), according to one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

After identifying separate ATP concentrations to selectively activate P2X4 and P2X7 receptors we tested cholesterol metabolites to identify current modulation. We set an experimental protocol using the synthetic testosterone derivative, which is already known to positively modulate P2X4 current in a micromolar range (1-30 μM) (Sivcev et al., 2019). To evaluate the modulation of P2X4 receptor, BV2 cells were exposed to multiple stimulations to ATP 5 μM for 5 s, with an interval of 25 s, to help

stabilizing P2X4 current. Afterwards, cells were exposed for 25 s to TB-T 20 μM followed by the co-application with ATP 5 μM (Fig. 3A, upper panel). Peak current obtained during co-application of TB-T and ATP was normalized to the peak current of stable ATP current before TB-T application. Such comparison showed a potentiation in P2X4 current with TB-T, as expected (Fig. 3B). Since it is known that NSs affect the P2X4 current in a nanomolar range (Codocedo et al., 2009), we also explore such possibility in our model. BV2 cells were then exposed to TB-T 200 nM using the same experimental protocol described and, also in this conditions, P2X4 current was potentiated (Fig. 3, A-B).

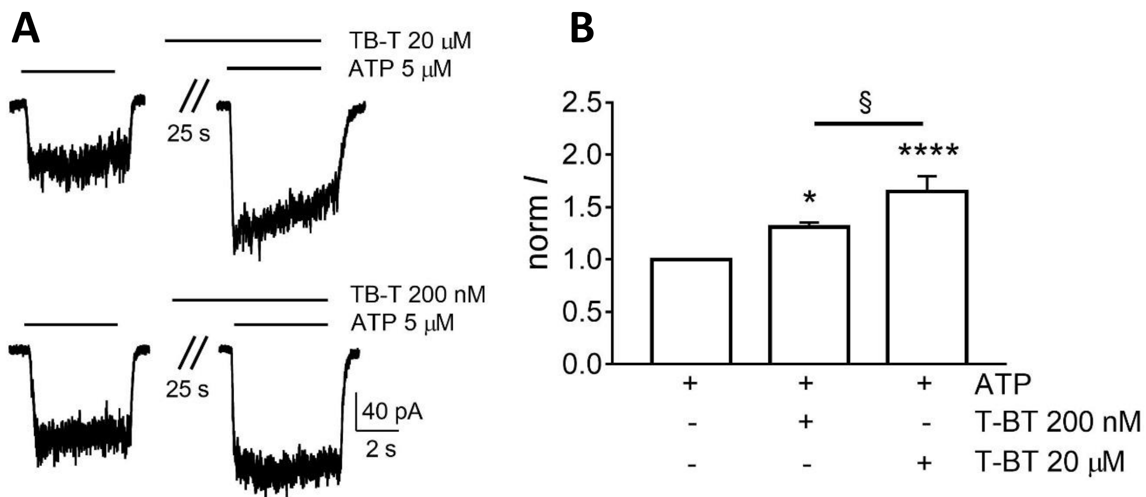

Figure 3. *Effects of synthetic TB-T on P2X4 current.* ***A***. *Representative traces of basal P2X4 receptor current elicited by ATP 5 μM (left trace), followed by co-application with TB-T (right trace), pre-applied for 25 s at indicated concentrations (top vs bottom panel) and co-applied with ATP.* ***B.*** *Quantification of P2X4 current obtained from recordings in A. Peak current obtained in presence of TB-T was normalized to basal P2X4 current (indicated as 1). Bars are average ± SEM, n = 7-8, * $p < 0.05$, **** $p < 0.0001$ (vs ATP bar), § $p < 0.05$, according to one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

In a different set of cells, we tested the effect of TB-T on P2X7 current. BV2 cells were activated twice with ATP 300 μM (Fig. 4A), while, in a separate group, cells were exposed to TB-T 20 μM only before

the second ATP stimulation (with 25 s pre-exposure, Fig. 4B). Therefore, peak current obtained in the second ATP stimulation was normalized to the peak obtained in the first ATP application (Fig. 4C). This protocol allowed us to compare the increase in the second stimulation in both conditions, ATP only (in control group) and co-application of ATP and TB-T, despite the natural increase in current elicited by the second P2X7 receptor stimulation. Comparing normalized peak current, we observed no hint of P2X7 current potentiation by TB-T (Fig. 4C). However, observing traces obtained during ATP and TB-T co-application, we noticed that the P2X7 receptor kinetic changed (Fig. 4A vs 4B). Therefore, we measured the rise time (s) of the channel using the standard exponential function and the Chebyshev method to find the best fit for each trace elicited by P2X7 receptor activation, in both control and treated groups. We observed that TB-T increased the opening rate of P2X7 receptor compared to the control group (Fig. 4D).

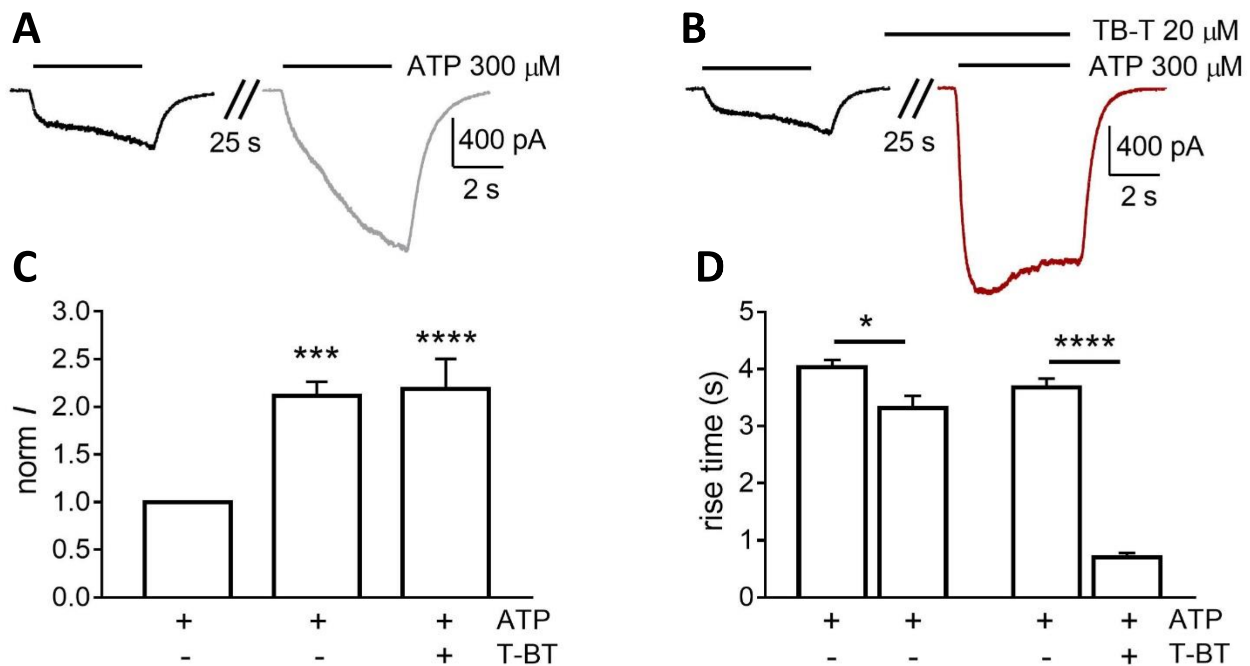

Figure 4. *Effects of synthetic TB-T on P2X7 current.* ***A.*** *Representative traces of P2X7 receptor current elicited by ATP 300 μM. Control cells were stimulated twice by ATP with 25 s of interval.* ***B.***

Representative traces of P2X7 receptor current of a separate cells group. They were first stimulated with ATP 300 μM for 5 s, exposed to TB-T 20 μM for 25 s, and finally challenged with ATP and TB-T. ***C.*** *Quantification of P2X7 current from recordings in A and B. Peak current elicited in second stimulation in both groups was normalized to peak current elicited in each group by ATP first stimulation (indicated as 1). Bars are average ± SEM, n = 10-13, *** $p < 0.001$ and **** $p < 0.0001$ (vs ATP bar).* ***D.*** *Comparison of the rise time of P2X7 receptors in all conditions recorded in A and B. Bars are average ± SEM, n =10-13, * $p < 0.05$ and **** $p < 0.0001$ for opening time, 2nd stim vs 1st stim in each group. Significance was attributed by one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

Using the same protocol shown for TB-T, we tested the steroid hormone 17β-estradiol 1 μM, and we did not observe any modulations in either P2X4 and P2X7 current (data not shown). Afterwards, we tested both natural cholesterol metabolites expressed in the brain, alloP and 25-HC. Based on evidences that alloP potentiates $GABA_A$ receptor current, we used the same concentration in our model, 10 μM. P2X4 current was potentiated by pre-applying alloP 10 μM for 25 s and then co-applying it along with ATP 5 μM (Fig. 5A-B, as indicated). Therefore, we reduced alloP concentration to 100 nM and P2X4 current was highly potentiated (~30% more than alloP 10 μM, Fig. 5A-B). BV2 cells were then exposed to 25-HC and this oxysterol did not show any P2X4 current modulations in either tested concentrations, 10 μM and 100 nM (Fig. 5, C-D).

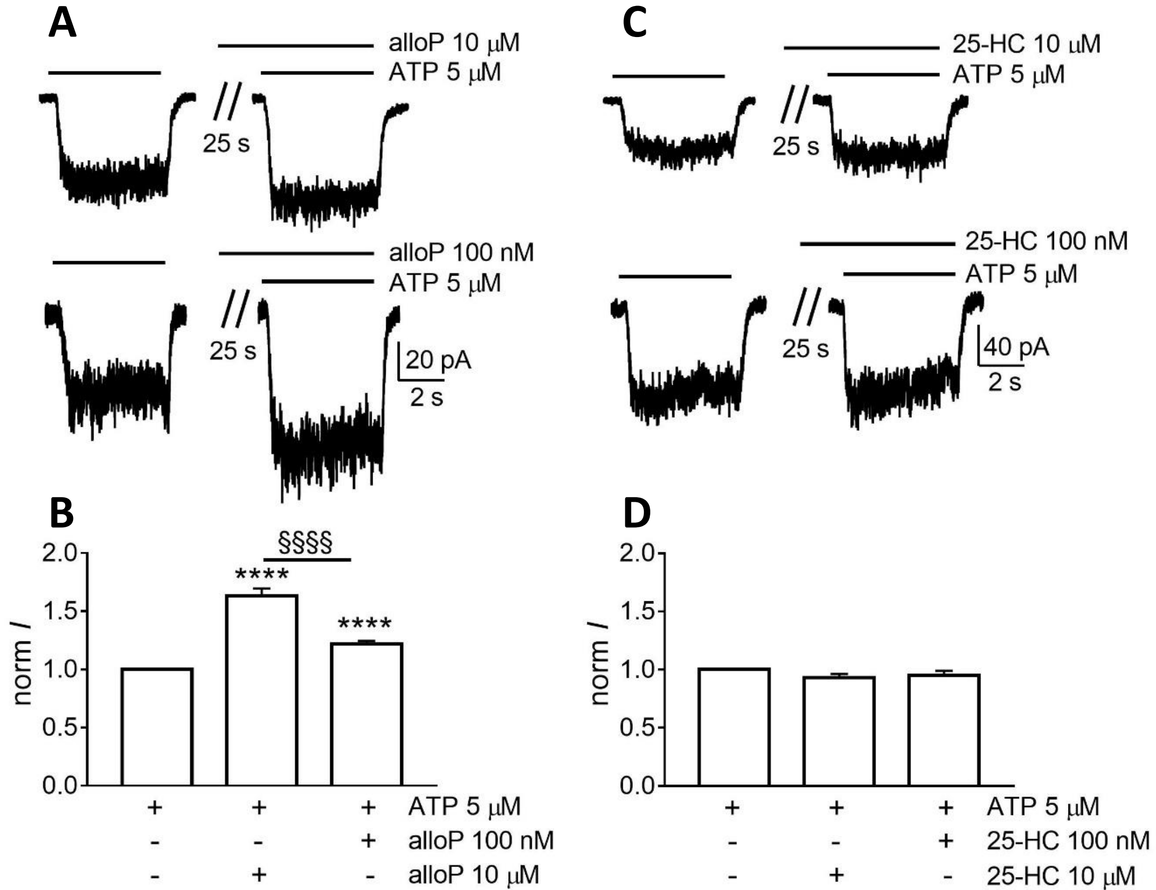

Figure 5. *Effects of cholesterol metabolites alloP and 25-HC on P2X4 current.* **A and C**. *Representative traces of basal P2X4 receptor current elicited by ATP 5 μM, followed by co-application with alloP and 25-HC. Cholesterol metabolites were pre-applied for 25 s at indicated concentrations.* ***B and D.*** *Bar diagrams show the quantified P2X4 current from traces in A and C, respectively. Basal P2X4 current was plotted as 1 and peak current elicited in presence of each compound was normalized to corresponding basal current. Bars are average ± SEM. In B, n = 5-8,* **** *$p < 0.0001$ (vs ATP bar),* §§§§ *$p < 0.0001$; in D, n = 7. Significance was attributed by one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

Both alloP and 25-HC were tested to evaluate P2X7 current modulation. Natural NS alloP 10 μM and oxysterol 25-HC 10 μM significantly increased P2X7 current compared to the control group (Fig. 6).

However, decreasing concentration to 100 nM for both compounds, only 25-HC positively modulates P2X7 current, even if quantification did not show any significant differences to potentiation elicited by 25-HC 10 μM (Fig. 6F).

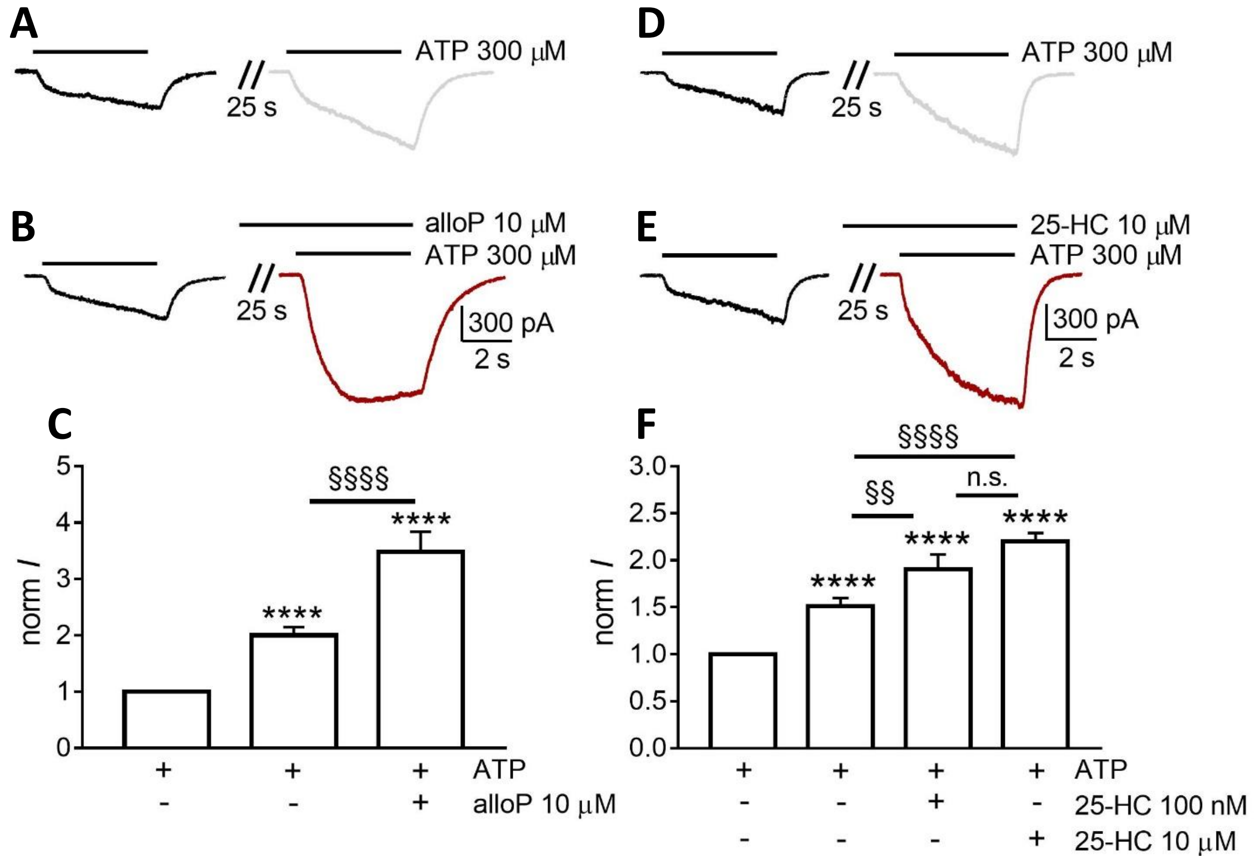

Figure 6. *Effects of natural cholesterol metabolites on P2X7 current.* ***A*** *Representative trace of P2X7 receptor current elicited by ATP 300 μM. Control cells were stimulated twice by ATP with 25 s of interval.* ***B.*** *Representative traces of P2X7 receptor current of a separate cells group. They were first stimulated with ATP 300 μM for 5 s, exposed to alloP 10 μM for 25 s, and finally challenged with ATP and alloP.* **C.** *Quantification of P2X7 current from recordings in A and B. Peak current elicited in second stimulation in both groups was normalized to peak current elicited in each group by ATP first stimulation (indicated as 1). Bars are average ± SEM, n = 11,* **** *p < 0.0001 (vs ATP bar),* §§§§ *p < 0.0001.* ***D and E****. Representative traces of P2X7 receptor current elicited by ATP 300 μM in control (D) and 25-HC 10 μM treated (E) group, using the same protocol shown in A and B.* **F.** *Quantification*

of P2X7 current from recordings in D and E. Bars are average ± SEM, n = 5-8, **** $p < 0.0001$ *(vs ATP bar),* §§ $p < 0.01$ *and* §§§§ $p < 0.0001$*. Significance was attributed by one-way ANOVA for repeated measurements and Tukey's multiple comparisons test.*

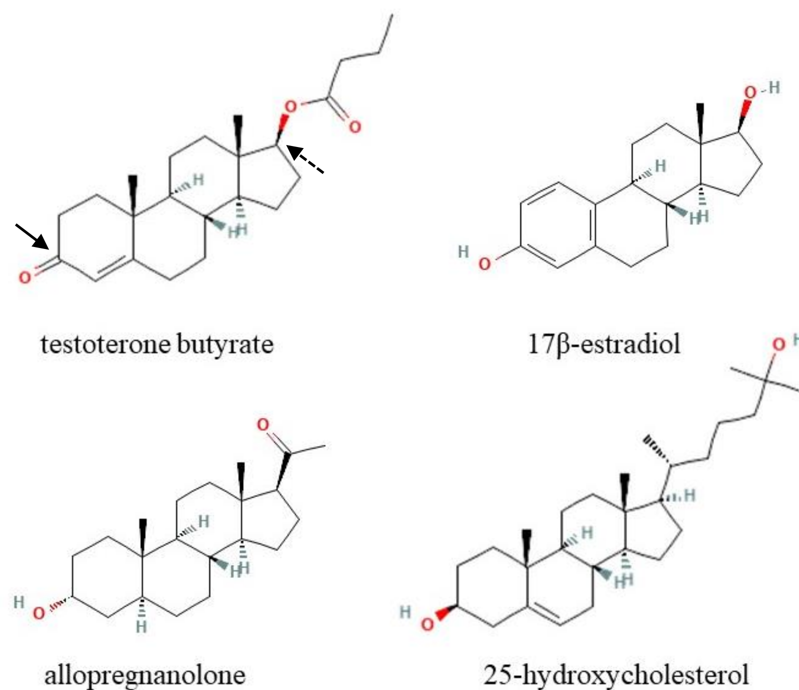

Figure 7. *Chemical structure of cholesterol metabolites tested. In testosterone butyrate structure, arrows indicate positions that might influence ionotropic purinergic modulation (C-3, solid arrow, C-17, dotted arrow).*

Discussion

In this study, we show for the first time the modulation of cholesterol metabolites in native ionotropic purinergic receptors express in BV2 microglia cells. Among several receptors, BV2 cells express P2X4 and P2X7 receptors (Trang et al., 2020). In our model, using patch-clamp experiments, we can separate the activation of each receptor subtype. Both receptors are ligand-gated ion channels, activated by ATP at different concentrations (5 μM for P2X4 and 300 μM for P2X7 receptor). Another important feature that allows to separate individual activation of each receptor is that P2X7 receptor activated by multiple ATP application shows a current increase, because the pore size is widening in these conditions (Yan et al., 2008). On the other hand, after several ATP applications, P2X4 current shows a desensitization (decrease in current) until current becomes stable. Each ionotropic current was finally blocked by selective antagonists for each receptor subtypes, A438079 for P2X7 and 5-BDBD for P2X4. However, in our model, P2X4 current was not fully abolished by 5-BDBD. In heterologous system, exposure to 5-BDBD 10 μM and subsequent co-application with ATP 10 μM is shown to inhibit the ~90% of P2X4 current (Coddou et al., 2019). In BV2 cells, we observed that the same antagonist concentration co-applied with ATP blocked ~ 60% of the P2X4 current. Increasing 5-BDBD concentration up to 30 μM did not show any further inhibition. We speculate that in our model P2X4 might be forming heterotrimers with other P2X receptors subtypes (e.g., P2X4/1, P2X4/6, P2X4/7) (Saul et al., 2013), that can be insensitive to the selective P2X4 receptor antagonist. Still, the contribution from P2X4 current seems predominant at ATP concentration used to activate it.

Since cholesterol metabolism impairment it is now considered a feature that leads to brain neuroinflammatory response, cholesterol metabolites might play a role in microglia functions that are involved in inflammation. Based on their chemical structure, we show in this study that modulation differs between P2X4 and P2X7 receptors.

As previously reported, steroid hormones such as testosterone and 17β-estradiol do not show any effect on either P2X4 or P2X7 current. When a linear alkyl chain (4 carbons) was added at C-17 position of testosterone structure through an ester moiety, obtaining TB-T, this change allowed to positively modulate P2X4 current but not P2X7, in a heterologous system (Sivcev et al., 2019). Here, in a native system, we confirmed these observations, but our experiments provide extra information. Our data show that P2X4 receptor can be activated also by a TB-T concentration in the nanomolar range, with an increase of ~ 20% compared to basal current. This effect was not found in the heterologous system (Sivcev et al., 2019), suggesting that an endogenous receptor might have a higher sensitivity to this compound.

On the other hand, our data show that P2X7 peak current was not affected by TB-T in the micromolar range, but the opening channel kinetics was 3-fold faster compared to control cells, suggesting that P2X7 receptor function might be affected by TB-T.

Another molecule that presents an alkyl chain at C-17, longer than TB-T, is 25-HC, where the ester group is absent, and the 6-carbon chain presents a methyl group in position 1 and an -OH and 2 more methyl groups at the end. Experiments shown here, demonstrate that the P2X4 current is not affected by 25-HC neither in micromolar nor nanomolar range, suggesting that the length of side chain might be critical for P2X4 activity. Despite the lack of effect by TB-T, 25-HC enhanced the P2X7 current at both tested concentrations, suggesting that a longer alkyl chain might be necessary to modulate P2X7 response. About alloP, we confirmed P2X4 current potentiation already at nanomolar concentration in BV2 cells, as shown previously in a heterologous system (Codocedo et al., 2009). Comparing chemical structures of TB-T and 25-HC to alloP, the NS has a short chain in C-17 position, whereas 17β-estradiol present an -OH group in the same position and it lacks P2X4 current potentiation. Taken together, these data suggest that a carbon chain at C-17 is important to retain ionotropic receptor potentiation. It has been shown that extrasynaptic $GABA_A$ current is modulated by alloP when the

orientation of -OH group in C-3 is kept in α, while the β orientation of the same group lacks such effect (Carver and Reddy, 2016). Apparently, such observation might explain the lack of effect by 25-HC and 17β-estradiol (-OH group in C-3 with β orientation) compared to alloP (-OH group in C-3 with α orientation). On the other side, the presence of a ketone group in TB-T (that does not allow the α orientation) still potentiate P2X4 current. In this study, effects by alloP on P2X7 receptor are more difficult to reconcile with a structure-activity relationship of tested compounds. Potentiation of P2X7 current is observed with alloP only at micromolar concentration and the group in C-17 shown a shorter chain than 25-HC that potentiates the same receptor already at nanomolar concentration. The longer carbon chain in TB-T compared to alloP at C-17 is expected to allow P2X7 current potentiation but the peak current was not modified by TB-T and only channel opening kinetics were affected by this compound. Therefore, we hypothesized that other chemical features such as the -OH group in C-3 is required to position the drug in the putative binding site on P2X7 receptor, more effectively when in β orientation as in 25-HC.

Another feature that might affect ionotropic purinergic current modulation is the capability of each cholesterol metabolite to permeate the plasma membrane and reach a putative binding site for either P2X4 or P2X7 receptors. Such capability is measured by the octanol-water partition coefficient (logP). Negative values of this parameter indicate higher affinity for aqueous phase, suggesting that such compounds interact with receptors in the extracellular domain, whereas positive logP values indicate the plasma membrane permeation (Chisari et al., 2009; Vincent et al., 2016). All tested compounds show a positive logP value (calculated by Molinspiration online software): 17β-estradiol, 3.43; alloP, 4.17; TB-T, 4.87; 25-HC, 6.42. Based on these values we can speculate that the lower logP value showed by 17β-estradiol justify the lack of effects in modulating either P2X4 or P2X7 current. Among other cholesterol metabolites, the higher value showed by 25-HC seems important to effectively activate P2X7 receptor, even at nanomolar concentration, while such feature did not affect P2X4

current potentiation. Therefore, we can suggest that the interaction with cholesterol metabolites and P2X7 receptor is favored by compounds that might be constrained in the plasma membrane for a longer time, and this capability might be required to interact with receptor portions not easily accessible by compounds with lower logP values. On the other hand, interaction with P2X4 receptors by cholesterol metabolites might be favored by less hydrophobic compounds that easily reach receptor transmembrane domains. Based on these differences, drug development might design molecules that selectively modulate P2X4 and P2X7 receptors. This requirement might be useful to pursue based on different effects that each receptor has in healthy and pathological conditions.

Since extracellular ATP concentrations vary in the brain, accordingly to physiological or inflammatory conditions (Di Virgilio et al., 2022), our study might suggest that purinergic ionotropic activation and cholesterol metabolites current modulation might act on different conditions. P2X4 facilitates BDNF microglial release and synaptic plasticity (Parkhurst al., 2013), whereas, during inflammation, higher extracellular ATP levels promote the release of pro-inflammatory mediators, such as IL-1β, by P2X7 receptor activation (Dubyak, 2012). Although alloP is shown to have neuroprotective effects (Divaccaro et al., 2021), we provide evidence this NS might exacerbate the microglia-mediated inflammation by P2X7 receptor activation. About 25-HC, it has been shown a change in pore size of the P2X7 receptor following a long exposure (48h) to this oxysterol with no ATP activation in a non-immune cell line (Olivier et al., 2017), suggesting a genomic pathway activation. On the other hand, during inflammation, elevated 25-HC concentrations were found (Reinmuth et al., 2021). Here, data provide direct evidence that 25-HC affects the P2X7 current already in nanomolar range concentration. Taken together, these observations suggest that 25-HC might be mainly involved in neuroinflammation where high ATP concentrations are elevated. On the other side, alloP synthetic analogues that potentiate P2X4 current at lower ATP extracellular level, might be useful to promote neuronal survival and functions mediated by microglia.

In summary, our data provide new evidences about modulation of P2X4 and P2X7 receptor current in microglial cells, strongly connected to the molecular structure of compounds tested in this study. Our observations allow to conclude that, among cholesterol metabolites, the length of an alkyl chain in C-17 is crucial for a P2X4 receptor modulation. Moreover, the spatial orientation of -OH group in C-3 increases the sensitivity of P2X4 receptor when it is in α conformation, while in β is more effective on P2X7 receptors. These data suggest important implications in drug design to obtain molecules modulating ionotropic P2X current in microglia.

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General discussion

Nowadays, neuroinflammation is recognized as a hallmark for neurodegenerative diseases (Stephenson et al. 2018). Since many inflammation mediators are released by microglia, these cells are certainly a key in the onset and progression of inflammatory responses (Kwon and Koh, 2020). ATP and glutamate extracellular levels are elevated during neuroinflammation, therefore ionotropic purinergic receptor, P2X4 and P2X7, and metabotropic glutamate $mGlu_5$ receptor, all expressed in microglia might have a role in such conditions. In this scenario, activation of P2X7 receptors lead to the release of pro-inflammatory mediators by microglia such us IL-1β (Clark et al., 2010), ROS (Bartlett et al., 2013) and NO (Gendron et al., 2003). On the other hand, controversial roles have been attributed to $mGlu_5$ receptor. The selective activation of $mGlu_5$ receptor decreased both ROS and NO levels (Byrnes et al., 2009), suggesting an anti-inflammatory function, supported also by use of selective $mGlu_5$ receptor antagonist, which in turn induced an increase of both ROS and iNOS expression (Chantong et al., 2014). In contrast to these results, it has been shown that $mGlu_5$ is a direct target for LPS (Liu et al., 2014) and $mGlu_5$ receptor activation may leads to release microvesicles containing inflammatory molecules, such as IL-1β and miRNA (Beneventano et al., 2017), leading to a pro-inflammatory condition. All these findings focused on the independent effects of $mGlu_5$ and P2X7 receptors. Data shown in Chapter I, recently published (Chisari et al., 2023), give a new perspective in studying neuroinflammation because indicate a crosstalk of both receptors. Data are obtained in BV2 cells, a murine continuous cell lines abundantly used as model for microglia studies (Horvath et al., 2008; Henn et al., 2009; Stansley and Hensley, 2012), because it avoids several weaknesses shown by microglia primary cultures. Results in chapter I show that a simultaneous activation of P2X7 and $mGlu_5$ receptors induces a higher amount of P2X7 current compared to current measured with no activation of $mGlu_5$ receptor. The sensitized P2X7 current elicited with multiple stimulations (Martínez-cuesta et al., 2020) is also potentiated by $mGlu_5$ receptor activation. The pharmacological

block of $mGlu_5$ using the selective antagonist MTEP (Lea IV and Faden, 2006), prevents such increase. A possible explanation of this modulation might be that more P2X7 receptors are recruited in plasma membrane after $mGlu_5$ activation, because it is known that in other cells, group I mGlu receptors activation promote NMDA trafficking in the plasma membrane (Lan et al., 2001). However, $mGlu_5$ activation might increase the open channel probability of P2X7 receptors that is already known to occur by the interaction with TMEM16 (Dunning et al., 2021). The interaction between P2X7 and $mGlu_5$ receptors has also a functional outcome in the increased NO production and release, responsible for the exacerbation of neuroinflammation. It is important to note that NO levels in this study are not affected by $mGlu_5$ activation alone, suggesting a non-inflammatory role for this receptor itself. Interestingly, the potentiation of P2X7 current is mediated by $mGlu_5$ receptors and not by others Gq-coupled receptors, suggesting that different signal transduction pathways might be responsible for such modulation. Among those, a bridge in this interaction might be played by adenosine A2A receptors that are shown to interact with both P2X7 (Dias et al., 2021) and $mGlu_5$ receptors (Borroto-Escuela et al., 2018). Another interesting link is played by the phosphorylation state of AKT, reduced by purinergic ionotropic P2X7 (He et al., 2017) and enhanced by metabotropic glutamate $mGlu_5$ receptors when a positive allosteric modulator is used to activate this receptor (Bhat et al., 2021). In this study, CHPG was used as orthosteric agonist, therefore it is possible to assume that AKT phosphorylation state was decreased in these experimental conditions. Nevertheless, metabotropic glutamate $mGlu_5$ receptor signaling might involve other effectors that in microglia are not yet identified, requiring further studies to clarify interaction with P2X7 receptor.

An interesting elucidation on P2X7 current potentiation could be possible by the identification of a binding site responsible for such outcome. It is possible to imagine that a specific receptor portion could be involved in the current potentiation, regardless of stimuli responsible for it. Since no information are available so far about a site responsible for ionotropic purinergic potentiation, a second

approach during this project aimed to elucidate if different drugs showing specific features in their chemical structures might affect purinergic current. Data in Chapter II (manuscript in preparation) suggest a structure-activity relationship on P2X7 and P2X4 current modulation. Results show that a putative binding site might be located in different portions of ionotropic purinergic receptor subtypes, P2X4 and P2X7. Compounds used in the study shown in Chapter II are cholesterol metabolites and they were selected based on evidences that these compounds are involved in neuroinflammation (Divaccaro et al., 2021; Reinmuth et al., 2021) and on their capability to modulate several ionotropic receptors (Codocedo et al., 2009; Paul et al., 2013; Carver and Reddy, 2016). Experiments were performed in BV2 cells and using different ATP concentrations, it was possible to selectively activate P2X4 or P2X7 receptors. On P2X4 receptors, TB-T and alloP showed an increase in current, while P2X7 current was potentiated by alloP and 25-HC. Based on their chemical structures, it is possible to identify specific features that selectively affect purinergic current. About P2X4 receptor, the side chain and its length in C-17 position of both TB-T and alloP are critical structural elements in current potentiation. Furthermore, the -OH group in α orientation at C-3 position, as in alloP structure, is another important feature to enhance P2X4 current. Moreover, compounds with a logP included between 4 and 5 are more efficient in activating this receptor. About P2X7 receptor, the side chain in C-17 remains an important part of steroid molecule to potentiate ion flux through this channel, showing that a longer chain as in 25-HC is required to activate the receptor at nanomolar concentration. Ultimately, the higher logP showed by 25-HC (6.42) suggest that the higher capability of this compound to permeate the plasma membrane is an important feature to effectively potentiate P2X7 current.

Conclusions

Results achieved in this project provide new insights on how microglia in the inflammatory process respond to glutamate, ATP and cholesterol metabolites (Fig. 6). Here, it is shown that the concomitant presence of these transmitters in the CNS extracellular space has important implications in the modulation of microglia activity. The well-known role of P2X7 receptor in exacerbating of microglial-mediated neuroinflammation is further favored by simultaneous activation of $mGlu_5$ receptor. This finding highlights a new pathway that might promote the harmful effects of microglia in several neurodegenerative diseases during inflammation. Therefore, these data suggest that the identification of P2X7 and $mGlu_5$ receptors crosstalk and further knowledge on how this communication is pursued might be useful to identify new targets to help reducing neuroinflammation and preserve neuronal viability. In the second part of this project, the structure-activity relationship among cholesterol metabolites on their ionotropic purinergic transmission modulation suggests that specific substituents of the steroid molecule play a kye role in the modulation of both P2X4 and P2X7 receptors. Observations in this study highlight peculiar features important for drug design, aiming to obtain molecules acting as agonists or positive allosteric modulators for P2X4 receptor and antagonists or negative allosteric modulators for P2X7 receptor. Thus, this strategy might help to treat neuroinflammation mediated by microglia and prevent detrimental consequences for human health.

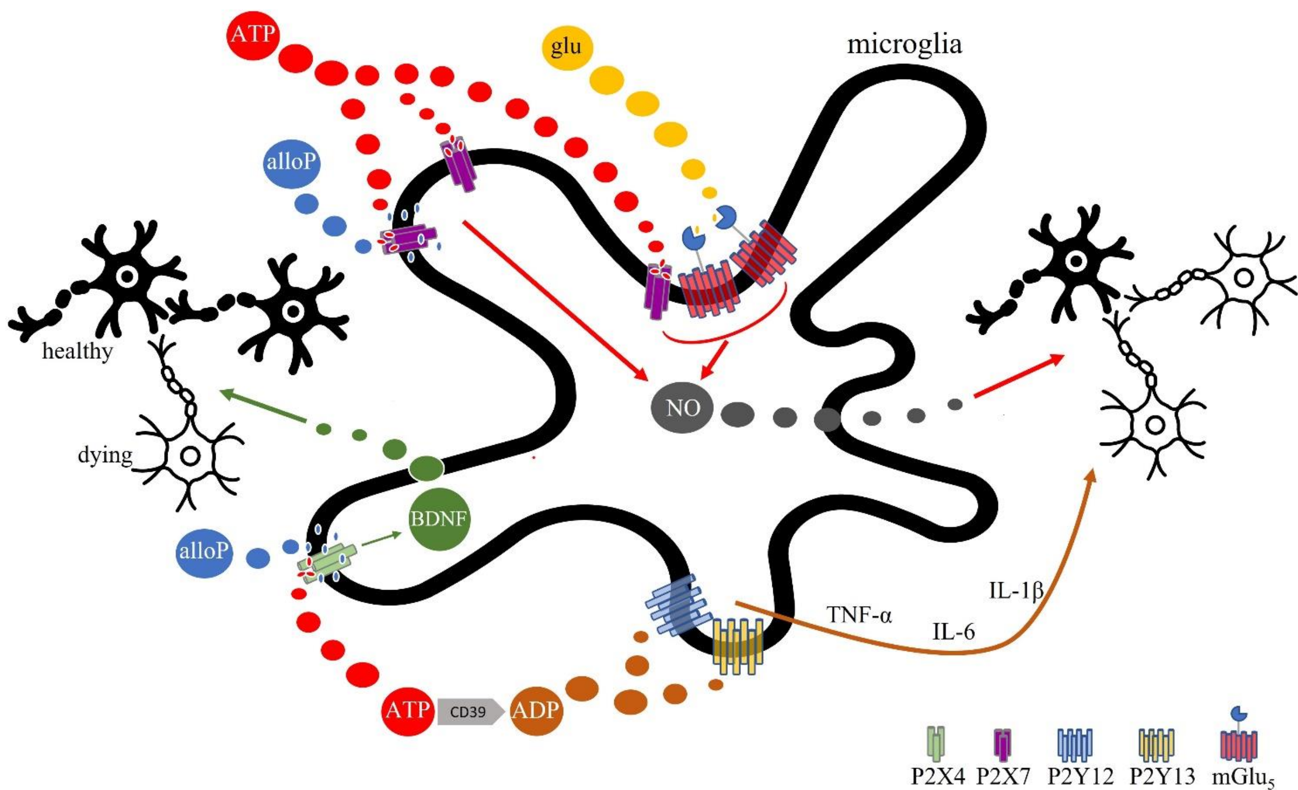

Figure 6. *Potential implications of microglial purinergic transmission in neuronal survival. Cartoon depicts how different ATP concentrations activate P2X4 and P2X7 receptors separately, indicating their role in neuronal survival, based on data reported in this study. The crosstalk between P2X7 and mGlu$_5$ receptors is also indicated, as well as the modulation by neuroactive steroid alloP. Moreover, the contribution by purinergic metabotropic P2Y12 and P2Y13 receptors, activated after ATP conversion by CD39 enzyme in ADP, is shown.*

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Contributions to scientific meetings

Poster presentation:

- **M. BARRACO**, M. A. SORTINO, M. CHISARI. Exploring P2X4 purinergic receptor modulation by allopregnanolone. *Federation of European Neuroscience Societies Forum, Paris, France; July 9-13, 2022.*

- **M. BARRACO**, E. KUDOVA, M. A. SORTINO, M. CHISARI. Purinergic signaling in microglia and potential role of neurosteroids in current modulation. *50th Annual Meeting of the Society for Neuroscience; November 8-11, 2021.*

- **M. BARRACO**. Modulation of purinergic current by glutamate signaling in microglia cells. *V Department Retreat, Capo Peloro, Messina; December 1, 2019.*

Abroad internship

Visiting Scholar at Prof. Steven Mennerick's lab, Department of Psychiatry, Washington University Medical Campus, St Louis MO. USA, August-December 2021.

Prof. Mennerick is an expert in neurosteroids and electrophysiology techniques and this visit allowed to improve technical skills and knowledge.

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