Adrenergic modulation of nucleotide receptors in rodent and human microglia

Thomas Deluc

Integrated Program in Neuroscience

McGill University
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List of abbreviations

AD	Alzheimer's disease
ADP	Adenosine diphosphate
ADRB2	β2 adrenergic receptor
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BzATP	Benzoyl adenosine 5'-triphosphate
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DAM	Disease-associated microglia
DAMPs	Damage-associated molecular patterns
GPCR	G protein-coupled receptor
IBMX	3-isobutyl-1-methylxanthine
iMGL	Human iPSC-derived microglia
hMGL	Human primary microglia
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iPSC	Induced pluripotent stem cells
ISO	Isoproterenol
LC	Locus coeruleus
LPS	Lipopolysaccharide
MS	Multiple sclerosis
NLRP3	Nucleotide-binding, leucine-rich repeat, pyrin domain containing 3

P2X	P2X receptor
P2Y	P2Y receptor
PAM or PAM3CSK4	TLR1/2 agonist
PAMP	Pathogen-associated molecular pattern
PD	Parkinson’s disease
qPCR	Quantitative polymerase chain reaction
THIK-1	TWIK-related halothane-inhibited K+ channel
TLR1/2	Toll-like receptor 1/2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha
TWIK2	Two-pore domain weak inwardly rectifying K+ channel 2
UDP	Uridine diphosphate

Abstract

Chronic neuropathic pain and neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease are characterized by central inflammation and neuronal death with no effective treatment. Microglia, the resident immune cells of the central nervous system (CNS), have received much attention in the past decades, due to their significant role in these pathologies. In healthy conditions, microglia are constantly surveying their environment and represent the first line of defense as they are the first cells to respond to any kind of disturbance in the CNS. Microglia have the capacity to detect danger signals and activate intracellular pathways to protect the CNS. However, dysregulation of these microglial pathways has been shown to be a major issue in neuropathological conditions. Extracellular levels of nucleotides, such as ATP and its metabolites, are increased in pathological conditions and act as danger signals for microglia through various purinergic/pyrimidinergic (P2) receptors. P2 receptors are composed of seven ionotropic ATP-gated channels (P2X) and eight metabotropic (P2Y) G protein-coupled receptors (GPCRs) which are widely expressed in mammals and have important physiological functions. A subset of P2 receptors expressed in microglia perform essential functions such as the release of growth factors (P2X4) or pro-inflammatory cytokines (P2X7), chemotaxis (P2Y12/13), and phagocytosis (P2Y6). These microglial P2 receptors play significant pathological roles in inflammatory pathways, pain sensitization, maintenance of chronic pain and neurodegenerative diseases. Yet, endogenous modulation of these receptors by monoamines has not been studied in depth. The development of novel analgesic approaches is contingent on our

understanding of the regulatory pathways involving the microglial P2 receptors that contribute to the pathophysiology of chronic pain. The metabotropic Gs-coupled β2 adrenergic receptor (ADRB2) is expressed in microglia and exerts a modulatory role on P2Y12-dependent chemotaxis, therefore it represents a promising candidate regarding a functional modulation of microglial P2 receptors relevant to chronic inflammatory and neuropathic pain states. This thesis focuses on crosstalk between ADRB2 and specific P2 receptors in microglia, and consequently on the role of ADRB2 in key functions that are dysregulated in pathological pain. We provide convincing evidence that ADRB2 is a modulator of microglial P2Y6-dependent phagocytosis and P2X7-dependent IL-1β release. We demonstrate that ADRB2 activation inhibits the calcium transients evoked by activation of P2Y6 receptors in primary mouse microglia, and this modulation is conserved in human iPSC-derived microglia. Furthermore, we found that phagocytosis induced by P2Y6 was reduced by ADRB2 activation in mouse and human microglia, in agreement with our calcium imaging results. However, we did not observe a significant effect of P2Y6 or ADRB2 on the release of inflammatory cytokines. Our qPCR results show that ADRB2 microglial activation reduces P2Y6 expression level. We also observed that ADRB2 can reduce the P2X7-dependent release of IL-1β in human iPSC-derived microglia and in human primary microglia. However, calcium imaging, electrophysiology, and qPCR experiments demonstrated that ADRB2 did not affect the calcium transients, the currents, or the expression of P2X7 in primary mouse or human microglia. Based on our findings, we conclude that ADRB2 1) decreases the expression level of P2Y6 and exerts a negative modulation on P2Y6-dependent phagocytosis, and 2) modulates the release of IL-1β evoked by P2X7 activation. Further research will be required to better

understand the intracellular mechanisms involved in these modulations, and whether the functional impact of ADRB2 on nucleotide receptors in rodent and human microglia is relevant to the pathophysiology of chronic pain and neurodegenerative diseases.

Résumé

La douleur chronique neuropathique et les maladies neurodégénératives, telles que la maladie d'Alzheimer ou la maladie de Parkinson, se caractérisent par une inflammation centrale et une mort neuronale sans traitement efficace. Les microglies, cellules immunitaires résidentes du système nerveux central (SNC), sont au cœur des études en raison de leur rôle crucial dans ces pathologies. En conditions normales, les microglies surveillent constamment leur environnement, constituant la première ligne de défense face à toute perturbation dans le SNC. Elles ont la capacité de détecter des signaux de danger, activant des voies intracellulaires pour protéger le SNC. Toutefois, la dysrégulation de ces voies microgliales est problématique dans les conditions pathologiques. Les niveaux extracellulaires de nucléotides, tels que l'ATP et ses métabolites, augmentent dans les conditions pathologiques, agissant comme des signaux de danger pour les microglies via divers récepteurs purinergiques/pyrimidinergiques (P2). Les récepteurs P2 sont composés de sept récepteurs ionotropes (P2X) et huit récepteurs métabotropes couplés aux protéines G (P2Y). Un sous-ensemble de récepteurs P2 est exprimé dans les microglies, assurant des fonctions essentielles telles que la libération de facteurs de croissance (P2X4), de cytokines pro-inflammatoires (P2X7), la chimiotaxie (P2Y12/13), et la phagocytose (P2Y6). Ces récepteurs P2 microgliaux sont impliqués dans les voies inflammatoires, la sensibilisation à la douleur, le maintien de la douleur neuropathique et des maladies neurodégénératives. Cependant, la modulation endogène de ces récepteurs par les monoamines n'a pas été pleinement explorée. Le développement de nouvelles

approches analgésiques dépend de notre compréhension des voies régulatrices affectant les récepteurs P2 microgliaux qui contribuent à la pathophysiologie de la douleur chronique. Le récepteur adrénergique bêta-2 couplé à la protéine Gs (ADRB2), exprimé dans les microglies, exerce un rôle modulateur sur la chimiotaxie dépendante de P2Y12, et émerge comme un candidat prometteur pour la modulation des récepteurs P2 microgliaux liés aux états inflammatoires chroniques et à la douleur neuropathique. Cette thèse se focalise sur les intéractions entre l'ADRB2 et certain récepteurs P2 microgliaux, et par conséquent sur le rôle de ADRB2 sur les fonctions clés qui sont dérégulées dans la douleur pathologique. Nous présentons des preuves convaincantes montrant que l'ADRB2 est un modulateur de la phagocytose dépendante de P2Y6 et de la libération d'IL-1β dépendante de P2X7 dans les microglies. Nous montrons que l'activation de l'ADRB2 inhibe les réponses calciques provoquées par l'activation de P2Y6 dans les microglies primaires de souris, une modulation conservée dans les microglies dérivées d'iPSC humaines. De plus, l'activation de l'ADRB2 réduit la phagocytose induite par P2Y6, chez la souris et l’humain, corroborant nos résultats d'imagerie calcique. Cependant, nous n'avons pas observé d'effet significatif sur la libération de cytokines inflammatoires. Nos résultats de qPCR démontrent que l'activation de l'ADRB2 réduit le niveau d'expression de P2Y6. Nous démontrons également que l'ADRB2 réduit la libération d'IL-1β dépendante de P2X7 dans les microglies humaines. Cependant, des expériences d'imagerie calcique, d'électrophysiologie et de qPCR ont démontré que l'ADRB2 n'affectait pas les réponses calciques, les courants ou l'expression de P2X7 dans les microglies primaires de souris ou humaines. Nos conclusions indiquent que l'ADRB2 diminue l'expression de P2Y6, exerçant une modulation négative sur la

phagocytose dépendante de P2Y6 et module la libération d'IL-1β induite par P2X7. Des recherches supplémentaires seront nécessaires pour comprendre les mécanismes intracellulaires impliqués dans ces modulations et pour déterminer si l'ADRB2 a un impact fonctionnel sur d'autres récepteurs nucléotidiques microgliaux.

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Contributions to original knowledge

This thesis aims to fill the gaps in knowledge concerning the role of norepinephrine in modulating nucleotide receptors in microglia, focusing on P2Y6-dependent phagocytosis and P2X7-dependent IL-1β release. Below are the contributions to original knowledge in Chapters 2 and 3 of this thesis, which correspond to research articles in final preparation for submission.

Chapter 2

Deluc T, Dorion M-F, Maussion G, Tang Y, Lo R.T.M, Ase A, Durcan T.M, Stifani S and Séguéla P. (2024) **Adrenergic control of phagocytosis in rodent and human microglia.**

This chapter characterizes, for the first time, the neuromodulatory effect of norepinephrine on P2Y6 in rodent and human microglia. We demonstrate that activation of the beta-2 adrenergic receptor (ADRB2) in microglia reduces the microglial UDP-P2Y6-evoked phagocytosis in rodents and in humans. As P2Y6 activation has been associated with diseases, the identification of a novel modulation mechanism could be relevant in disease conditions. Moreover, we show that ADRB2 activation also reduces the calcium transients of P2Y6 in both rodents and humans. Finally, we show that the expression of P2Y6 decreases with microglial ADRB2 activation. This chapter identifies a novel crosstalk between ADRB2 and P2Y6 in microglia, reducing phagocytosis by potentially acting on P2Y6 gene expression and UDP-evoked calcium transients.

Chapter 3

Deluc T, Dorion M-F, Ase A, Maussion G, Durcan T.M and Séguéla P (2024) **Adrenergic control of P2X7-dependent IL-1β release in microglia.**

Based on the findings of the previous chapter and the literature, this chapter investigates the neuromodulatory effect of norepinephrine on P2X7 in rodent and human microglia. We identify a reduction in P2X7-dependent IL-1β release following ADRB2 activation in microglia. Yet, we show that ADRB2 does not exert this modulation directly on P2X7, suggesting a modulation downstream P2X7. This chapter identifies, for the first time, a modulation of P2X7-dependent IL-1β release by ADRB2, a pathway fundamental to inflammation and pathological conditions. Yet, additional research is required to elucidate this modulation.

Author contributions

Chapter 1

T.D. wrote all sections, P.S. edited the text.

Chapter 2

Deluc T, Dorion M-F, Maussion G, Tang Y, Lo R.T.M, Ase A, Durcan T.M, Stifani S and Séguéla P. (2024) **Adrenergic control of phagocytosis in rodent and human microglia.**

T.D. and P.S. designed the project and all the experiments. T.D. was involved in all experiments. T.D. performed all calcium imaging, phagocytosis, and qPCR experiments. M.-F.D. performed the cytokine experiments. Y.T. and R.T.M.L. provided the iPSC-derived microglia. T.D. and P.S. wrote the manuscript.

Chapter 3

Deluc T, Dorion M-F, Ase A, Maussion G, Durcan T.M and Séguéla P. (2024) **Adrenergic control of P2X7-dependent IL-1β release in microglia.**

T.D. and P.S. designed the project and all the experiments. T.D. was involved in all experiments. T.D. performed all calcium imaging, and qPCR experiments. M.-F.D. performed the cytokine experiments. A.A. performed the electrophysiology experiments. T.D. and P.S. wrote the manuscript.

Chapter 4

T.D. wrote all sections, P.S. edited the text.

Chapter 1. General Introduction

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Objectives

Rationale

According to Health Canada, one in five Canadians was living with chronic pain in 2022 (HealthCanada, 2022). 4% of Canadians of age 40 and older were diagnosed with Parkinsonism in 2013/2014 (HealthCanada, 2018). The World Health Organization counted 55 million people in the world living with dementia in 2023, including the most common form of dementia, Alzheimer's disease (AD) which represents 60-70% of cases (WorldHealthOrganization, 2023). With a growing and aging population, the estimation of people living with chronic pain, Parkinsonism, or dementia is drastically increasing, and in some cases, estimations predict that the number will double (50% increase of Parkinsonism patients in 2031). In addition, for chronic pain, there is no effective treatment besides opioids and gabapentin, two pharmacological approaches with severe side effects (Quintero, 2017; Speed et al., 2018). In the absence of effective treatments for neurodegenerative diseases either, it is imperative that we better understand these complex pathologies to either develop treatments that are more effective and have less side effects or, at least, provide therapeutic options for improving the quality of life for patients.

Pain has a basic protective function, alerting the body of any tissue damage and acting as a survival mechanism. However, dysregulation of somatosensory pathways following tissue/nerve injury may lead to chronic neuropathic pain. Neurodegenerative diseases, such as AD or Parkinson’s disease (PD), are characterized by neuronal loss leading to progressive disabilities. Both chronic pain and neurodegenerative diseases are associated with neuroinflammation, neurotoxicity and cell death.

In search of a better understanding of the mechanisms and novel therapeutic targets for chronic pain and neurodegenerative diseases, recent studies targeted microglia, resident immune cells of the central nervous system (CNS), as major contributors of these pathological conditions over the past two decades (Haight et al., 2019; Stefanova, 2022).

Microglia represent the first line of defense in the CNS. Purines (including ATP and ADP) and pyrimidines (such as UDP) act as danger signals through specific purinergic receptor subtypes (P2Y12, P2Y13, P2Y6, P2X4, and P2X7) expressed in microglia. These surface receptors contribute to or modulate key microglial functions. However, dysregulation of microglial pathways involving these purinergic receptors can cause excessive neuroinflammation and death in chronic pain or neurodegenerative diseases. In light of this, it is important to identify the dysregulated purinergic pathways involved in each pathological conditions, as they represent potential therapeutic targets.

To this end, this dissertation focuses on novel functional crosstalk between adrenergic and purinergic receptors in rodent and human microglia.

Literature review

1.1 Introduction to microglia

Microglia, the resident immune cells of the CNS, originate from the yolk sac during the embryonic phase (day 8.5 in mice and around 13 weeks of gestation in humans) and represent around 10% of the cells in the CNS. They have received much attention in the past decades due to their importance in major central and immune functions, and the growing evidence of their involvement in most neurodegenerative diseases as well as chronic pain. Microglia are constantly surveying their environment (Arcuri et al., 2017; Inoue & Tsuda, 2018) and are among the first cells to respond to any perturbation in the CNS. A recent literature review (Paolicelli et al., 2022) adjusted the nomenclature in the field of microglial research. The classical terms used to describe microglial states, such as resting versus activated microglia and M1 versus M2 states are becoming outdated. The idea of a limited number of microglial states is no longer valid, since microglia display a wide repertoire of states, depending on their environment and cellular activity. It is now recognized that microglia are the most dynamic cells of the healthy mature brain. Thus, it is more appropriate to allude to surveying activity instead of resting state. The emergence of in vivo two-photon imaging approaches provides increasing evidence of various microglial activation states beyond the restricted M1 and M2 states. Microglia respond to fluctuations in their local environment owing to their constant movement, with their processes extending and retracting. Their processes contain phagocytosis material for rapid activation in physiological conditions. Thereby, they are always active regardless of a pathogenic context. M1 was considered a classical activation state of microglia, involving pro-inflammatory and neurotoxic phenotypes (detrimental), while M2 was

considered an alternative activation state, involving anti-inflammatory and neuroprotective phenotypes (beneficial). The association of M1 and M2 to distinct markers has become irrelevant. Microglia often co-express markers associated with M1 and M2, suggesting flexibility and plasticity, to react dependently to environmental clues in order to adapt their morphology and deliver appropriate responses. Microglial heterogeneity is gaining more attention, especially in pathological conditions, where identification of specific states will help to better grasp microglial complexity.

1.1.1 Microglia in the healthy CNS

The last two decades have seen a massive growth of knowledge on the importance of microglia in neurodevelopment, brain homeostasis, and synaptic plasticity. In neurodevelopment, microglia are essential in building efficient neuronal networks. Microglia play a major role in neuronal survival by maintaining active synapses or performing synaptic pruning, a process of removing non-functional, redundant, or underused synaptic connections. Synaptic pruning is a phenomenon dependent on the complement factors C1q/C3 that tag the synapses for elimination. Microglia use this process not only during neurodevelopment, but also throughout adult life for maintaining neuronal plasticity (Woodburn et al., 2021).

Chemotaxis, characterized by the motility of microglia toward damaged cells, is an essential homeostatic function in healthy and pathological conditions. Neuronal activity, via the release of molecules, influences the motility of microglia, in particular to consolidate or eliminate synapses. Microglia are also the first to respond to traumas and cell death in the CNS by performing phagocytosis and by releasing growth factors and cytokines. These functions, generally associated with pathological conditions and

inflammation, are essential to maintain the stability of neuronal circuits. For example, microglia release brain-derived neurotrophic factor (BDNF) to ensure the functioning of synapses (Parkhurst et al., 2013; Woodburn et al., 2021), or cytokines for the modulation of synaptic plasticity (Bourgognon & Cavanagh, 2020). Microglia express a core of genes that are essential to CNS integrity (Sideris-Lampretsas & Malcangio, 2021). Nonetheless, under physiological conditions, microglia constantly modify their genetic profile according to the needs of their surroundings.

Phagocytosis, a major microglial function, involves different molecular components associated with various functions. Phagocytosis is associated with chemotaxis, as it is crucial for the phagocytotic cells to reach the site that needs to be cleared. CX3CL1 (fractalkine) or adenosine diphosphate (ADP) are two signaling molecules released by neurons and recognized by microglia due to the expression of CX3CR3 and P2Y12/13 receptors (P2Y12/13) on their plasma membrane. A knockout of these receptor genes induces a delay in the phagocytosis process (Fuhrmann et al., 2010; Pagani et al., 2015). At the site of injury, “eat-me” and “don’t-eat-me” are two categories of signals that are important in determining whether phagocytosis occurs or not. “Eat-me” signals such as phosphatidylserine, calreticulin or UDP tag neuronal targets to be phagocytosed by microglia. Phosphatidylserine directly activates triggering receptor expressed on myeloid cells 2 (TREM2) or G protein-coupled receptor 56 (GPR56) transduction in microglia to induce phagocytosis (Li et al., 2020; Wang et al., 2015). Calreticulin acts through activation of the microglial lipoprotein receptor-related protein 1 (LRP1) to initiate phagocytosis (Gardai et al., 2005). Finally, UDP is released from stressed neurons to activate P2Y6 receptor (P2Y6) and induce microglial phagocytosis (Inoue, 2007; Koizumi

et al., 2007). Activation of these different receptors will lead to the reorganization of actin proteins leading to morphological changes in the plasma membrane of microglia to facilitate the engulfment of cellular particles. “Don’t-eat-me” signals such as CD47 and sialic acid expressed on neurons inhibit phagocytosis by microglia. CD47 interacts with signal-regulatory protein alpha (SIRPα), and sialic acid with sialic acid-binding immunoglobulin-type lectins (Siglecs) on microglia to prevent phagocytosis (Brown & Frazier, 2001; Gardai et al., 2005; Puigdellivol et al., 2021).

1.1.2 Microglia in the diseased CNS

In pathological states, microglial dysfunction contributes to the central inflammation typical of chronic neuropathic pain and neurodegenerative diseases such as AD, PD, multiple sclerosis (MS), or amyotrophic lateral sclerosis (ALS) (Abe et al., 2020; Du et al., 2017; Xu et al., 2016).

The proliferation of microglia, referred to as microgliosis, is a marker of their abnormal activity. Colony stimulating factor 1 receptor (CSF1R) is an essential receptor for microglial development and survival. Inhibition of CSF1R has been shown to induce the death of 99% of microglia in the CNS. However, upon removal of CSF1R inhibition, the remaining microglia can proliferate and replenish the entire CNS within one week (Elmore et al., 2014). Microglia have the ability to self-renew, generally at a low rate due to their long life (Ajami et al., 2007; Tewari et al., 2024). Microgliosis, considered as a critical step in mounting an innate immune response in the CNS, has been observed in many diseases including chronic neuropathic pain. Activated by the neuronal release of CSF1 or IL-34, CSF1R signaling is essential to promote microgliosis in neuropathic pain models. Indeed, absence of CSF1R has been reported to be sufficient to prevent

microgliosis and consequently to decrease pain hypersensitivity (Greter & Merad, 2013; Inoue & Tsuda, 2018; Masuda et al., 2020).

1.1.3 Microglia in pain

Pain is a normal protective mechanism, notifying the body of potential or real tissue damage. According to the International Association for the Study of Pain, pain is defined as *"an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"* (Raja et al., 2020). From a basic mechanistic standpoint, pain is initiated when a noxious stimulus is transduced by specialized receptors found on the surface of neuronal sensory terminals at the site of injury. This nociceptive signal is sent through specific sensory fibers to neurons located in the dorsal horn of the spinal cord, and then relayed to various brain regions in charge of processing pain perception and nocifensive response. Yet, disruption of this somatosensory pathway, occurring after tissue or nerve injury, can result in chronic pain, a significant public health issue affecting at least 20% of the population (Gereau et al., 2014). Chronic pain is defined by abnormal hyperexcitability leading to persistent pain lasting more than three months. It can be characterized either by an increase in response to noxious stimuli (hyperalgesia) or by innocuous stimuli becoming painful (allodynia) (Jensen & Finnerup, 2014; Meacham et al., 2017).

Microglia have been extensively well documented on their crucial role in the development and maintenance of chronic/neuropathic pain. Following nerve injury, microglia proliferation and activation are involved in the expression of neuropathic pain. Ablation or inhibition of microglia by the antibiotic medication Minocycline precludes the onset of neuropathic pain (Ward & West, 2020). Most of the studies investigating the role

of microglia in chronic pain have focused on spinal microglia. Several pathways and molecules have been shown to participate in the hyperexcitability of spinal somatosensory neurons. The abnormal release of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), or tumor necrosis factor-alpha (TNF-α), and the release of chemokines by microglia have been shown to cause neuronal hyperexcitability. The modulatory effects of BDNF and nucleotide-binding, leucine-rich repeat, pyrin domain containing 3 (NLRP3) inflammasome, as well as clearance functions carried out by microglia, have been associated with pathological disruption of spinal circuits and neuropathic pain. The following sections will describe some of these mechanisms in more detail.

1.1.4 Microglia in neurodegenerative diseases

AD, the most common neurodegenerative disease that causes dementia in the world, is characterized by amyloid plaques of Aβ peptide in the brain and intracellular neurofibrillary tangles of tau protein (Duyckaerts et al., 2009). Microglia are considered essential in the development or resolution of AD. Indeed, high levels of microglial activation have been found near Aβ plaques (Schlachetzki & Hull, 2009). These microglia have been characterized as disease-associated microglia (DAM) and participate in the clearance of Aβ (Keren-Shaul et al., 2017). Studies have demonstrated that microglia could have beneficial effects through uptake and degradation of Aβ, and limiting the propagation of tau pathology, but also have detrimental effects by promoting plaque development, neuroinflammation, and tau spreading (Gao et al., 2023).

PD, another prevalent neurodegenerative disease, is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of

misfolded α-synuclein in Lewy bodies. Similarly to AD and most neurodegenerative diseases, an increase in microglia activation is observed in PD. Microglia seem to have beneficial effects for PD by promoting the clearance of α-synuclein. However, this clearance can also be detrimental because α-synuclein, when internalized in microglia, causes neuroinflammation in PD.

In addition to AD and PD, microglia are involved in most other known neurodegenerative diseases. Briefly, in MS microglia activation leads to chronic inflammation and deficits in phagocytosis of cellular debris contribute to the onset of MS. Depletion of microglia in the experimental autoimmune encephalomyelitis (EAE) mouse model decreases inflammation and improves clinical symptoms (Wies Mancini et al., 2023). Microglia are also involved in ALS where they are protective at early stages but then become detrimental in later stages due to impairment of phagocytosis, pro-inflammatory activity, and secretion of neurotoxic factors that induce motor neuron death (Geloso et al., 2017; Henkel et al., 2009).

No effective treatment devoid of severe side effects is currently available regarding chronic pain or neurodegenerative diseases.

1.2 Damage-associated molecular patterns (DAMPs)

The constant surveying activity of microglia is essential to detect any stressed, damaged, or dead cells due to their ability to recognize danger signals released in the extracellular space such as damage-associated molecular patterns (DAMPs). An abnormal increase in a molecule that is normally present at very low levels in the extracellular space characterizes a DAMP. DAMPs include mitochondrial and nuclear RNA and DNA, nucleosides, and nucleotides (Murao et al., 2021).

1.3 Purinergic and pyrimidinergic signaling

Adenosine triphosphate (ATP) is the first metabolite of a group of four purines. ATP is rapidly hydrolysed by the ectonucleoside triphosphate diphosphohydrolase (ENTPDase1/CD39) to ADP, which is then hydrolysed by ectonucleotide pyrophosphatase/phosphodiesterases (E-NPP) to adenosine monophosphate (AMP), itself finally hydrolysed by ecto-5-nucleotidase (E-5-nucleotidase/CD73) to adenosine. ATP was discovered in 1929 by Karl Lohmann and identified as “the universal energy reservoir of the cell” (Khakh & Burnstock, 2009; Langen & Hucho, 2008). ATP was later demonstrated to also have an important signaling role outside the cell.

Burnstock, in 1972, reported the discovery that ATP is not only a source of energy but also an extracellular messenger (Burnstock, 1972; Burnstock et al., 1972; Khakh & Burnstock, 2009). In 1976, Burnstock also proved that in the nervous system, ATP can be released as a co-transmitter. This was the first demonstration of corelease of neurotransmitters, indicating that neurons have the capacity to synthesize, store, and release more than one type of neurotransmitter (Khakh & Burnstock, 2009). Nowadays, we know that ATP can be co-released with other transmitters such as norepinephrine or acetylcholine. For example, noradrenergic neurons that project to the cortex can co-release norepinephrine and ATP (Poelchen et al., 2001). ATP has also been recognized as a single transmitter. Thus, ATP can either be released as the main neurotransmitter to contribute to synaptic excitation or as a co-transmitter to act as a modulator with an impact on synaptic plasticity. For example, ATP can modulate glutamatergic transmission by acting on AMPA receptors, or on GABAergic transmission by acting on GABA-a receptors

that control excitatory and inhibitory inputs in the CNS, respectively (Jo et al., 2011; Pougnet et al., 2014).

Uracil nucleotides, including uridine diphosphate (UDP) and uridine triphosphate (UTP), belong to the chemical family of pyrimidines. Uridine undergoes several sequential phosphorylation to become uridine monophosphate (UMP), UDP and UTP. Similarly to purines, pyrimidines have also been shown to be release extracellularly, (Lazarowski et al., 1997; Saiag et al., 2009), participating in the regulation of essential transductions.

1.3.1 Sources of extracellular nucleotides

Concentrations of extracellular nucleotides such as ATP are low (in the nanomolar range) compared to their intracellular concentrations (in the millimolar range). Small increases in extracellular ATP can induce a healthy stimulation to perform cell differentiation, motility, proliferation or secretion of neurotransmitters and growth factors (Di Virgilio, 2000; Di Virgilio et al., 2023).

The source of ATP is more complex than what researchers initially thought. As mentioned in the previous paragraph, ATP can be released or co-released via synaptic vesicles by neurons. Nevertheless, ATP can also be released through passive leakage, the vesicular nucleotide transporter (VNUT), or other channels: connexins, pannexin (PANX1), calcium homeostasis modulator 1 (CALHM1), volume-regulated anion channels (VRACs), and maxi-anion channels (MACs). In addition, mechanisms of extracellular ATP release through astrocytes or P2X7 receptors (P2X7) have been described (Vultaggio-Poma et al., 2020). In opposition to the controlled mechanisms mentioned above, damaged, or stressed cells can leak large amounts of nucleotides including ATP, UTP, or UDP, leading to a drastic increase in the concentration of nucleotides in the extracellular

space. This abnormal concentration indicates the presence of danger, thus, these nucleotides are considered DAMPs. ATP has the particularity to be also a pathogen-associated molecular pattern (PAMP), i.e. a molecule secreted by foreign organisms such as bacteria and acting as a danger to microglia. Extracellular ATP/UTP/UDP can be detected through the activation of various purinergic and pyrimidinergic (P2) receptors expressed at the surface of microglia.

However, uncontrolled accumulation of ATP in the extracellular space in the CNS can be detrimental by causing cytotoxicity or by initiating neuroinflammation (Vultaggio-Poma et al., 2022). Extracellular concentrations of ATP/UDP are increased in pathological conditions (Di Virgilio & Adinolfi, 2017; Li et al., 2014), reaching abnormal levels in many neurodegenerative diseases and in chronic neuropathic pain.

1.3.2 The family of purinergic receptors

As mentioned above, Burnstock played a central role in the discovery of purinergic signaling, as he coined the term “purinergic transmission”. The concept of purinergic transmission through the release of extracellular ATP implies the presence of purinergic receptors. In the late seventies, the first purinergic receptors were identified (Burnstock, 1976) and in 1978, Burnstock classified purinergic receptors into two types, P1 and P2 receptors, standing respectively for adenosine and ATP/ADP receptors. About a decade later, several groups (including ours) used molecular approaches to clone ATP receptors and identify a large family of purinoceptors with distinct properties.

P1 receptors comprise four subtypes of G protein-coupled receptors: A1, A2A, A2B and A3. A1 and A3 are coupled to Gi proteins (inhibition of adenylate cyclase), while A2A

and A2B are coupled to Gs proteins (activation of adenylate cyclase). P1 receptors are widely expressed in the CNS.

P2 receptors are subdivided into two gene families (P2X and P2Y) with different transduction mechanisms activated by nucleotides. P2X are ATP-gated cation channels (Khakh et al., 2001; North, 2002), permeable to sodium, potassium, and calcium ions. The P2X gene family consists of seven subunits (P2X1-7) that can assemble into homomeric or heteromeric combinations. P2Y are metabotropic G protein-coupled receptors. P2Y receptors are composed of eight different G protein-coupled receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) that trigger a variety of downstream intracellular pathways (Abbracchio et al., 2009; Inoue, 2006). The missing numbers in the P2Y nomenclature are either non-mammalian orthologs or orphan receptors.

1.3.3 Physiology and pathophysiology of purinergic receptors

In mammals, P2 receptors are widely expressed throughout the body. They play a role in numerous physiological functions, and have both short-term and long-term effects, generally performed by P2X and P2Y respectively. In the CNS, P2 receptors are expressed in all cell types where they participate in neuroinflammation, neurotransmission, synaptic plasticity, and neuromodulation. They are also involved in development, cell proliferation and differentiation, cell death, motility, regeneration, and repair. ATP and P2 receptors even play a role to the five senses. However, they also contribute to many neurodegenerative diseases and chronic pain. This thesis is mainly focusing on two P2 receptor subtypes described in more detail in the next sections.

1.4 Microglial purinergic receptors

A subset of P2 receptors is expressed in microglia, i.e. P2Y12, P2Y13, P2Y6, P2X4 and P2X7. They participate in microglial crucial functions such as surveillance, chemotaxis, motility, activation, proliferation, inflammation, and phagocytosis. Nevertheless, they have been involved in the development of pathology such as chronic neuropathic pain.

1.4.1 Microglial P2Y12 and P2Y13 receptors

We previously mentioned that microglia possess the ability to adjust their genetic profile based on the needs of their surroundings. However, despite these changes, microglia express their own specific genetic profile. P2Y12 receptor (P2Y12) is now considered as one of these signature genes and it is recognized as a genetic marker of microglia. P2Y12 and P2Y13 (P2Y12/13) are selectively activated by ADP and are coupled to Gi proteins, their activation resulting in the inhibition of adenylate cyclase and a decrease of cyclic adenosine monophosphate (cAMP).

P2Y12/13 have been shown to be involved in the surveillance and the motility of microglia. Nevertheless, two different mechanisms of motility have been described (Madry et al., 2018; Smolders et al., 2019). Under physiological conditions, as previously discussed, microglia are in constant extension and retraction of their processes to survey their local environment. Under pathological conditions or in the presence of a danger signal, microglia move toward the site of injury by sensing chemoattractant molecules that are released. P2Y13 has been shown to be critical in the surveillance of the environment operated by microglia (Kyrargyri et al., 2020), whereas P2Y12 is involved in the motility of microglia (Agostinho et al., 2020; Fan et al., 2017). The mechanism of active

surveillance depends on the coupling between P2Y13 and a two-pore domain potassium channel, THIK-1 (TWIK-related halothane-inhibited K+ channel), a microglial function that is not P2Y12-dependent (Sipe et al., 2016). Nevertheless, activation of P2Y12 is necessary for the motility of microglia toward the site of injury, a mechanism that is termed chemotaxis. The molecular mechanism of chemotaxis is not totally understood, yet it has been demonstrated that P2Y12 can induce the activation of phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) signaling pathways, leading to an increase in intracellular calcium concentration and activation of protein kinase B (Akt) to induce movement and the phenotypic transition from surveying to amoeboid microglia (Irino et al., 2008). Intriguingly, the participation of another P2 receptor has been showed to be involved in this function, as the ATP-gated channel P2X4 receptor (P2X4) is interacting with P2Y12 to regulate chemotaxis (Ohsawa et al., 2007).

P2Y12 is also involved in physical interactions with neurons, a fundamental step in the direct communication of microglia with neurons (Agostinho et al., 2020). It has been shown to be responsible for microglial interaction at the synapse (Schafer et al., 2013) and at the neuronal cell body (Cserép et al., 2020). The interaction and direct communication of microglia with neurons through P2Y12 confirm the key role of microglia not only in pathological conditions but also in physiological conditions. These discoveries highlight the contribution of microglia to the synapse, supporting the concept of a quadripartite structure. The classical tripartite synapse involves the presynaptic neuron, the postsynaptic neuron, and an astrocyte, forming a functional unit modulating the release of synaptic neurotransmitters (Araque et al., 1999). However, recent evidence suggests that microglia could constitute a key fourth element at the synapse, due to their

capacity to influence and interact dynamically with both neurons and astrocytes (Schafer et al., 2013).

P2Y12 has been shown to play a role in neurodegenerative diseases such as MS (Amadio et al., 2010), brain ischemia (Gelosa et al., 2014; Webster et al., 2013), and chronic neuropathic pain (Kobayashi et al., 2008).

Interestingly, there is a functional complementarity between microglial P2Y12 and P2Y6 receptors in the presence of an injury in the CNS. First, microglia move toward the site of injury upon P2Y12 activation, then P2Y6 activation induces phagocytosis to clear cellular debris. This duality is characterized by a high expression of P2Y12 and a low expression of P2Y6 during surveying activity and chemotaxis, followed by low expression of P2Y12 and high expression of P2Y6 during phagocytosis.

1.4.2 Microglial P2Y6 receptors

In the CNS, P2Y6 is almost exclusively expressed in microglia. P2Y6 is selectively activated by UDP (UDP > UTP > ADP) and is coupled to Gq proteins which results in the activation of the PLC-β/IP3 pathway, leading to the rise of intracellular calcium and PKC activity. Microglial P2Y6 has been shown to induce phagocytosis (Inoue, 2007; Koizumi et al., 2007).

1.4.2.1 P2Y6 and phagocytosis

Phagocytosis is a cellular process, characterized by the engulfment of particles (>0.5 µm) such as neuronal debris within a plasma-membrane envelope. This mechanism is required to eliminate pathogens, cellular debris, or dead cells, and it contributes to synaptic pruning. Phagocytosis consists of surrounding the target with the cell's plasma membrane to form a vesicle (phagosome). This vesicle then fuses with lysosomes,

cytoplasmic organelles that contain digestive enzymes. This fusion finally leads to lysis and elimination of the targeted cellular material within the microglia, a critical process for immune defense and tissue homeostasis.

As discussed above, UDP can be released by stressed or damaged neurons in the extracellular space. The increase of extracellular UDP, leads to an increase of microglial P2Y6 expression and its activation. It has been demonstrated that microglial P2Y6 activated by UDP induces phagocytosis and clearance of neuronal debris (Koizumi et al., 2007). In addition, blocking P2Y6 activation by the selective antagonist MRS2578 or through genetic ablation prevents microglial phagocytosis (Neher et al., 2014; Puigdellivol et al., 2021; R. X. Wen et al., 2020). Recently, it has been proposed that microglial P2Y6 is involved in phagocytosing stressed but viable neurons. However, P2Y6 activation would not induce the elimination of healthy cells, dead cells, or cellular debris, as these processes do not entail the release of UDP (Puigdellivol et al., 2021). In addition, microglial P2Y6 has been shown to participate in the phagocytosis of synapses during development (Dundee, Puigdellivol, Butler, & Brown, 2023) and aging (Dundee, Puigdellivol, Butler, et al., 2023). Moreover, the stimulation of P2Y6 by UDP is also able to block ATP-dependent migration of microglia through the inhibition of P2X4 (Bernier et al., 2013), confirming close functional links between several P2 receptors, that influence each other to perform different tasks. This is in concordance with the differential expression observed between P2Y12 and P2Y6.

1.4.2.2 P2Y6 and cytokines

It remains an open question whether microglial P2Y6 is involved in the production and release of cytokines and chemokines. It has been shown, that UDP, through

activation of P2Y6, promotes the synthesis and genetic expression of CCL2 in microglia obtained from the CNS and spinal cord of rodents (Kim et al., 2011; Morioka et al., 2013). Another study demonstrated that, in a rat model of mechanical allodynia and thermal hyperalgesia, IL-6 release was triggered by P2Y6 through recruitment of the JAK/STAT pathway (Bian et al., 2019). Finally, another group showed that inhibition of P2Y6 decreases the mRNA levels of various cytokines such as TNF-α, IL-6, or MIP-2/IL-8 in primary microglia primed by LPS (Yang et al., 2017). However, MRS2578, a selective antagonist of P2Y6, did not influence the mRNA levels of several cytokines (Ruo-Xue Wen et al., 2020) and it did not have a measurable effect on the proliferation or release of cytokines (Neher et al., 2014). These contradictory results highlight the complexity of microglial physiology and could be explained by the known phenotypic and genetic heterogeneity of microglia.

1.4.2.3 P2Y6 and neuropathology

There is evidence for a role of P2Y6 in various neurodegenerative diseases or in chronic pain. Yet, it is not completely understood if P2Y6 plays beneficial or detrimental roles in specific neurological disorders. P2Y6 expression is increased after ischemic stroke, likely to clear the debris through the mediation of microglial phagocytosis (Ruo-Xue Wen et al., 2020). In AD, P2Y6 activation improves microglial clearance of amyloid debris. The elimination of neuronal debris by P2Y6 activation-dependent phagocytosis might be beneficial to control neuroinflammation; however, this phagocytotic pathway could also be detrimental by eliminating viable neurons, leading to neurodegeneration (Anwar et al., 2020; Woods et al., 2016). In PD, a correlation has been observed between the upregulation of P2Y6 and the upregulation of inflammatory cytokines, suggesting that

P2Y6 not only participates in phagocytosis but also contributes to neuroinflammation (Yang et al., 2017). It has also been demonstrated that activation of P2Y6, via the “eat-me” signal phosphatidylserine, eliminates viable neurons in a Parkinson model induced by rotenone (Emmrich et al., 2013). In addition, P2Y6 inhibition by the antagonist MRS2578 prevented dopaminergic neuron death (Oliveira-Giacomelli et al., 2019) and inhibited LPS-induced neuronal loss, suggesting that inhibition of P2Y6 could be beneficial to limit neuronal loss in PD (Milde et al., 2021). In neuropathic pain, P2Y6 has been involved in the release of IL-6 via JAK/STAT pathway activation, a pathway that may be involved in inflammation and induction of chronic neuropathic pain (Bian et al., 2019). In addition, an upregulation of the P2Y6 receptor after peripheral nerve injury has been reported, and P2Y6 is involved in mechanical allodynia and thermal hyperalgesia (Huang et al., 2018). Inhibition of this UDP receptor can alleviate the pain response (Huang et al., 2018) and have an antiallodynic effect (Wang et al., 2019). Boosting the activation of P2Y6 could be a therapeutic strategy to improve the beneficial effects of clearing cellular debris and facilitating tissue repair. Moreover, P2Y6 may participate in the release of cytokines in specific disease states (Bian et al., 2019; Yang et al., 2017). Therefore, the use of a modulatory P2Y6 ligand in function of the disease stage could represent a relevant approach to promote productive inflammation (Parisien et al., 2022). However, if the clearance of debris can be beneficial, it may become counter-productive at later stages (Anwar et al., 2020). More research on P2Y6 is needed to better comprehend its different roles leading to specific cellular responses.

1.4.3 Microglial P2X4 receptors

The ATP-gated receptor-channel P2X4 is largely expressed throughout the CNS in neurons but also in glia and in particular in microglia. P2X4 is activated by extracellular ATP, which opens its non-selective cation channel to allow calcium, sodium, and potassium ions to pass through. P2X4 is predominantly expressed intracellularly in the lysosomal compartments (Qureshi et al., 2007) and is in constant trafficking to the plasma membrane following inflammatory stimuli (Raouf et al., 2007). My first publication as a first co-author underlies the importance of the internalization of P2X4. We created a transgenic knock-in mouse line expressing a mutated P2X4 to prevent its constitutive endocytosis, producing a genetic model that mimics pathological states in which P2X4 is upregulated. This resulted in impairment of synaptic plasticity in the hippocampus and caused memory deficits as well as anxiety (Bertin et al., 2021). Though, little is known about the intracellular role of P2X4, it might have more of a biological importance intracellularly than researchers previously had thought.

Activation of microglia during pathological conditions, such as chronic neuropathic pain, is mostly dependent on the recruitment of P2X4 at the cell surface. Upregulation of the transcription factor interferon regulatory factor (IRF) 8 in microglia induced by nerve injury leads to the translocation of IRF5 via fibronectin that binds to the P2X4 promoter and increases its expression (Masuda et al., 2014). The IRF8/IRF5/P2X4 pathway participates in the etiology of chronic neuropathic pain and pain hypersensitivity. Indeed, in neuropathic pain models, P2X4 is upregulated in the dorsal horn of the spinal cord where activated microglia promote hyperexcitability. The activation of P2X4 receptors by ATP leads to the calcium-dependent release of the neurotrophin BDNF, which activates

its neuronal cognate receptor tyrosine receptor kinase B (TrkB) expressed in secondary sensory neurons in lamina I of the spinal cord. Engagement of this P2X4/TrkB pathway leads to downregulation of the potassium chloride transporter 2 (KCC2), producing an increase of intracellular levels of chloride ions and resulting in disinhibition due to suboptimal GABAergic inputs. This pathway also potentiates postsynaptic ionotropic glutamatergic signaling via the NMDA receptor, leading to postsynaptic hyperexcitability (Malcangio, 2017; Stokes et al., 2017). These P2X4-dependent mechanisms mediate allodynia and hyperalgesia, as selective inhibition of P2X4 signaling alleviates these symptoms (Inoue, 2019). Accordingly, downregulation of P2X4 expression or genetic ablation of P2X4 also reduces allodynia (Inoue, 2019). P2X4 has been shown to be upregulated in various neurodegenerative diseases (such as in AD or PD) and could have an impact on neuroinflammation, chemotaxis (post ischemic inflammation), or phagocytosis of myelin in MS (Montilla et al., 2020).

1.4.4 Microglial P2X7 receptors

The receptor-channel P2X7 is a cation-selective channel that is gated by high concentrations of extracellular ATP (EC50= 780 µM) or by the exogenous agonist benzoyl adenosine 5'-triphosphate (BzATP, EC50= 285 µM for mouse and 7 µM for human). Microglia is the cell type where P2X7 is expressed the most in the CNS; however, this receptor is also expressed in other glial cells, including astrocytes (Bhattacharya & Biber, 2016; Sperlagh & Illes, 2014).

1.4.4.1 P2X7 and inflammation

The central role of P2X7 in driving inflammation has been well-documented. As previously mentioned, microglia can detect PAMPs and DAMPs, a crucial step in

mediating inflammation through P2X7 activation. Indeed, recruitment of the P2X7 pathway leads to the activation of the NLRP3 inflammasome. Following major tissue damage, high concentrations of ATP (DAMPs) in the extracellular space can bind and activate the P2X7 receptor. Calcium and sodium ions (inward), as well as potassium ions (outward), flow through the P2X7 channel. The efflux of potassium ions is a crucial step in activating the NLRP3 inflammasome, though it is not entirely clear which molecular component is primarily responsible for this efflux. It was suggested that two-pore domain potassium (K2P) channels including THIK1 and TWIK2 were the main mediators of this drop in intracellular potassium (Di et al., 2018; Drinkall et al., 2022). However, a more recent study establishes that P2X7 is mainly responsible for the potassium efflux, while THIK1 may have another intracellular role (Rifat et al., 2024). Despite doubts regarding what mediates this potassium efflux, this step is fundamental for inducing a gradual recruitment of pore-forming pannexin 1 and activating the NLRP3 inflammasome. The NLRP3 inflammasome activates caspase1 (by cleavage of the immature caspase1), which finally leads to the release of the pro-inflammatory cytokine IL-1β and IL-18 (Di et al., 2018; Giuliani et al., 2017). It is now admitted that two consecutive steps involving PAMPs, and DAMPs are required for the effective release of IL-1 β from microglia. Activation of toll-like receptor 4 (TLR4) or TLR1/2 by PAMPs (such as lipopolysaccharide LPS and Pam3CSK4) leads to the intracellular accumulation of pro-IL-1β in the cytosol, then activation of P2X7 by the DAMP ATP produces the release of mature IL-1β in the extracellular space. P2X7 activation can also promote the formation of reactive oxygen species (ROS) via activation of the p38 MAPK-dependent NADPH oxidase pathway

(Parvathenani et al., 2003) and has been shown to be involved in the release of other pro-inflammatory cytokines such as TNF-α and IL-6 (Shieh et al., 2014).

1.4.4.2 Other functions of P2X7

P2X7 has the capacity to conduct large organic cations by forming a large conductance pore permeable to large fluorescent molecules such as Yo-Pro (Di Virgilio et al., 2018; Harkat et al., 2017; Karasawa et al., 2017). This is a property that P2X7 shares with a few ionotropic P2 receptors such as P2X4 (Wei et al., 2016). A sustained activation of P2X7 by ATP, leading to the opening of the large pore, induces a significant rise in intracellular calcium ions and a subsequent ionic imbalance that causes cell death. In addition, P2X7 is considered as one “death/suicide” receptor (Virgilio et al., 1998) as the recruitment of caspases through P2X7 signaling is able to induce apoptosis (Kopp et al., 2019; Savio et al., 2018). It should be noted, however, that, in opposition to cell death, this large pore function has been associated with promoting microglial proliferation (Monif et al., 2016). Preventing P2X7 pore dilation has been shown to stop the proliferation of microglia and P2X7 participates in the growth of microglial cell populations during development via an IL-1β-dependent mechanism (Rigato et al., 2012). Depending on the amount of ATP present, P2X7 could facilitate phagocytosis (in the absence of ATP), contribute to proliferation (with low ATP levels), or induce cell death (with high ATP levels) (Kanellopoulos & Delarasse, 2019).

1.4.4.3 P2X7 and neuropathology

The release of pro-inflammatory cytokines and ROS induced by P2X7 signaling has been involved in the pathophysiology of neurodegenerative diseases and chronic pain (Savio et al., 2018). Cell death mediated through P2X7 has also been linked to

several neurodegenerative diseases. Generally, there is increasing evidence that inhibiting the activity of P2X7 in pathological conditions is beneficial in order to limit excessive inflammation and neurotoxicity.

In AD, P2X7 expression is upregulated in activated microglia that surround Aβ plaques (Parvathenani et al., 2003). In addition, Aβ plaques cause an increase in IL-1β, which is P2X7-dependent (Sanz et al., 2009). Pharmacological inhibition or genetic silencing of P2X7 is sufficient to prevent both the activation of microglia and the neuroinflammation that is linked to the presence of Aβ plaques (Carvalho et al., 2021; Y. H. Chen et al., 2021; Francistiova et al., 2020; Thawkar & Kaur, 2019). Moreover, the ATP/P2X7 pathway enhances microglial migration toward Aβ plaques and reduces microglial phagocytic capability (Martinez-Frailes et al., 2019), an effect that is reversed by the silencing of P2X7 function (Martin et al., 2019).

In PD, P2X7 is upregulated and participates in gliosis, synaptotoxicity, and neurotoxicity (Carmo et al., 2014; Ren et al., 2021; Van Weehaeghe et al., 2019). α-synuclein binds and activates P2X7 in microglia, leading to the generation of ROS through the PI3K/AKT pathway (Jiang et al., 2015), which impair dopaminergic and glutamatergic transmissions and cause neurotoxicity. In addition, inhibition of P2X7 has been shown to attenuate the symptoms of PD (Carmo et al., 2014) and to reduce the loss of dopaminergic neurons in the substantia nigra (Dutta et al., 2008).

P2X7 is upregulated in MS and appears to be deleterious, contrary to P2X4 (Domercq & Matute, 2019). Exposure to an antagonist of P2X7 in a preclinical model of MS partially inhibits the symptoms and reduces the level of the cytokines IL-1β, TNF-α, and IL-6 (Sharp et al., 2008). Similarly, P2X7 is upregulated in ALS and is involved in

chronic central inflammation and neurotoxicity, two key features of the disease prevented by blocking P2X7 receptor activity (Apolloni et al., 2013; D'Ambrosi et al., 2009).

It has been demonstrated that P2X7 activation by endogenous ATP contributes to the development of chronic inflammatory and neuropathic pain (Tsuda, 2017). Inhibition of P2X7 by the antagonist A-740003 reduces neuropathic pain (Burnstock & Knight, 2018). In particular, the release of IL-1β through the P2X7 pathway has been shown to be a key event in chronic inflammation and pain. Indeed, blockade of P2X7 reduces both mechanical allodynia and thermal hyperalgesia in a rodent model of pain (Clark et al., 2010; Honore et al., 2009). Recently, an interesting hypothesis has identified P2X7 as one of the candidate receptors responsible for tolerance following chronic morphine use. Indeed, upregulation of P2X7 expression has ben observed following opioid treatment and treatment with a P2X7 antagonist prevents the analgesic tolerance to morphine (Leduc-Pessah et al., 2017). It was reported that morphine treatment after peripheral nerve injury acts as a persistent DAMP via recruitment of P2X7, TLR4, and caspase1, all involved in the formation and activation of the inflammasome NLRP3 (Grace et al., 2018).

1.5 The noradrenergic system

The main source of central norepinephrine comes from a small nucleus in the brainstem called the locus coeruleus (LC). From this small population of neurons originate projections that target many diverse regions in the CNS to exert neuromodulation through the release of norepinephrine. Norepinephrine acts on a set of metabotropic adrenergic receptor subtypes that are widely expressed throughout the CNS (in various regions and cellular types). The noradrenergic system modulates numerous major central functions such as sleep and wake states, sensory discrimination, intrinsic excitability,

neuroinflammation, and pain. Adrenergic receptors are composed of two main groups of GPCRs: α and β receptors. α receptors include three α1 subtypes (α1A, α1B, and α1D) coupled to Gq/11 proteins and three α2 subtypes (α2A, α2B, and α2C) coupled to Gi/o proteins. β receptors include the β1, β2, and β3 subtypes, all coupled to Gs proteins. Through activation of these adrenergic receptors in different targets of LC projections, the noradrenergic system has been shown to be affected in neurodegenerative diseases such as AD and PD.

1.5.1 The antinociceptive effect of the noradrenergic system

In the pain circuit, the noradrenergic descending fibers originating from the LC are activated in response to noxious stimuli mediating spinal antinociceptive effects (Pertovaara, 2006). When these noradrenergic descending pathways are activated, norepinephrine released in the dorsal horn of the spinal cord inhibits the transmission of nociceptive signals coming from the dorsal root ganglia. By activating presynaptic α2 adrenergic receptors that inhibit voltage-gated calcium channels and postsynaptic α2 adrenergic receptors that open potassium channels, norepinephrine decreases glutamatergic transmission (Kawasaki et al., 2003; Sonohata et al., 2004). It was also suggested that adrenergic descending pathways mediate indirect inhibition in the spinal cord via GABAergic interneurons located in the superficial lamina of the dorsal horn (Pertovaara, 2006). Altogether, these noradrenergic mechanisms prevent the transmission of abnormal nociceptive inputs and control the spread of noxious signals by differentiating the site of injury from adjacent areas. Moreover, it is thought that the gabapentinoids, commonly used in chronic pain treatment, are achieving their analgesic effects through the noradrenergic descending pathway from the LC to the dorsal horn of

the spinal cord (Hayashida & Obata, 2019). Additionally, stimulation of the noradrenergic descending pathway has been shown to inhibit mechanical pain and thermal hypersensitivity, whereas a lesion of the LC induces an increase in pain responses (Hayashida et al., 2008).

1.5.2 The β2 adrenergic receptor and microglia

Interestingly, norepinephrine can also regulate microglial activity, in particular its activation and the release of cytokines. Indeed, microglia express noradrenergic receptors, specifically the β2 adrenergic receptor subtype (ADRB2). In the CNS, the expression of the ADRB2 is typical of the microglial genetic profile as shown in the RNA sequencing data from the Linnarsson lab (Zeisel et al., 2018). Through ADRB2 signaling, norepinephrine has been shown to inhibit the activation of microglia in various regions of the brain (Sugama et al., 2019). Moreover, selective ADRB2 agonists have been found to suppress LPS-induced release of microglial cytokines such as IL-6, IL-1β, TNF-α (Heneka et al., 2010; Qian et al., 2011), or CSF-1 (Damo et al., 2023).

Recently, microglial ADRB2 has been reported to influence microglial filopodia extension (Bernier et al., 2019). Filopodia, located at the tip of microglial processes, scan the environment for cues at a nanoscale level. The growth of these filopodia is driven by intracellular cAMP. Norepinephrine, through ADRB2, has been shown to contribute to cAMP-driven filopodia extension (Bernier et al., 2019). In addition, ADRB2 has been shown to regulate microglial dynamics differently in awake and anesthetized mice (Liu et al., 2019; Stowell et al., 2019). Lower levels of norepinephrine and reduced neuronal activity in anesthetized mice increase microglial arborization and surveillance. Treatment with the selective ADBR2 agonist clenbuterol in anesthetized mice reduces microglial

processes and surveillance. Selective blockade of ADRB2 or inhibition of the LC increases the monitoring range of microglia. Furthermore, norepinephrine has been shown to reduce contact areas between microglia and neurons (Liu et al., 2019; Mercan & Heneka, 2019; Stowell et al., 2019). Taken together, these findings on the noradrenergic modulation of microglial arborization and surveillance suggest a functional interaction between Gs-coupled ADRB2 and Gi-coupled P2Y12.

New evidence suggests an upregulation of ADRB2 in microglia after peripheral nerve injury. The authors reported that activation of microglial ADRB2 suppresses pro-inflammatory signaling and attenuates mechanical and cold allodynia in chronic neuropathic pain (Damo et al., 2023). In addition, duloxetine, an inhibitor of norepinephrine reuptake, has been shown to inhibit microglial P2X4 by reducing the ATP-evoked increase in intracellular calcium levels (Yamashita et al., 2016), indicating that the noradrenergic system is able to modulate pain-related P2X4-mediated neuroinflammation.

Taken together, there is increasing evidence that ADRB2 represents a physiologically relevant candidate for modulating purinergic signaling in microglia.

Objectives

Based on our review of literature, which critically outlines the need for a better understanding of microglial mechanisms that contribute to clinically relevant pathological states, the goal of this dissertation is to decipher the modulation of purinergic signaling involved in chronic pain and/or neurodegenerative diseases to identify new potential therapeutic targets.

Nucleotides such as ATP and UDP are released in the extracellular space in large amounts following cell stress, cell damage or cell death, and they act as danger signal to microglia. P2Y6, a pyrimidinergic GPCR mainly expressed in microglia in the mammalian CNS, is the UDP sensor linked to phagocytosis (Koizumi et al., 2007). Modulation of P2Y6 activity to improve the beneficial effects and reduces the detrimental effects is promising as therapeutic target, thus, we aim to investigate a potential candidate as a modulator of microglial P2Y6.

Although Chapter 2 will investigate P2Y6 modulation, other P2 receptors carry important physiological functions in microglia. In particular, the ionotropic purinergic receptor P2X7 plays a major role in the release of inflammatory cytokines. That is why Chapter 3 will focus on P2X7 and the need to better understand mechanisms that could regulate its activity, knowing that several antagonists targeting P2X7 fail to pass clinical studies (Territo & Zarrinmayeh, 2021).

Thus, our main objective is to identify in microglia a signaling pathway involved in the modulation of P2Y6-dependent phagocytosis and P2X7-dependent cytokine release.

Based on expression data and physio-pharmacological evidence, our working hypothesis states that the norepinephrine/ADRB2 signaling pathway is a relevant candidate for regulating key P2Y6 and P2X7 functions in microglia. As primary objectives, we want to determine if the GPCR ADRB2 modulates the activity of P2Y6 receptors and P2X7 receptors in rodent microglia and if so, whether this modulation also impacts key P2Y6- and P2X7-dependent functions, i.e. phagocytosis and pro-inflammatory cytokine release, respectively. A second objective is to investigate if the modulation of P2Y6 and P2X7 by ADRB2 is conserved in human microglia too. Finally, to identify possible mechanisms, quantitative PCR experiments have been performed to evaluate the expression levels of P2Y6 and P2X7 after ADRB2 activation.

To this end, the main objectives of this dissertation will be:

1) Determine whether ADRB2 has a neuromodulatory effect on microglial P2Y6 function.
    a. Whether P2Y6-dependent phagocytosis is regulated by ADRB2.
    b. Whether P2Y6 is involved in cytokine release.
    c. Whether this modulation is conserved in human.
    d. Investigate a possible mechanism.
2) Determine whether ADRB2 has a neuromodulatory effect on microglial P2X7 function.
    a. Whether P2X7-dependent cytokine release is regulated by ADRB2.
    b. Whether this modulation is conserved in human.
    c. Investigate a possible mechanism.

Chapter 2

Adrenergic control of phagocytosis in rodent and human microglia

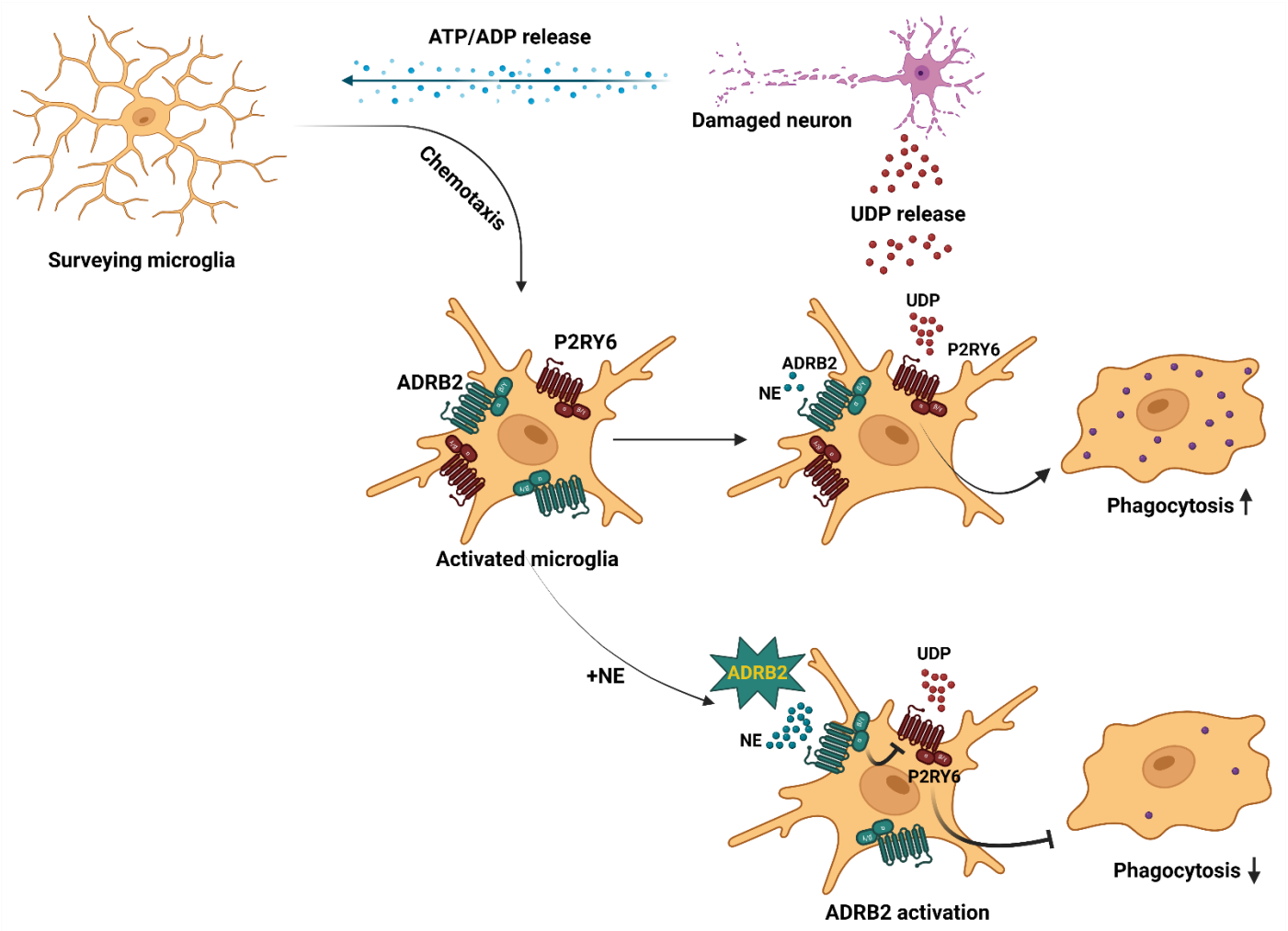
Thomas DELUC[1,2], Marie-France DORION[1], Gilles MAUSSION[1], Yeman TANG[1], Rita T.M. LO[1], Ariel ASE[1,2], Thomas M. DURCAN[1], Stefano STIFANI[1] and Philippe SÉGUÉLA[1,2,3]

[1] Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Canada

[2] Alan Edwards Centre for Research on Pain, McGill University, Montreal, Canada

[3] Correspondence: philippe.seguela@mcgill.ca

Graphical abstract



Abstract

Microglia, the resident immune cells of the CNS, are in constant survey of their environment. Extracellular nucleotides, released by stressed or damaged neurons, act as danger signals to microglia through various purinergic/pyrimidinergic (P2) receptors. P2 receptors are essential to key microglial functions such as chemotaxis, phagocytosis, and release of cytokines. They have been shown to play significant role in neuroinflammation and chronic pain. In the CNS, the UDP receptor P2Y6 is mostly expressed in microglia where its activation induces phagocytosis, a function dysregulated in most neurodegenerative diseases and in chronic pain. Yet, regulation of P2Y6 activity has not been studied in depth. The microglial β2 adrenergic receptor (ADRB2) represents a promising candidate for modulation of P2Y6 receptors by norepinephrine. Our calcium imaging data indicate that the ADRB2 agonist isoproterenol inhibits the calcium transients evoked by activation of P2Y6 receptors in primary mouse microglia, and this functional modulation is conserved in human iPSC-derived microglia. In agreement with these results, we also found that the phagocytotic activity induced by P2Y6 is reduced by ADRB2 signaling in both primary mouse microglia and human iPSC-derived microglia. Finally, we report that activation of ADRB2 is linked to a decrease in the expression levels of P2Y6 mRNA. These findings provide evidence that selective intracellular crosstalk between nucleotidic and adrenergic transductions control innate immune responses in the CNS, potentially contributing to the pathophysiology of neurodegenerative disorders and chronic pain.

Keywords: Microglia, Purinergic receptor, Phagocytosis, Adrenergic receptor

Introduction

Microglia, resident immune cells in brain and spinal cord, are emerging as central actors in the healthy and disease states of the central nervous system (CNS) by performing key functions such as secretion of growth factors and cytokines, chemotaxis, and phagocytosis (Butovsky & Weiner, 2018). Microglial contribution is essential to maintain brain homeostasis, for the neurodevelopment, synaptic plasticity, and respond to injury by mediating synaptic pruning. Microglial phagocytosis, a cellular process that consists of engulfing particles (>0.5 μm) within a plasma-membrane envelope, is necessary for immune defense and tissue homeostasis by eliminating pathogens, cellular debris, or dead cells, and contributing to synaptic pruning. One major function of microglia involves their ability to identify danger signals (such as ATP) and “eat-me” signals (such as UDP) to initiate phagocytosis. However, dysregulation of microglial mechanisms has been demonstrated to contribute to central neuroinflammation seen in many neurodegenerative conditions and chronic pain.

The P2Y6 receptor, mainly expressed in microglia in the CNS, is selectively activated by UDP and is coupled to Gq proteins, resulting in the recruitment of the PLC/IP3 pathway. UDP, which is released by stressed or damaged neurons, acts as an “eat-me” signal that results in increased expression and activation of microglial P2Y6 to induce phagocytosis and clearance of neuronal debris (Inoue, 2007; Koizumi et al., 2007). Moreover, inhibition of P2Y6 activity by the selective antagonist MRS2578 or by genetic ablation prevents microglial phagocytosis (Neher et al., 2014; Puigdellivol et al., 2021; R. X. Wen et al., 2020), confirming the importance of P2Y6 in the regulation of this function. It has been suggested that P2Y6 mediates phagocytosis of stressed but viable neurons,

however, microglia are not able to engulf healthy cells, dead cells, cellular debris, or beads since these processes do not involve the release of UDP (Puigdellivol et al., 2021). Recent studies reported the involvement of microglial P2Y6 in the phagocytosis of synapses during development (Dundee, Puigdellivol, Butler, & Brown, 2023) and aging (Dundee, Puigdellivol, Butler, et al., 2023). In addition, microglial P2Y6 has been associated with the release of cytokines, yet its role is not completely clear.

P2Y6 has been shown to be involved in various neurodegenerative diseases and chronic pain, however, it is not completely understood whether P2Y6 is beneficial or detrimental. P2Y6 is upregulated in pathological states such as ischemic stroke, Parkinson's disease, and neuropathic pain. P2Y6-evoked phagocytosis has been shown to be important in the clearance of cellular debris after ischemic stroke (R. X. Wen et al., 2020), or amyloid debris in Alzheimer's disease (Anwar et al., 2020). However, even if P2Y6-induced phagocytosis appears beneficial in neurodegenerative diseases or chronic pain, this phagocytotic pathway could also be harmful, as it may remove healthy neurons, ultimately contributing to neurodegeneration (Anwar et al., 2020; Woods et al., 2016). For example, the inhibition of P2Y6 prevented dopaminergic neuronal death in PD (Oliveira-Giacomelli et al., 2019) and may represent a good strategy in PD (Silva et al., 2023). P2Y6 has also been associated with neuroinflammation by contributing to the release of cytokines in PD (Yang et al., 2017) and neuropathic pain (Bian et al., 2019). After nerve injury, upregulated P2Y6 has been shown to participate in mechanical allodynia and thermal hyperalgesia (Huang et al., 2018). Its inhibition alleviates pain response (Huang et al., 2018) and induces antiallodynic effects (Wang et al., 2019). Promoting the activation of P2Y6 could be a therapeutic approach to enhance the beneficial effects of

productive inflammation, clearing cellular debris, and facilitating tissue repair. However, since promoting inflammation and excessive phagocytosis could have adverse effects in the long-term, further research on P2Y6 is essential to fully understand its various roles and contributions to specific cellular responses. In light of this, it is crucial to investigate modulatory mechanisms of microglial P2Y6 to better regulate its activity.

Release of norepinephrine by noradrenergic fibers originating from a small nucleus of the brainstem, the locus coeruleus (LC), is known to play a major neuromodulatory role by acting on adrenergic receptors widely expressed throughout the CNS. Several studies have revealed that norepinephrine can modulate microglial cells via the β2 adrenergic receptor (ADRB2), which is the major adrenergic receptor expressed in this cell type (Zeisel et al., 2018). Activation of ADRB2 by norepinephrine inhibits microglial activation across different brain regions (Sugama et al., 2019) and suppresses the release of microglial cytokines such as IL-6, IL-1β, TNF-α (Heneka et al., 2010; Qian et al., 2011), or CSF-1 (Damo et al., 2023) induced by LPS. Microglial phagocytosis has also been shown to be decreased by the stimulation of the non-selective β-adrenergic receptor agonist isoproterenol (Steininger et al., 2011). Interestingly, microglial ADRB2 has been reported to regulate cAMP-driven filopodia extension (Bernier et al., 2019), and to inhibit microglial surveillance (Liu et al., 2019; Stowell et al., 2019), suggesting a link between ADRB2 and P2Y12-dependent function. Furthermore, administration of the norepinephrine reuptake inhibitor duloxetine to microglia has been shown to reduce calcium levels triggered by ATP via P2X4 receptor-channels (Yamashita et al., 2016). Altogether, adrenergic transduction via ADRB2 represents a physiologically relevant candidate to modulate the functions driven by P2Y6 in microglia.

Materials and Methods

Mouse primary microglia

Primary microglia were collected from the brains of P1 to P4 C57BL/6 wild-type mice (Charles River Canada). Briefly, cortices were isolated and trypsinized using 0.25% trypsin-EDTA for 30 minutes at 37 °C. After successive centrifugations, washes in microglia medium (composed of DMEM/F12 (1:1), FBS and penicillin/streptomycin), and trituration, cells were filtered through 100-µm pore size nylon strainers to obtain a glial mix. This glial mix, in presence of microglia medium, was cultured in flasks at 37 °C and 5% CO_2. After two weeks, the flasks were shaken for 2 hours to detach microglia. After centrifugation and resuspension, microglia (95% purity) were plated in glass-bottom dishes (MatTek) for use 24 to 48 hours.

Human iPSC-derived microglia

Differentiation of human iPSC (cell line CS29) into microglia was obtained as previously described (Douvaras et al., 2017).

Human primary microglia

Human brain tissues were collected from non-malignant cases of temporal lobe epilepsy (2–71-year-old female and male patients), at sites distant from suspected primary epileptic foci. Microglial isolation was performed as previously described (Durafourt et al., 2013). Briefly, trypsin (Invitrogen) and DNase (Roche) treatment were used to digest tissues and then dissociated mechanically through a nylon mesh filter. Tissue homogenate was then subjected to Percoll (Sigma-Aldrich) gradient centrifugation to isolate the glial cells. Differential adhesive properties of glial cells were used to purify microglia. Microglia were cultured in Minimum Essential Medium (MEM; Sigma Aldrich)

supplemented with 1% GlutaMAX (ThermoFisher Scientific), 1% penicillin/streptomycin (P/S; ThermoFisher Scientific), 0.1% glucose (Sigma Aldrich) and 5% fetal bovine serum (FBS; Wisent Bioproducts) and kept at 37 °C under a 5% CO2 atmosphere. Use of all human cells was approved by the McGill University Health Centre Research Ethics Board.

Calcium imaging

We used Fura2-based ratiometric measurement of [iCa^{2+}] to functionally assess the modulation of ATP receptors by ADRB2 in various cellular models. Primary mouse microglia or human iPSC-derived microglia were loaded with the calcium-sensitive fluorescent dye fura-2 AM for 40 minutes, in a solution at pH 7.4 containing (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, with 1% BSA. After 40 minutes of incubation, cells were incubated for 30 minutes at 37 °C to hydrolyze the acetoxymethyl ester of the dye. Loaded cells were imaged using an inverted microscope (Nikon Eclipse TE300) and were excited every 2 seconds with 340 nm and 380 nm excitation filters controlled with Metafluor (Molecular Devices) to generate ratio (340/380) images corresponding to intracellular calcium concentrations. During experiments, microglial cells were constantly perfused with external solution containing or not the ligands of interest. Double applications (20 seconds) were used to compare second responses to the first ones for intra-cell normalizations. Experiments without treatment between two applications were considered control experiments and are further compared to experiments with a treatment inter-stimulation, to assess a quantitative modulation of ATP receptors by ADRB2 activation. Peak amplitude of the responses was subtracted to baseline ratios (mean of 20 seconds prior the stimulation) and statistical comparisons

were determined using Student's t-tests. Second responses in each experiment (control versus treatments) were compared using one-way ANOVA.

Phagocytosis assay

Our phagocytosis assay was modified from a previously published protocol (Lian et al., 2016). Briefly, after pre-opsonization of red fluorescent latex beads (1 µM, Sigma) in FBS for 1 hour and dilution in microglia medium, beads were added to the glass bottom MatTek dishes and incubated for 2 hours at 37 degrees Celsius. Then, dishes were incubated with treatment (Control, UDP 100 µM, UDP 100 µM /Isoproterenol 20 µM, or Isoproterenol 20 µM) for 45 minutes. At the end of the treatment, cells were washed with cold PBS in presence of the live microglial marker Isolectin GS-IB4-Alexa Fluor™ 488 conjugate for 10 minutes to label microglia. Two additional washes with PBS to remove the excess of beads, then fixation with PFA 4% for 15 minutes were performed before the acquisition of the images using Metamorph (Molecular Devices). A minimum of 3-5 ROIs per dish were imaged to calculate the mean of number of IB4+ cell with beads / number of total IB4+ cells. The mean of each dish was used to make a general mean of a specific experimental group. One way ANOVA and unpaired Student's t-tests were used to statistically compare the groups.

Cytokines assay

Human primary microglia were incubated with treatment (Vehicle, UDP 100 µM, UDP 100 µM /Isoproterenol 20 µM, or Isoproterenol 20 µM alone) for 3 hours and then supernatants were collected. Cytokine measurements were performed using the Human Inflammatory Cytokine Cytometric Bead Array (CBA) kit from bdBiosciences to assess the levels of interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor

necrosis factor alpha (TNF-α). As per the instructions, beads, and detection reagent (PE) were mixed and incubated with the treated microglial cells for 3 hours in the dark at room temperature. After wash, centrifugation and resuspension, the samples were analyzed by flow cytometry to measure the levels of cytokines.

qPCR

RNA was extracted from either control, Pam3CSK4-treated or isoproterenol-treated primary mouse microglia using the RNeasy Mini Kit (Qiagen). After cDNA synthesis, qPCR was carried out using TaqMan universal master mix and gene specific primers (P2Y6: Mm01275472_m1 and ADRB2: Mm02524224_s1). The mean cycle threshold (CT) values were normalized to GAPDH (Mm99999915_g1) and ACTB (Mm01205647_g1). Fold changes were calculated using the delta CT method.

Statistical analysis

Prism 6 (GraphPad) was used for quantitative analysis, statistics, and graphs. One-way ANOVA or unpaired t-tests were performed for statistical analysis.

Results

3.1 Calcium transients evoked by UDP/P2Y6 are inhibited by ADRB2 activation in primary mouse microglia.

The activation of the Gq-coupled P2Y6 receptor by UDP leads to the stimulation of phospholipase Cβ isoforms, followed by the production of inositol trisphosphate, ultimately leading to the release of calcium from intracellular stores. To address whether microglial P2Y6 activity can be modulated by ADRB2, we took advantage of P2Y6 signaling and the fact that activation of ADRB2 by isoproterenol does not influence the calcium status of the cell. We conducted calcium imaging experiments to measure intracellular changes in calcium levels evoked by the endogenous P2Y6 agonist UDP in mouse microglial cells in primary culture. The typical intracellular calcium status before and after stimulation (using 340/380 ratio) is represented in Figure 1A, with average recordings in each condition of the double application protocol (described in the method section) shown in Figure 1B. The functional expression of P2Y6 in mouse microglia was confirmed by recording robust UDP-evoked calcium transients in most cells. Our control experiment, consisting of a double application of UDP (50 µM), showed a decrease in the second response (63 %) indicating desensitization (Figures 1B). To investigate whether ADRB2 can modulate UDP-evoked P2Y6-mediated calcium responses, we exposed the microglial cells to isoproterenol (10 µM), an agonist of β-adrenergic receptors, between two applications of UDP (Figure 1A-B), resulting in a decrease in the second response (30 %). The comparison of second responses with control experiments revealed an inhibitory effect of ADRB2 on P2Y6 calcium response (Figure 1C). To investigate this modulation further, we used IBMX, a phosphodiesterase inhibitor, to mimic the activation of the ADRB2/Gs/adenylate cyclase pathway by increasing intracellular cAMP levels.

Application of IBMX (100 µM) between the stimulations appeared to be comparable to those obtained with isoproterenol treatment, i.e. there was a decrease in the second response compared to the first response (26 %) and a significant inhibitory effect when the second responses were compared to control experiments (Figure 1A-C). Together, these results indicate that calcium transients mediated by P2Y6 and evoked by UDP are inhibited by ADRB2 activation through the Gs-coupled cAMP/PKA pathway in mouse microglia.

3.2 Calcium transients evoked by UDP/P2Y6 are inhibited by ADRB2 in human iPSC-derived microglia.

To determine whether ADRB2 modulation of UDP-evoked P2Y6 calcium transients is conserved in human microglia, we used calcium imaging in iPSC-derived microglia. The average traces of both conditions are shown in Figure 2A. Using the same parameters as in the mouse experiments, we used the double application protocol to first validate the functional expression of P2Y6 by recording robust UDP-evoked transients (Figure 2A). Similarly to the experiment in mice, we observed that the second response was desensitized (63 %) compared to the first response (Figure 2A). Activation of ADRB2 with isoproterenol induced an inhibition of the second UDP-evoked P2Y6 calcium response (41 %), compared to the first response (Figure 2A). The comparison of second responses with control experiments indicated a significant inhibitory effect induced by isoproterenol treatment on the UDP-evoked P2Y6 calcium second response (Figure 2B). Together, these results support the inhibitory effect of ADRB2 on P2Y6 calcium responses and indicate that this crosstalk is conserved between rodents and humans.

3.3 Phagocytosis evoked by UDP/P2Y6 is inhibited by ADRB2 in primary mouse microglia.

Our next goal was to investigate whether key microglial functions mediated by P2Y6 are impacted by ADRB2 modulation. UDP is released by stressed or damaged neurons, acting as a “eat-me” signal through microglial P2Y6 to initiate phagocytosis (Koizumi et al., 2007). Microglial phagocytosis is essential for the clearance of cellular debris, damaged/dead cells, or synaptic pruning (Inoue et al., 2009). Dysfunction of phagocytosis has been reported in neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and chronic pain. To investigate whether UDP-evoked P2Y6-dependent phagocytosis is impacted by ADRB2 modulation, we performed a phagocytosis assay in primary mouse microglia based on the uptake of fluorescent latex beads. Typical images of phagocytosed beads by microglia cells for different treatments are shown in Figure 3A. UDP treatment showed a higher percentage of phagocytosing cells compared to the control condition (Figure 3B), confirming the role of the UDP/P2Y6 pathway in inducing phagocytosis. Co-treatment of UDP and the ADRB2 agonist isoproterenol showed a significant decrease in the number of active phagocytosing cells compared to the group treated with UDP alone (Figure 3B). In addition, treatment with isoproterenol alone did not increase the number of phagocytosing cells compared to the control group (Figure 3C), suggesting that recruitment of the isoproterenol/ADRB2 pathway does not have an impact on the baseline level of phagocytosis in microglia. Together, these data confirm the inhibitory modulation of UDP/P2Y6-induced phagocytosis by ADRB2, in agreement with our calcium imaging results.

3.4 Phagocytosis evoked by UDP/P2Y6 is inhibited by ADRB2 pathway in human microglia.

We then interrogated if P2Y6-dependent phagocytosis can also be modulated by ADRB2 in human microglia by performing a phagocytosis assay with the same parameters as in the mouse experiments, using human iPSC-derived microglia. Typical images of phagocytosed beads by microglial cells for different treatments are shown in Figure 4A. UDP treatment induced a higher percentage of phagocytosing cells compared to the control condition (Figure 4B), confirming the phagocytic role of P2Y6 in human iPSC-derived microglia. Co-treatment of UDP and the ADRB2 agonist isoproterenol significantly decreased the index of phagocytosing cells compared to UDP treatment alone (Figure 4B). Furthermore, isoproterenol treatment without UDP did not increase the number of phagocytosing cells compared to the control group (Figure 4C). Altogether, these results confirmed the modulatory role of ADRB2 on P2Y6-dependent phagocytosis, a modulation that is conserved in human microglia.

3.5 P2Y6 signaling does not modulate the release of cytokines in human primary microglia.

The involvement of microglial P2Y6 in the production and release of cytokines in the CNS is still unclear. To investigate whether microglial P2Y6 could be involved in cytokine release, we performed a cytokine assay to measure the levels of pro-inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), as well as the anti-inflammatory cytokine interleukin 10 (IL-10) in human primary microglia. In addition, we used isoproterenol to see if ADRB2 activation could affect P2Y6 involvement in this function. Although we observed a slight trend of an increase in IL-6 and TNF-α release, acute treatment with UDP (100 μM) did not have a

significant effect on the release of IL-1β, IL-6, TNF-α, or IL-10 compared to the basal level of cytokine release (Figure 5A). Acute treatment with isoproterenol also did not affect cytokine release or influence P2Y6-dependent involvement in cytokine release (Figure 5A). We further investigated whether P2Y6 could influence cytokine release by priming microglia with Pam3CSK4, a TLR1/2 agonist that turns microglia to a pro-inflammatory state. Pam3CSK4 priming significantly increased the release of the cytokines compared to non-primed microglia. However, UDP treatment did not have a significant impact on cytokine release in microglia primed with Pam3CSK4 (Figure 5B). Together, our results demonstrate that P2Y6 is not involved in the release of cytokines (IL-1β, IL-6, TNF-α, or IL-10) in both surveillant and Pam3CSK4-primed human primary microglia.

3.6 ADRB2 downregulates P2Y6 mRNA expression in mouse primary microglia.

Finally, to explore the mechanism involved in the modulation of P2Y6-dependent phagocytosis by ADRB2, we assessed whether P2Y6 gene expression could be affected by microglial priming with Pam3CSK4, a TLR1/2 agonist that activates microglia toward a pro-inflammatory state, or by recruitment of the ADRB2 pathway. We used qPCR in pretreated primary mouse microglia for 24 hours either with Pam3CSK4, or with the ADRB2 agonist isoproterenol. Pretreatment with Pam3CSK4 did not change the expression of P2Y6 compared to the control basal expression. Interestingly, isoproterenol pretreatment significantly decreased P2Y6 expression level compared to the control basal expression (Figure 6A), suggesting that ADRB2 downregulates P2Y6 expression. In parallel, we found that ADRB2 expression was drastically reduced in microglia primed with Pam3CSK4, indicating a lower expression in activated microglia (Figure 6B).

Discussion

We provided evidence that recruitment of the ADRB2 pathway decreases P2Y6 activity in rodent and human microglia. We demonstrated that the activation of ADRB2 by isoproterenol inhibits UDP-evoked P2Y6 calcium transients in both mouse and human microglia. We also used IBMX, a phosphodiesterase inhibitor resulting in an increase of cyclic AMP that mimics the Gs-coupled activation of ADRB2, to confirm the inhibitory effect on P2Y6 calcium transients. Intracellular and extracellular calcium play crucial roles in the regulation of microglial functions (Brawek & Garaschuk, 2013; Sharma & Ping, 2014). Microglial P2 receptors are essential for the fluctuation of intracellular calcium, either through ATP-gated P2X receptors (such as P2X4 and P2X7) that are calcium-permeable cation channels or through Gq-coupled P2Y6 signaling which recruits the PLC/IP3 pathway and leads to transient increases of cytoplasmic calcium ions. Recently, a study demonstrated that the calcium changes generated through UDP-P2Y6 activation is necessary for microglial phagocytosis during epileptogenesis (Umpierre et al., 2024). However, the specific mechanism of UDP/P2Y6-induced phagocytosis is still unknown and needs to be addressed.

Phagocytosis is a protective mechanism carried out by microglia that contributes to shape the neuronal network during neurodevelopment by pruning redundant, underused, or malfunctioning synapses and by eliminating cellular debris and pathogens throughout life. UDP, acting as an “eat-me” signal transduced by microglial P2Y6, has been shown to induce phagocytosis (Inoue, 2007; Koizumi et al., 2007). We provide evidence that UDP-evoked P2Y6-dependent phagocytosis is decreased following ADRB2 activation by isoproterenol in both rodent and human microglia, confirming the effect of

ADRB2 on the calcium response of P2Y6. We can hypothesize that ADRB2 controls P2Y6-dependent phagocytosis through the regulation of P2Y6 calcium transients.

P2Y6, upregulated in several pathological states, has been shown to participate in the clearance of debris in Alzheimer's disease, Parkinson's disease or after an ischemic stroke. Yet, the role of P2Y6 is unclear, as it seems to become detrimental at later stages of these diseases by eliminating viable neurons and participating in neurodegeneration (Anwar et al., 2020; R. X. Wen et al., 2020; Woods et al., 2016). Controlling microglial P2Y6-dependent phagocytosis with adrenergic modulation could help to maintain an homeostatic level of elimination without affecting viable neurons, thereby limiting neuronal loss.

Microglia play an important role in the elaboration and maintenance of neuropathic pain. P2Y6 has been shown to be upregulated and involved in neuropathic pain, as pharmacological or knockout of P2Y6 reduced the pain phenotype (Huang et al., 2018; Wang et al., 2019). However, little is known regarding the importance of microglial P2Y6-dependent phagocytosis in neuropathic pain. A recent study demonstrated that microglia selectively engulf spinal inhibitory synapses at the early onset of the disease, favoring the imbalance of excitation (Yousefpour et al., 2023). Furthermore, it has been shown that microglial P2Y6 participates in the pruning of synapses during aging (Dundee, Puigdellivol, Butler, et al., 2023) and development (Dundee, Puigdellivol, Butler, & Brown, 2023). We could hypothesize that P2Y6 may be involved in the pruning of inhibitory interneurons in neuropathic pain, and ADRB2 can be beneficial in controlling P2Y6 activity.

Several studies have shown the involvement of P2Y6 in the production and release of various cytokines and chemokines: it has been showed to be involved in the expression of CCL2 (Kim et al., 2011; Morioka et al., 2013), IL-6 (Bian et al., 2019), as well as TNF-α, and IL-8 mRNA expression (Yang et al., 2017). However, other groups have reported that the selective P2Y6 antagonist MRS2578 had no effect on mRNA expression levels of various cytokines (R. X. Wen et al., 2020) or on the production and release of cytokines (Neher et al., 2014). In our hands, treatment with UDP to activate P2Y6 does not affect the release of IL-1β, IL-6, IL-10, and TNF-α in both Pam3CSK4-primed or non-primed human primary microglia. An acute treatment with isoproterenol did not influence the UDP/P2Y6 effect regarding the release of these cytokines. Our results suggest that microglial P2Y6 is not involved in the release of pro-inflammatory cytokines in our experimental conditions.

Finally, we showed that a prolonged 24-hour treatment with isoproterenol decreased the expression level of P2Y6 in microglia. This result strongly supports the inhibitory effect of the ADRB2 signaling pathway on P2Y6-dependent calcium transients and P2Y6-dependent phagocytosis. The downregulation of P2Y6 likely results in lower phagocytosis activity through a decrease in P2Y6-dependent calcium transients. Interestingly, priming microglia with Pam3CSK4 did not influence P2Y6 expression level, but it downregulated ADRB2 expression level. Downregulation of ADRB2 might facilitate the induction of phagocytosis through disinhibition of P2Y6 signaling in active microglia.

Altogether, this study reveals a novel modulation of microglial P2Y6-dependent phagocytosis through ADRB2 and noradrenergic input. Norepinephrine can be co-released with ATP in the cortex (Poelchen et al., 2001) and it has been shown that

adrenergic signaling in the cortex can be modulated by purinergic signaling to regulate key neuronal functions (Pinho et al., 2013; Quintas et al., 2023; von Kugelgen et al., 1994). For example, P2Y6 has been shown to inhibit NMDA-evoked norepinephrine release in the cortex (Quintas et al., 2023). This novel functional crosstalk between adrenergic and P2 receptors, specifically between microglial ADRB2 and P2Y6, may participate in the regulation of several other basic microglial functions. Further investigations will be needed to better understand the molecular mechanisms underlying this crosstalk, as well as its involvement in pathological states.

Figures and legends

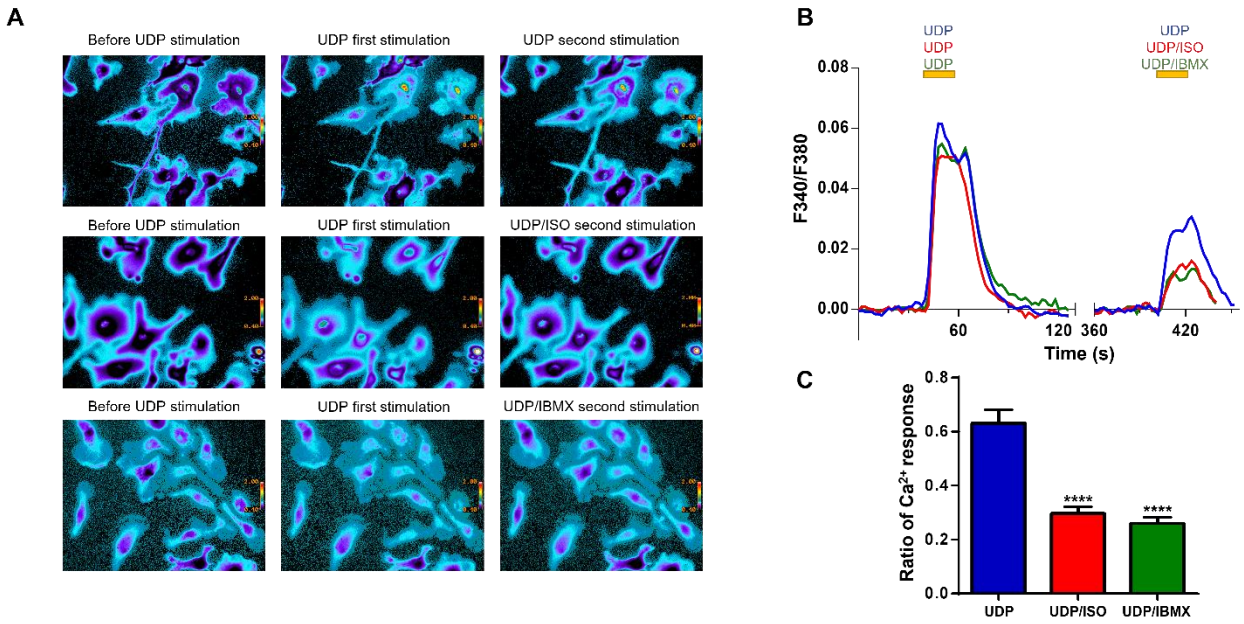


Figure 1. ADRB2 signaling inhibits UDP-evoked calcium transients in mouse primary microglia.

(**A**) Representative examples of Fura2-based ratiometric imaging showing the intracellular calcium status of microglial cells before and after stimulation with P2Y6 agonist UDP in each experimental condition. (**B**) Averages traces showing calcium transients evoked by P2Y6 activation in primary mouse microglia. The 340/380 ratio (F340/F380) reflects the intracellular calcium status of the cells. Traces on the left represent averaged first stimulations, showing UDP-evoked calcium responses. Traces on the right side represent averaged second stimulations with or without treatment (50 µM UDP alone, 50 µM UDP + 10 µM isoproterenol, or 50 µM UDP + 100 µM IBMX) applied between stimulations (interval = 5 min). (**C**) Quantitative comparisons of second responses. One-way ANOVA was performed followed by Tukey's *post hoc* test. Mean ± SEM of n = 98 for control (UDP), n = 89 for UDP + ISO, n = 138 for UDP + IBMX. ****P < 0.0001.

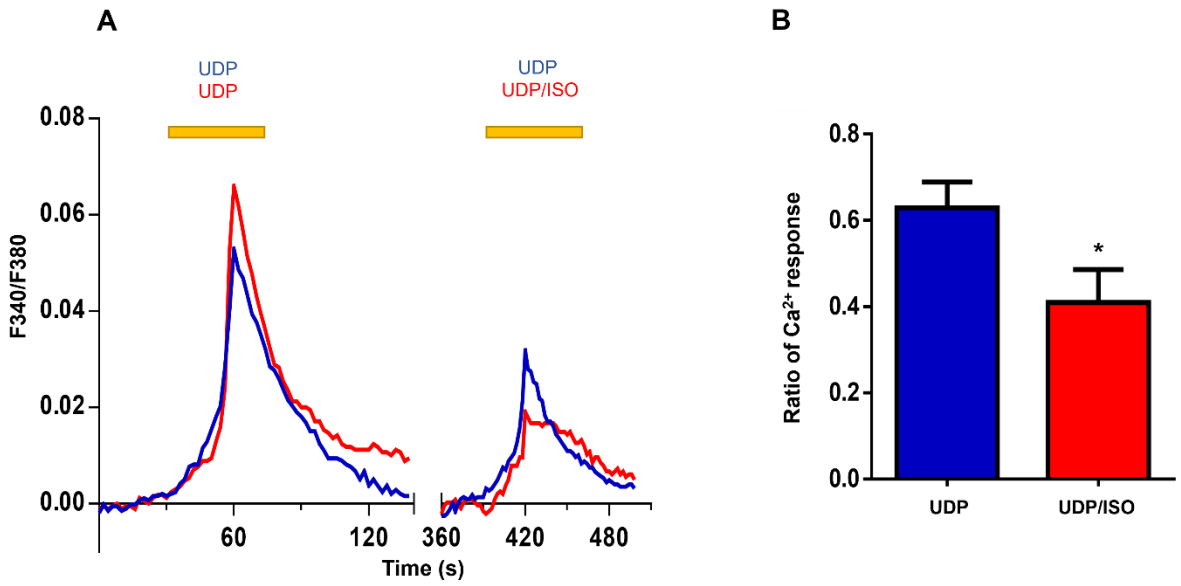


Figure 2. ADRB2 signaling inhibits UDP-evoked calcium transients in human iPSC-derived microglia.

(**A**) Averaged traces showing calcium transients evoked by P2Y6 activation with UDP in human iPSC-derived microglia. The 340/380 ratio (F340/F380) represents the intracellular calcium status of the cells. Traces on the left show first averaged 100 μM UDP-evoked calcium responses. Traces on the right represent second averaged calcium responses, with or without 20 μM isoproterenol applied between stimulations (interval = 5 min). (**B**) Quantitative comparison of second calcium responses. Unpaired t-test was performed. Mean ± SEM of n = 23; for control (UDP alone), n = 17 for UDP/ISO. *P < 0.05.

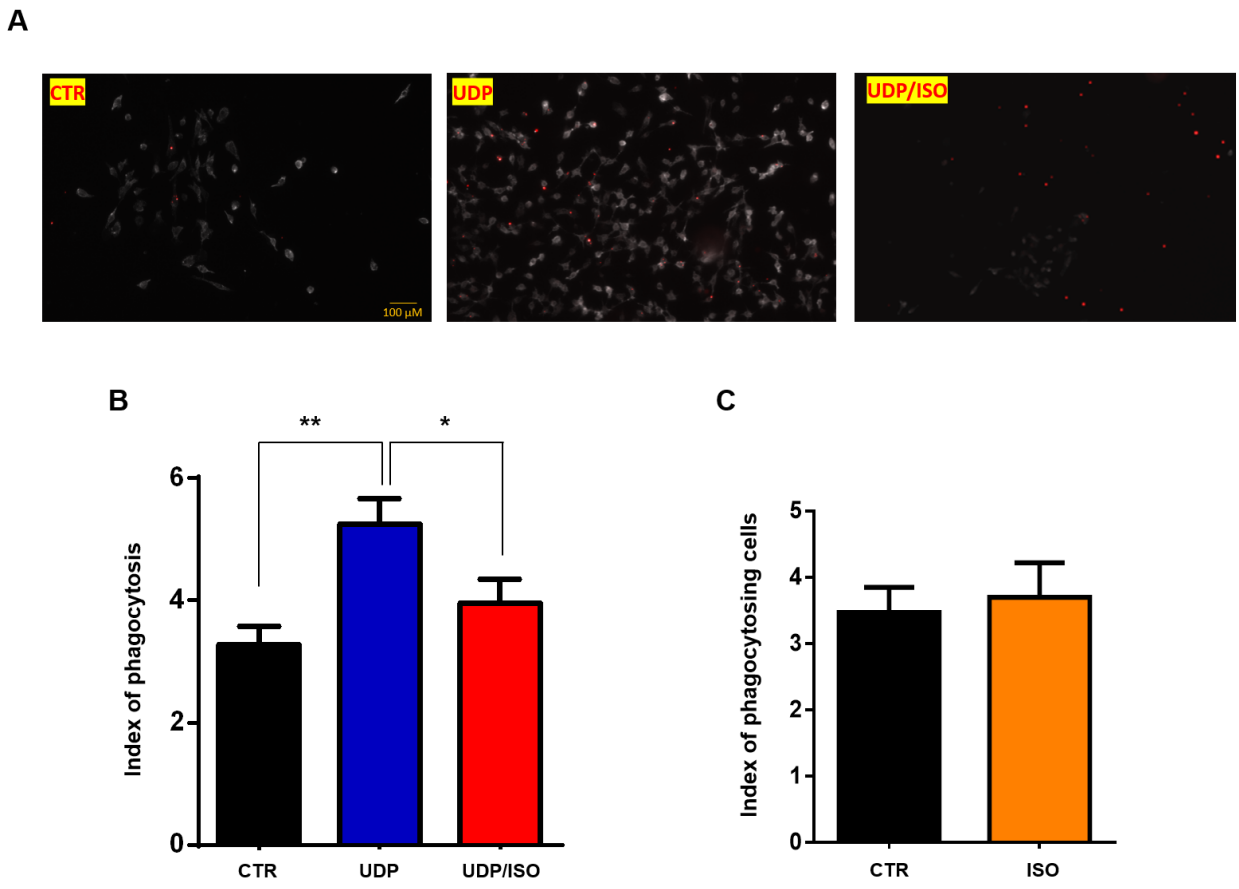


Figure 3. ADRB2 inhibits UDP-induced P2Y6-dependent phagocytosis in mouse primary microglia.

(**A**) Typical images of phagocytosed fluorescent beads by mouse primary microglia in each experimental condition. Quantitative comparison of the percentage of phagocytosing cells treated in (**B**) with vehicle (n = 15), 100 µM UDP (n = 15), 100 µM UDP + 20 µM ISO (n = 18), or in (**C**) vehicle (n = 11), ISO (n = 10). In B, one-way ANOVA was performed followed by Tukey's post hoc test. Mean ± SEM *P < 0.05, **P < 0.01. In C, unpaired t-test was performed. Mean ± SEM. CTR= control; ISO=isoproterenol.

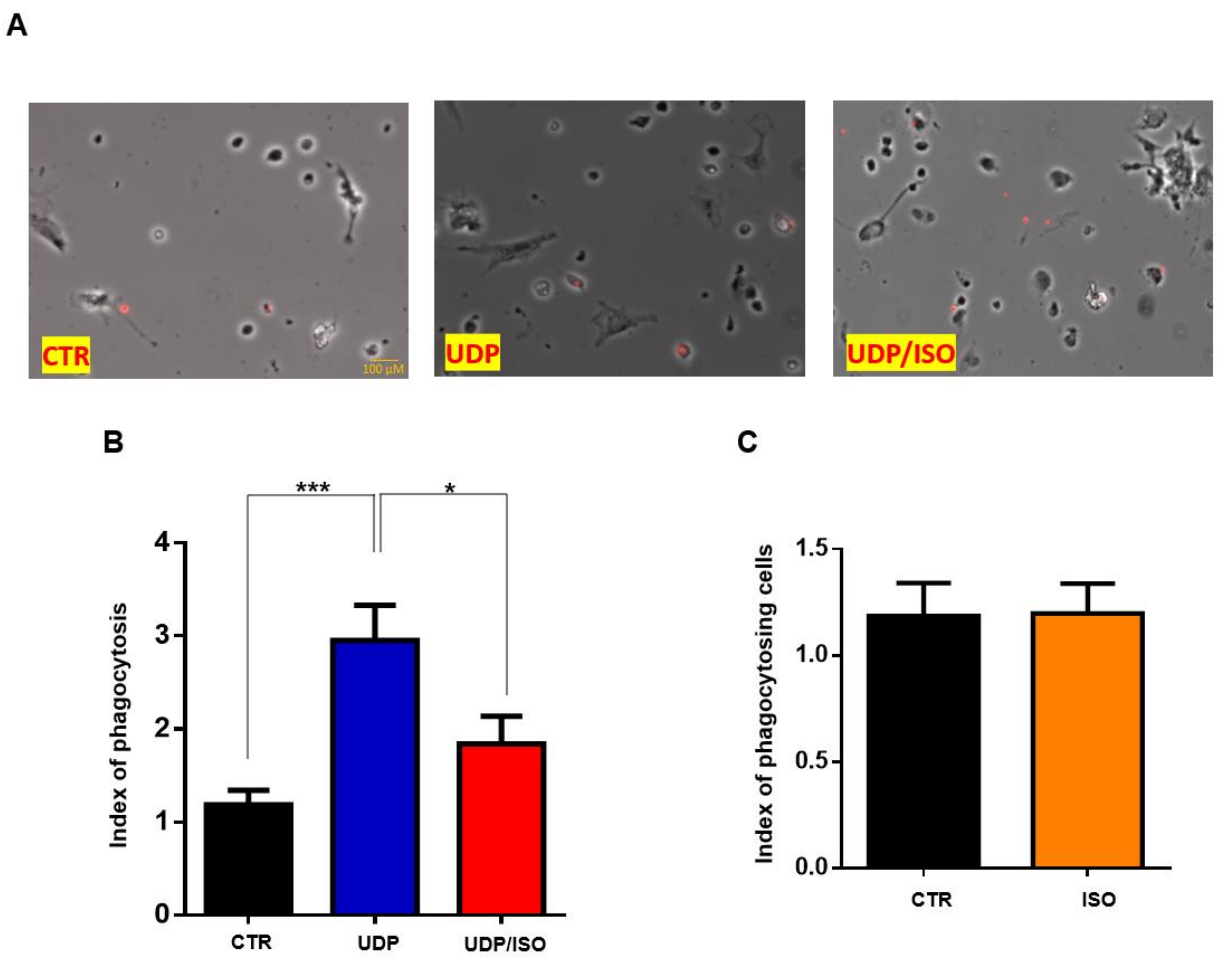


Figure 4. ADRB2 inhibits UDP-induced P2Y6-dependent phagocytosis in human iPSC-derived microglia.

(**A**) Typical images of phagocytosed beads by human iPSC-derived microglia in each experimental condition. Quantitative comparison of the percentage of phagocytosing cells treated with (**B**) vehicle (n = 15), UDP (n = 11), UDP + ISO (n = 11); or (**C**) vehicle (n = 15), ISO (n = 12). In B, one-way ANOVA was performed followed by Tukey's post hoc test. Mean ± SEM *P < 0.05, ***P < 0.00. In C, unpaired t-test was performed. Mean ± SEM. CTR= control; ISO=isoproterenol.

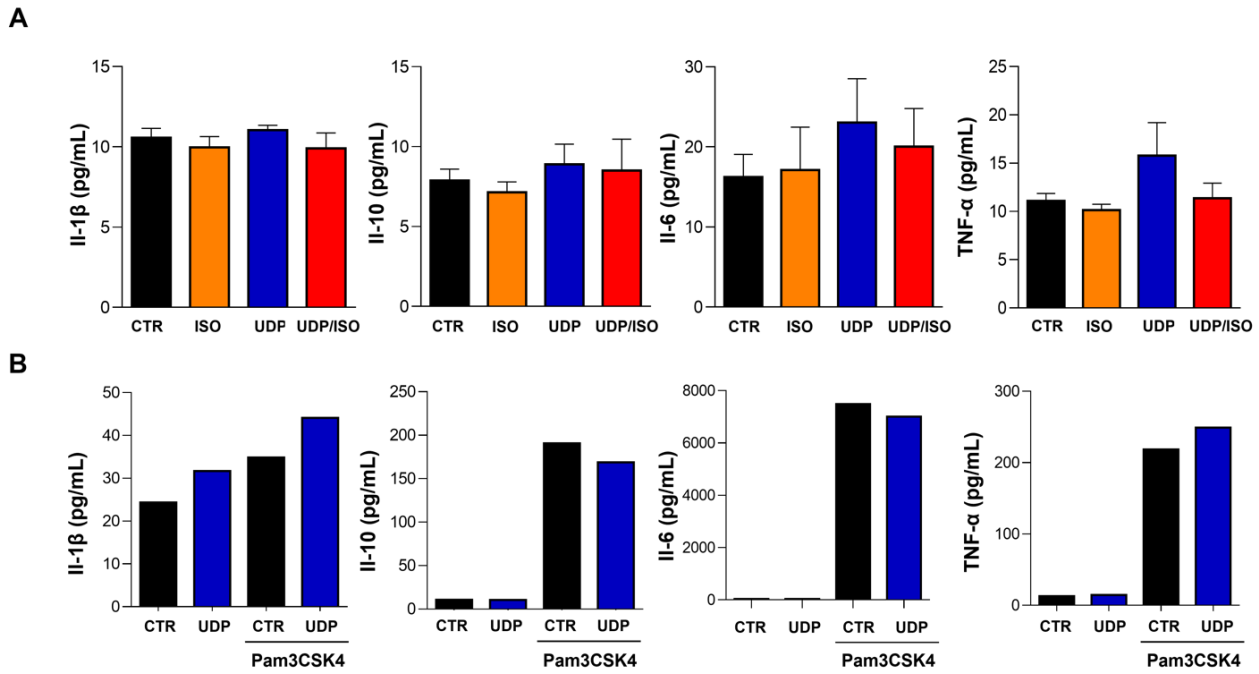


Figure 5. UDP-evoked P2Y6 activation does not impact cytokine release in human microglia.

Human primary microglia were treated with vehicle, P2Y6 agonist UDP (100 μM), and/or ADRB2 agonist isoproterenol (20 μM). (**A**) Measurements of IL-1β, IL-10, IL-6 and TNF-α release from human primary microglia in presence of UDP and/or isoproterenol (n=6). (**B**) IL-1β, IL-10, IL-6 and TNF-α release from human primary microglia primed with Pam3CSK4 in presence of UDP (n=1). One-way ANOVA was performed followed by Dunnett's post hoc test. Mean ± SEM. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; TNF-α = tumor necrosis factor α.

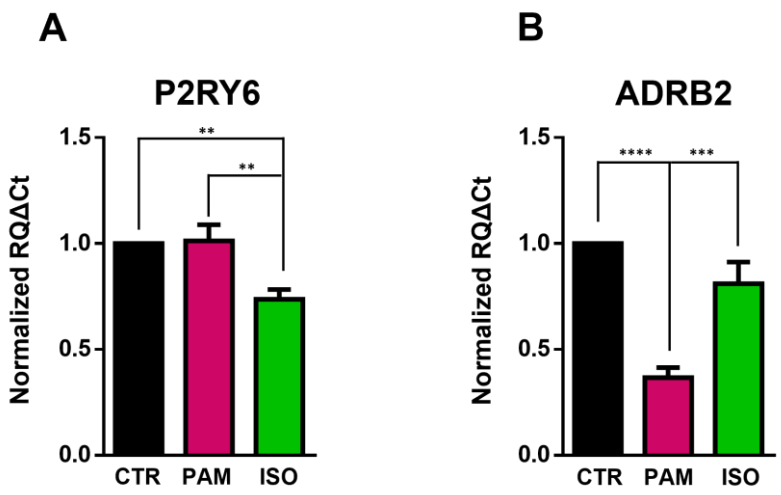


Figure 6. P2Y6 and ADRB2 gene expression is downregulated by ADRB2 and Pam3CSK4 signaling, respectively, in mouse primary microglia.

Real-time qPCR assessment of P2Y6 (**A**) and ADRB2 (**B**) mRNA expression levels in primary mouse microglia treated for 24 h with vehicle, Pam3CSK (100 ng/ml), or isoproterenol (20 μM). One-way ANOVA was performed followed by Tukey's *post hoc* test. Mean ± SEM of $n = 7$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. CTR= control; PAM=Pam3CSK4; ISO=isoproterenol.

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Linker

In Chapter 2, we provide conclusive evidence that the norepinephrine/ADRB2 signaling pathway modulates the function of the nucleotide receptor P2Y6 in microglia. We demonstrated that activation of ADRB2 reduced P2Y6-dependent phagocytosis, P2Y6-dependent calcium transients, as well as P2Y6 gene expression in both mouse and human microglia. In this first part of the thesis, we targeted one microglial P2 receptor involved in phagocytosis, a major function for a resident immune cell in the CNS that contributes to ensure homeostasis by eliminating dangerous elements and shaping neuronal networks by pruning.

Microglia are also involved in inflammation to minimize tissue damage and facilitate tissue healing. Interestingly, the ATP receptor P2X7 has been described as a central component in the release of the pro-inflammatory cytokine IL-1β. In addition, abnormal P2X7 signaling has been associated with most neurodegenerative diseases and with neuropathic pain, causing excessive neuroinflammation that damages the CNS. Yet, a regulatory mechanism of the pro-inflammatory ATP/P2X7 pathway, remains to be identified. Given the ADRB2's modulatory capacity documented in Chapter 2, we questioned whether ADRB2 could also influence the activity of other P2 receptors expressed in microglia. Chapter 3 will investigate whether ADRB2 can modulate the P2X7 function associated with the release of the major pro-inflammatory cytokine IL-1β.

Chapter 3

Adrenergic control of P2X7-dependent IL-1β release in microglia

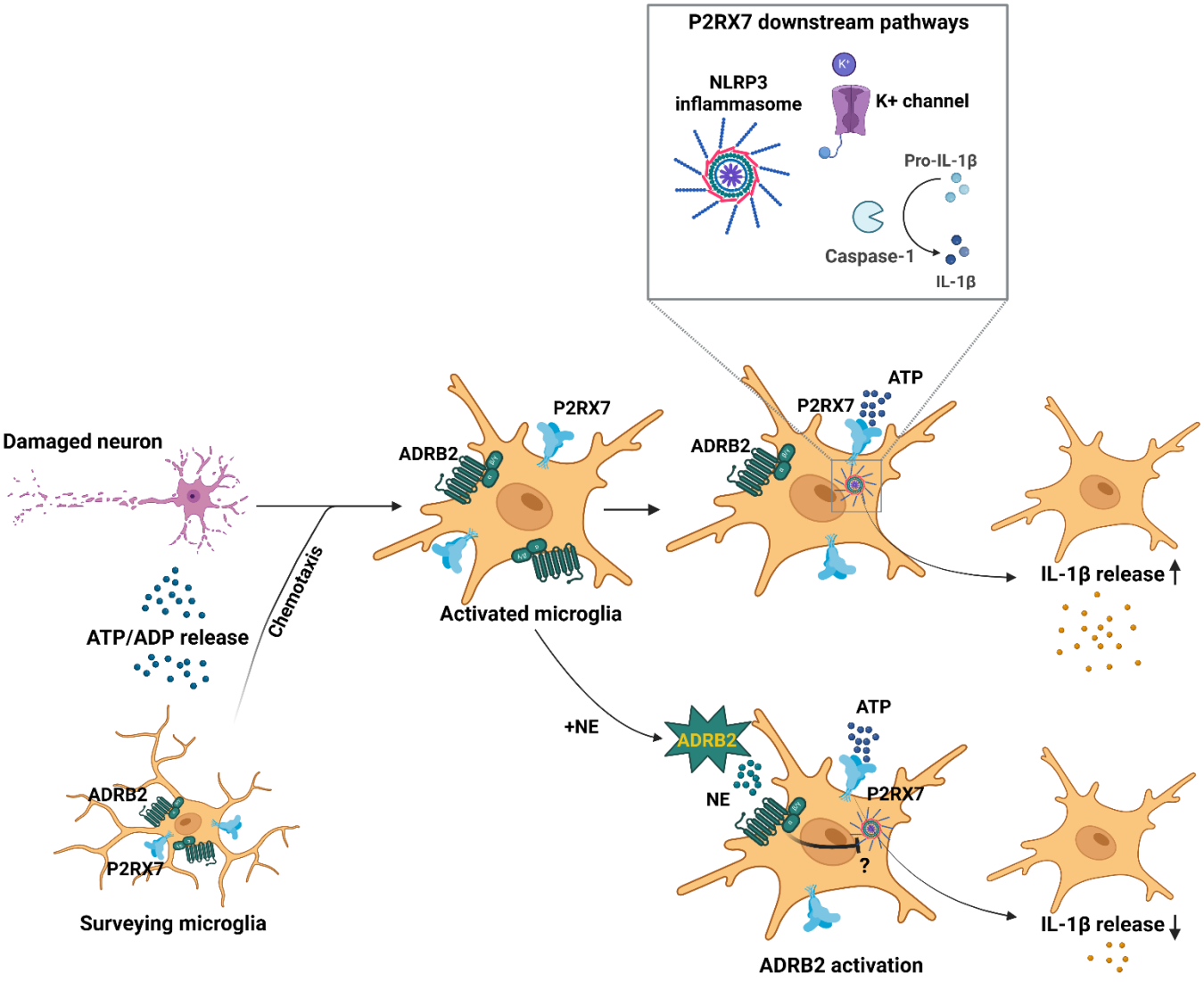
Thomas DELUC[1,2], Marie-France DORION[1], Ariel ASE[1,2], Gilles MAUSSION[1], Thomas M. DURCAN[1], and Philippe SÉGUÉLA[1,2,3]

[1] Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Canada

[2] Alan Edwards Centre for Research on Pain, McGill University, Montreal, Canada

[3] Correspondence: philippe.seguela@mcgill.ca

Graphical abstract



ADRB2 activation controls IL-1β release

Abstract

Microglia, the resident immune cells of the central nervous system (CNS), are constantly surveying their surroundings. Extracellular ATP, released by stressed or damaged neurons, acts as a danger signal to microglia through various purinergic/pyrimidinergic (P2) receptors to perform crucial functions such as chemotaxis, phagocytosis, and the release of transcription factors or cytokines. P2 receptors are known to have a substantial impact on inflammatory processes and neurodegenerative disorders. In the CNS, the ATP-gated receptor-channel P2X7 is expressed at high level in microglia, where it induces the release of the pro-inflammatory cytokine IL-1β through the NLRP3 inflammasome pathway. This function has been associated with various neurodegenerative diseases and with chronic pain, causing neuroinflammation and neurotoxicity. Yet, regulation of P2X7 has not been studied in depth. The β2 adrenergic receptor (ADRB2), the main adrenergic receptor in microglia, represents a promising candidate for modulation of P2X7. Cytokine measurements indicate that treatment with the ADRB2 agonist isoproterenol inhibits P2X7-dependent IL-1β release from Pam3CSK4-primed human microglia. We also report here that ADRB2 does not act directly through the P2X7 receptor-channel, as its activation did not affect BzATP-evoked calcium transients and cationic currents, or mRNA expression levels. Our findings provide evidence of a noradrenergic control of microglial immune responses triggered by P2X7 activation, yet the precise modulatory mechanism still remains to be elucidated.

Introduction

Over the past twenty years, there has been a rapid rise in discoveries concerning the roles of microglia, the resident immune cells of the central nervous system (CNS), in healthy or disease states. Microglia have been involved in preserving brain homeostasis, contributing to neurodevelopment, synaptic plasticity, and repair processes. One major function of microglia concerns their capacity to detect danger signals and initiate inflammation through the release of cytokines and chemokines. However, dysregulation of microglial mechanisms has been shown to contribute to central neuroinflammation observed in most neurodegenerative diseases and in chronic pain.

The ATP-gated receptor-channel P2X7 is expressed in glial cells in the CNS, with higher level of expression in microglia (Bhattacharya & Biber, 2016; Sperlagh & Illes, 2014). P2X7 has been associated with important microglial functions and represents an essential actor in the neuroinflammation induced by microglia. The well-characterized P2X7 pathway is a major contributor to the release of the pro-inflammatory cytokine IL-1β. P2X7 is activated by a high concentration of extracellular ATP, perceived as a danger signal to microglia (damage-associated molecular pattern or DAMP), leading to an influx of cations and an efflux of potassium ions. This efflux of potassium ions from microglia occurring through P2X7, as well as through two-pore domain potassium channels such as THIK1 or TWIK2 channels (Di et al., 2018; Drinkall et al., 2022), is necessary to induce the recruitment of the NLRP3 inflammasome. The inflammasome can then activate caspase-1 by cleaving its immature form, leading to the release of the pro-inflammatory cytokine IL-1β (Di et al., 2018; Giuliani et al., 2017). Extracellular IL-1β then binds to IL-1

receptors on target cells to initiate the expression of pro-inflammatory genes through the activation of NF-κB (Pinteaux et al., 2002).

Pro-inflammatory P2X7 function has been associated with several types of neurological disorders, including neurodegenerative diseases and chronic pain. Upregulated microglial P2X7 has been shown to be involved in multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), stroke, and neuropathic pain. One characteristic of these diseases is chronic inflammation. In AD, it has been demonstrated that Aβ plaques, a marker of the disease, induce the release of IL-1β which is dependent of P2X7 (Sanz et al., 2009). Genetic silencing or pharmacological inhibition of P2X7 prevents microglial activation and neuroinflammation caused by Aβ plaques (Carvalho et al., 2021; Y. H. Chen et al., 2021; Francistiova et al., 2020; Thawkar & Kaur, 2019). In MS, blockade of P2X7 activity decreases IL-1β levels and symptoms (Sharp et al., 2008). IL-1β has been associated with neuronal, vascular and oligodendrocyte damage in various neuropathological conditions such as PD (Mao et al., 2017; Yan et al., 2015), AD (Heneka et al., 2013) or MS (Lévesque et al., 2016; Mandolesi et al., 2013; Mendiola & Cardona, 2018). In addition, P2X7 has been shown to play a significant role in the development of chronic inflammatory and neuropathic pain (Tsuda, 2017), particularly through the NLRP3 pathway and IL-1β, causing chronic inflammation and pathological pain. Pharmacological blockade of P2X7 reduces neuropathic pain, including neuropathy-induced mechanical allodynia and thermal hyperalgesia (Clark et al., 2010; Honore et al., 2009). Chronic morphine treatment after peripheral nerve injury has been shown to act as a DAMP to microglia through P2X7, causing the formation of the NLRP3 inflammasome (Grace et

al., 2018) and P2X7 expression is therefore considered as one candidate for causing analgesic tolerance to morphine. Indeed, P2X7 antagonists prevent analgesic tolerance to morphine (Leduc-Pessah et al., 2017). Altogether, recruitment of microglial P2X7, in particular through the activation of the NLRP3 inflammasome and IL-1β release, can drive persistent neuroinflammation that causes neurodegeneration. Blockade of P2X7 activity has been shown to be beneficial by reducing inflammation and neurotoxicity in neurodegenerative diseases or neuropathic pain. Antagonists of P2X7 have failed clinically, however clinical trials using novel selective compounds are ongoing. A challenging question remains how to specifically modulate P2X7 rather than completely inhibit it response, as this could effectively control P2X7-dependent inflammation.

The role of the norepinephrine as a major neuromodulator in the CNS has been well investigated. The main source of noradrenergic fibers is located in a small nucleus of the brainstem, the locus coeruleus (LC), from where highly branched projections emerge to influence the activity of various regions and cell types due to the widespread expression of adrenergic receptors in the CNS. Interestingly, norepinephrine has been shown to modulate microglial cells through β2 adrenergic receptor (ADRB2), which is distinctly expressed in this cell type in the CNS. ADRB2 activation by norepinephrine has been shown to inhibit the activation of microglia in various brain regions (Sugama et al., 2019), and to supress LPS-induced microglial cytokine release, such as IL-6, IL-1β, TNF-α (Heneka et al., 2010; Qian et al., 2011) or CSF-1 (Damo et al., 2023). New evidence suggest ADRB2 is a modulator of P2Y12-dependent functions, as it has been shown to regulate cAMP-driven filopodia extension (Bernier et al., 2019) and to inhibit microglial process surveillance in awake mice (Liu et al., 2019; Stowell et al., 2019). Moreover,

treatment of microglia with the norepinephrine reuptake inhibitor duloxetine decreases calcium levels induced by ATP through P2X4 receptor-channels (Yamashita et al., 2016). Altogether, adrenergic transduction via ADRB2 represents a physiologically relevant candidate to modulate the inflammatory pathway driven by P2X7 in microglia.

In this study, we demonstrated that ADRB2 reduces the levels of IL-1β dependent on P2X7 activation. However, ADRB2 does not directly influence basic P2X7 receptor-channel functions such as calcium transients and membrane depolarization, and microglial P2X7 expression did not change following activation of ADRB2.

Materials and Methods

Mouse primary microglia

Primary microglia were collected from the brains of P1 to P4 C57BL/6 wild-type mice (Charles River Canada). Briefly, cortices were isolated and trypsinized using 0.25% trypsin-EDTA for 30 minutes at 37 °C. After successive centrifugations, washes in microglia medium (composed of DMEM/F12 (1:1), FBS and penicillin/streptomycin), and trituration, cells were filtered through 100-µm pore size nylon strainers to obtain a glial mix. This glial mix, in presence of microglia medium, was cultured in flasks at 37 °C and 5% CO_2. After two weeks, the flasks were shaken for 2 hours to detach microglia. After centrifugation and resuspension, microglia (95% purity) were plated in glass-bottom dishes (MatTek) for use 24 to 48 hours.

Human iPSC-derived microglia

iPSC lines were purchased (DYR0100 from American Type Cell Collection, GM25256 from Corriell Institute) or generated (3450) following McGill University Health Center's ethical guidelines (project# 2019-5374) with written consent from donors. Cells

were maintained in mTeSR™ Plus (STEMCELL Technologies) or in Essential 8™ media on Corning™ Matrigel™ hESC-Qualified Matrix -coated dishes, with subculturing every five to seven days using standard protocols (C. X.-Q. Chen et al., 2021).

Differentiation of iPSCs into microglia was carried out as previously described (Dorion et al., 2024). Briefly, hematopoietic progenitor cells were generated from iPSCs using STEMdiff Hematopoietic kit (STEMCELL Technologies) and subsequently cultured in Minimum Essential Medium alpha supplemented 1X with GlutaMAX 1X, 2X B27, 2X Insulin-Transferrin-Selenium, 1X penicillin/streptomycin (Thermo Fisher), 100 ng/mL interleukin-34, 50 ng/mL tumor growth factor-beta and 25 ng/mL macrophage colony-stimulating factor (Peprotech) for 25 days, following which 100 ng/mL C-X3-C motif chemokine ligand 1 (Peprotech) was also added to the culture. iMGL were used between 28 days and 42 days of differentiation.

Human primary microglia

Human brain tissues were collected from non-malignant cases of temporal lobe epilepsy (2–71-year-old female and male patients), at sites distant from suspected primary epileptic foci. Microglial isolation was performed as previously described (Durafourt et al., 2013). Briefly, trypsin (Invitrogen) and DNase (Roche) treatment were used to digest tissues and then dissociated mechanically through a nylon mesh filter. Tissue homogenate was then subjected to Percoll (Sigma-Aldrich) gradient centrifugation to isolate the glial cells. Differential adhesive properties of glial cells were used to purify microglia. Microglia were cultured in Minimum Essential Medium (MEM; Sigma Aldrich) supplemented with 1% GlutaMAX (ThermoFisher Scientific), 1% penicillin/streptomycin (P/S; ThermoFisher Scientific), 0.1% glucose (Sigma Aldrich) and 5% fetal bovine serum

(FBS; Wisent Bioproducts) and kept at 37 °C under a 5% CO2 atmosphere. Use of all human cells was approved by the McGill University Health Centre Research Ethics Board.

Calcium imaging

We used Fura2-based ratiometric measurement of [iCa²⁺] to functionally assess the modulation of ATP receptors by ADRB2 in various cellular models. Primary mouse microglia or primary human microglia were loaded with the calcium-sensitive fluorescent dye fura-2 AM for 40 minutes, in a solution at pH 7.4 containing (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, with 1% BSA. After 40 minutes of incubation, cells were incubated for 30 minutes at 37 °C to hydrolyze the acetoxymethyl ester of the dye. Loaded cells were imaged using an inverted microscope (Nikon Eclipse TE300) and were excited every 2 seconds with 340 nm and 380 nm excitation filters controlled with Metafluor (Molecular Devices) to generate ratio (340/380) images corresponding to intracellular calcium concentrations. During experiments, microglial cells were constantly perfused with external solution containing or not the ligands of interest. Double applications (20 seconds) were used to compare second responses to the first ones for intra-cell normalizations. Experiments without treatment between two applications were considered control experiments and are further compared to experiments with a treatment inter-stimulation, to assess a quantitative modulation of ATP receptors by ADRB2 activation. Peak amplitude of the responses was subtracted to baseline ratios (mean of 20 seconds prior the stimulation) and statistical comparisons were determined using Student's t-tests. Second responses in each experiment (control versus treatments) were compared using one-way ANOVA.

Electrophysiology

Whole-cell patch-clamp recording of transiently microglial cells (*V*hold = −40 mV) was performed using pipettes filled with internal solution, pH 7.2, containing (in mM): 120K-gluconate, 1 MgCl2, 5 EGTA and 10 HEPES. The recording solution, pH 7.4, comprised (in mM): 140 NaCl, 5 KCl, low divalent ions (0.2 CaCl2 and free MgCl2), 10 HEPES, and 10 glucose. Membrane currents were recorded using an Axopatch 200B amplifier and digitized at 500 Hz with a Digidata 1550B interface (Axon Instruments, Molecular Devices, Sunnyvale, CA). Only recordings with series resistance below 10 MΩ and stable for the duration of the recording were considered for analysis. Drugs were dissolved in a recording solution and applied using an SF-77B fast perfusion system (Warner Instruments, Hamden, CT) at a rate of 1 ml/minute. All experiments were performed at room temperature. For each individual experiment, 100 μM BzATP-evoked current amplitudes of fast cation channel opening.

Cytokines assay

Human iPSC-derived microglia or human primary microglia were incubated with treatment (vehicle, BzATP 200 or 500 μM alone, BzATP + Isoproterenol 50 μM, or Isoproterenol 50 μM alone) for 3 hours and then supernatants were collected. Cytokine measurements were performed using the Human Inflammatory Cytokine Cytometric Bead Array (CBA) kit from bdBiosciences to assess the levels of interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF-α). As per the instructions, beads, and detection reagent (PE) were mixed and incubated with the treated microglial cells for 3 hours in the dark at room temperature. After wash,

centrifugation and resuspension, the samples were analyzed by flow cytometry to measure the levels of cytokines.

qPCR

RNA was extracted from either control, Pam3CSK4-treated or isoproterenol-treated primary mouse microglia, using the RNeasy Mini Kit (Qiagen). After cDNA synthesis, qPCR was carried out using TaqMan universal master mix and gene specific primers (P2X7: Mm01199500_m1 and ADRB2: Mm02524224_s1). The mean cycle threshold (CT) values were normalized to GAPDH (Mm99999915_g1) and ACTB (Mm01205647_g1). Fold changes were calculated using the delta CT method.

Statistical analysis

Prism 6 (GraphPad) was used for quantitative analysis, statistics, and graphs. Repeated measures one-way ANOVA, standard one-way ANOVA, or two-way ANOVA tests were performed for statistical analysis.

Results

3.1 P2X7-dependent IL-1β release is decreased by long treatment with isoproterenol in human iPSC-derived microglia.

Activation of microglial P2X7 by the danger signal ATP has been shown to induce the release of IL-1β. This pathway has been involved in pathological conditions such as AD, PD and neuropathic pain, where it causes neuroinflammation. Using human iPSC-derived microglia, we first tested different concentrations of the P2X7 agonist BzATP and we observed that only BzATP at 500 µM induced a significant release of IL-1β (Figure 1A). This result was confirmed in human microglia primed with the pro-inflammatory lipopeptide Pam3CSK4, a TLR1/2 agonist that shifts microglial activation toward a pro-inflammatory state (Figure 1B). Cytokine levels were higher in primed microglia, in agreement with the known role of priming in amplifying microglial responses. Thus, for the following experiments using human iPSC-derived microglia, we used 500 µM BzATP.

Then, we demonstrated that BzATP treatment induced a significant release of IL-1β selectively through the activation of P2X7 (Figure 1C-D). Intriguingly, activation of P2X7 by BzATP did not induce the release of other cytokines (IL-6, IL-10 and TNF-α) in microglia primed or not with Pam3CSK4 (Supplemental figures 1A-D, 2A-D, 3A-D). The selective P2X7 antagonist A740003 supressed the release of IL-1β, confirming that the BzATP-evoked release is P2X7-dependent (Figure 1C). A740003 alone did not have any effect on the basal release of IL-1β. In parallel, we performed the same experiments with Pam3CSK4-primed microglia. We obtained similar results, i.e. an increase in IL-1β release in the presence of BzATP, supressed by A740003 (Figure 1D).

Our next goal was to see if we could influence the release of IL-1β, which is P2X7-dependent, by activating microglial ADRB2 with the beta-receptor agonist isoproterenol. We were able to induce the release of IL-1β with BzATP treatment as expected, however, a 3-hour acute treatment with isoproterenol (50 μM) did not have a significant effect on P2X7-dependent IL-1β release (Figure 1E). We obtained similar results when microglia were primed by Pam3CSK4 (Figure 1F). Isoproterenol by itself did not have an impact on the endogenous release of IL-1β. We conclude that an acute activation of microglial ADRB2 with isoproterenol does not modulate P2X7-dependent IL-1β release. Additionally, no effect of ADRB2 was observed on the release of other cytokines (Supplemental figures 1 E-F, 2E-F, 3E-F). We then decided to investigate whether a pretreatment with isoproterenol could influence P2X7 IL-1β release. We first used a short pretreatment (30 min or 1 h), but it did not affect IL-1β release (Supplemental Figure 4). Thus, we investigated whether a 24-hour pretreatment with isoproterenol could modulate P2X7-dependent IL-1β release (Figure 1G-H). We found that the pretreatment did not influence the endogenous release of IL-1β. Interestingly, we observed a trend towards a decrease in the P2X7-dependent release of IL-1β with the pretreatment (Figure 1G). In microglia primed with Pam3CSK4 and pretreated with isoproterenol, we discovered that only BzATP treatment was significantly different from the endogenous release of IL-1β. Indeed, the cotreatment of BzATP and isoproterenol did not differ from the control, meaning that activation of ADRB2 reduced the release of IL-1β induced by P2X7 (Figure 1H). In addition, in microglia primed with Pam3CSK4, our results revealed that ADRB2 activation significantly decreased the release of IL-6 and TNF-α but not IL-10 compared to its endogenous release (Supplemental figures 1G-H, 2G-H, 3G-H).

Finally, we used IBMX, a phosphodiesterase inhibitor, that can mimic the recruitment of adenylate cyclase following the activation of the Gs-coupled receptors, including ADRB2. A 24-hour pretreatment with IBMX (100 µM) induced a decrease in P2X7-dependent IL-1β release (Figure 1I). Interestingly, in microglia primed with Pam3CSK4 and pretreated with IBMX, only a BzATP treatment was significantly effective to induce the release of IL-1β compared to basal release. Similarly to microglia primed with Pam3CSK4 and pretreated with isoproterenol, cotreatment of BzATP and IBMX did not differ from control, indicating that recruiting the cAMP pathway reduces the release of IL-1β induced by P2X7 (Figure 1J). In addition, IBMX did not affect the release of other cytokines (Supplemental figures 1I-J, 2I-J, 3I-J).

Altogether, we concluded that only a 24-hour pretreatment of Pam3CSK4-primed microglia with isoproterenol or IBMX, inhibits the P2X7-dependent release of IL-1β, illustrated by the heat map (Figure 1K). Pretreatment with an ADRB2 agonist also impacted negatively the endogenous release of IL-6 and TNF-α but not IL-10 (Figure 1K).

3.2 P2X7-dependent IL-1β release is decreased by long treatment with isoproterenol in human primary microglia.

Next, we wanted to check if these results can be translated to human primary microglia. We first attempted to induce IL-1β release through P2X7 activation in human primary microglia. We treated the cells with 200 µM BzATP, as 500 µM was causing significant cell death. We were not able to induce IL-1β release via the activation of P2X7 with 200 µM BzATP. Other treatments (A740003, isoproterenol or IBMX) did not have any effect on the release of IL-1β (Figure 2A, 2B and 2C) or other cytokines (IL-6, IL-10 and TNF-α) (Supplemental figures 5A-C, 6A-C, 7A-C). Thus, we decided to prime the human

primary microglia with Pam3CSK4 and to pretreat them 24 hours with either isoproterenol or IBMX (Figure 2D-E). In this condition, we were able to induce the release of IL-1β through P2X7 activation. Interestingly, a 24-hour pretreatment of isoproterenol to activate ADRB2 reduced the P2X7-evoked IL-1β release, confirming our results obtained on human iPSC-derived microglia (Figure 2D). Treatment of Pam3CSK4-primed human primary microglia with IBMX did not influence P2X7-dependent IL-1β release (Figure 2E). Similarly to our results obtained with iPSC-derived microglia, only a 24-hour ADRB2 pretreatment (and not IBMX) decreased the release of IL-6 and TNF-α but not IL-10 compared to the endogenous release (Supplemental figures 5D-E, 6D-E, 7D-E).

We concluded that a priming of human primary microglia with Pam3CSK4 was necessary to induce IL-1β release through P2X7 activation, contrary to human iPSC-derived microglia. We also noticed that human iPSC-derived microglia needed a higher concentration of BzATP to induce the release of IL-1β, a concentration that appeared toxic to human primary microglia. Finally, we found that sustained ADRB2 activation, via a 24-hour pretreatment with isoproterenol, decreased the P2X7-dependent IL-1β release in Pam3CSK4-primed microglia, confirming our results obtained on human iPSC-derived microglia.

3.3 P2X7 calcium transients are not modulated by ADRB2 in mouse and human microglia.

Our next goal was to elucidate the mechanism that is involved in the modulation of P2X7-dependent IL-1β release by ADRB2. Our hypothesis was that ADRB2 exerts this modulation directly through the P2X7 channel. The non-selective P2X7 cation channel is highly permeable to calcium ions, resulting in an increase in intracellular calcium

concentration following its activation by ATP or BzATP. Ratiometric calcium imaging is a good technique to measure P2X7 calcium transients as activation of ADRB2 by isoproterenol does not influence the calcium status of the cell. First, we investigated whether ADRB2 could influence the calcium transients evoked by P2X7 in primary mouse microglia. We used a double application protocol (described in the method section) to compare the second response to the first response. Average recordings in each condition are represented in Figure 3A. Application of the P2X7 agonist BzATP (200 μM) induced a drastic augmentation of intracellular calcium, demonstrating by the first stimulation. Our control experiment consisting of a double application of BzATP, revealed that the second response was decreased (55 %) compared to the first calcium response, indicating desensitization (Figure 3B). We confirmed that the calcium responses were mediated by P2X7 by using the selective P2X7 antagonist A740003 (10 μM). Application of A740003 resulted in strong inhibition of the response to BzATP, confirming P2X7 activation (Figure 3B). To investigate whether ADRB2 can modulate P2X7 calcium transients, we applied the ADRB2 agonist isoproterenol (10 μM) between two applications, resulting in decrease of the second response (59 %). The comparison of second responses with control experiments did not show any significant difference (Figure 3B). Application of IBMX (100 μM) inter-stimulation resulted in similar results, i.e. a decrease in the second response compared to the first response (65 %) but no significant difference with our control experiments (Figure 3B). Our results suggest that ADRB2 activation with isoproterenol or treatment with IBMX does not modulate P2X7 calcium transients.

We also checked if P2X7 calcium transients could be modulated by the ADRB2 pathway in primary human microglia. Using the same parameters as in the mouse

experiments, we performed calcium imaging with the double application protocol in human primary microglia, illustrated by the average traces in Figure 4A. The results were very similar to those obtained in mouse microglia. In our control experiments, double application of the P2X7 agonist BzATP resulted in a decrease of the second response (52 %) compared to the first response (Figure 4B). Treatment with IBMX inter-stimulation did not show any significant change compared to control experiments (52 % vs 54 %), confirming our results in mice (Figure 4B). These results indicate that recruitment of the ADRB2 pathway does not modulate P2X7 calcium transients in rodent and human primary microglia.

3.4 P2X7 cation currents are not modulated by ADRB2 activation in primary mouse microglia.

We then investigated whether ADRB2 can modulate P2X7 currents by performing patch clamp electrophysiology experiments. Similar to our calcium imaging experiments, we used a double application. Typical recording traces are illustrated in Figure 5A. Double application of the P2X7 agonist BzATP induced a decrease in the second current response (72 %), comparable to what we observed in our calcium imaging experiments. Treatment with the ADRB2 agonist isoproterenol between two stimulations decreased the maximal current amplitude of the second response (82 %). However, comparison of the second responses in experimental and control conditions did not show any significant effect (Figure 5B), confirming our results in calcium imaging. We concluded that the ADRB2 pathway does not modulate P2X7 current responses.

3.5 Recruitment of the ADRB2 pathway does not modulate P2X7 mRNA expression in mouse primary microglia.

Finally, we interrogated whether the expression of the P2X7 gene could be influenced by microglial priming with Pam3CSK4 or by recruitment of the ADRB2 pathway. Using qPCR in primary mouse microglia, we pretreated the cells 24 hours either with Pam3CSK4 or with the ADRB2 agonist isoproterenol. Pretreatment with either Pam3CSK4 or isoproterenol did not change the expression level of P2X7 mRNA in mouse primary microglia (Figure 6A), suggesting that the ADRB2 pathway does not influence the expression of P2X7 in microglia. Interestingly, priming with Pam3CSK4 significantly reduced the expression level of the ADRB2 gene (Figure 6B).

Altogether, our results show that the ADRB2 pathway decreases IL-1β release, which is dependent on P2X7 in rodent and human microglia. Recruitment of the ADRB2 pathway also reduced the levels of IL-6 and TNF-α, while activation of P2X7 with BzATP did not modulate the release of the cytokines (IL-6, IL-10 and TNF-α). We conclude that ADRB2 is not acting directly on basic P2X7 receptor-channel functions, since ADRB2 activation did not show any effects on P2X7 gene expression, calcium transients, and cationic currents.

Discussion

We provided evidence that recruitment of the ADRB2 pathway decreases the release of IL-1β which is P2X7-dependent in rodent and human microglia. In all our microglial preparations (iPSC-derived, primaries, with priming or not), the mechanism of induction of IL-1β release by BzATP was P2X7-dependent as the release was supressed by the selective antagonist A740003. One interesting result was the higher concentration

of the P2X7 agonist BzATP required to induce the release of IL-1β in iPSC-derived microglia compared to primary microglia (500μM vs 200 μM respectively). In human primary microglia, 500 μM BzATP was causing significant cell death, highlighting another aspect of P2X7 function in immune cells. Sustained activation of P2X7 at high agonist concentration has been shown to result in the opening of a large pore associated to cell death (Kopp et al., 2019; Savio et al., 2018). Another interesting observation concerns the need for priming microglia by Pam3CSK4 in primary microglia, whereas the release of IL-1β via P2X7 could be induced without priming with Pam3CSK4 in iPSC-derived microglia. Our results in primary microglia confirm that depending on the cell type and TLR agonist used, P2X7-dependent release of IL-1β requires initial microglial activation by exposure to a pathogen-associated molecular pattern (PAMP) through Toll-like receptors (TLRs) to accumulate pro-IL-1β in the cytosol in order to be released following the activation of P2X7 by ATP (Di Virgilio et al., 2017). Interestingly, in our hands, activation of P2X7 by BzATP did not induce the release of several other cytokines (IL-6, IL-10, TNF-α) in all experimental conditions tested.

We found that although we could induce IL-1β release via P2X7 activation, this release was not affected by a 3-hour treatment with the ADRB2 agonist isoproterenol, regardless of Pam3CSK4 priming. We observed that only a 24-hour pretreatment with isoproterenol, could reduce P2X7-induced IL-1β release. In addition, ADRB2 activation alone led to a significant decrease in the release of IL-6 and TNF-α, but not IL-1β or IL-10, induced by priming with Pam3CSK4.

It has been previously reported that P2X7 is involved in the release of IL-6 and TNF-α in primary mouse microglia (Shieh et al., 2014), yet in our experimental settings,

we did not find any involvement of P2X7. In addition, ADRB2 has been reported to suppress the release of IL-6, IL-1β and TNF-α induced by LPS (Heneka et al., 2010; Qian et al., 2011). We found that ADRB2 reduced the Pam3CSK4-induced release of IL-6 and TNF-α but not IL-1β. Several experimental differences can explain these discrepancies, such as the different TLRs targeted (TLR4 vs TLR1/2), the culture conditions and the cell types used (mouse primaries vs human iPSC-derived microglia).

Furthermore, using 24-hour pretreatment with IBMX, a phosphodiesterase inhibitor that mimics the activation of the cAMP pathway by Gs-coupled ADRB2, we observed a tendency to reduce P2X7-dependent IL-1β release in iPSC-derived microglia. Interestingly, IBMX pretreatment on Pam3CSK4-primed microglia induces a significant decrease in P2X7-induced IL-1β release in iPSC-derived microglia but not in human primaries. IBMX has been shown to supress LPS-induced microglial TNF-α release but not IL-6, IL-1, or IL-10 (Yoshikawa et al., 1999). In our experiment, IBMX did not change the release of IL-6 or IL-10. However, there was a trend toward reduced TNF-α release in Pam3CSK4-primed primary human microglia. These findings suggest that the cAMP pathway of ADRB2 transduction might be involved. Further experiments are necessary to confirm the involvement of the cAMP pathway in the modulation of IL-1β release through P2X7 activation. Other ligands could be used to target intracellular pathways downstream of ADRB2. For instance, the β-arrestin pathway (Jean-Charles et al., 2017) may potentially play a role in this modulation.

Our calcium imaging, electrophysiology, and qPCR experiments on mouse and human microglia suggest that the norepinephrine/ADRB2 signaling pathway does not directly modulate P2X7 channel function and expression levels in microglia. Although our

findings show that ADRB2 can reduce IL-1β release dependent on P2X7 activation, this modulation does not directly impact P2X7 itself. The ATP/P2X7 signaling pathway leading to the release of IL-1β involves multiple components such as the NLRP3 inflammasome, background two-pore domain channels (TWIK2 and THIK1), and caspase 1 (Di et al., 2018; Di Virgilio et al., 2017; Drinkall et al., 2022). It would be interesting to investigate if ADRB2 acts on one or several of these components using pharmacological or genetic approaches. Additional research is also needed to determine which ADRB2-coupled intracellular pathway plays a major role in this modulation. The anti-inflammatory effect mediated by ADRB2 is not completely clear. Whether the signaling pathway involves the canonical pathway (cAMP/PKA or cAMP/EPAC) or the non canonical pathway (β-arrestin) is still debated (Albertini et al., 2020). In our conditions, mimicking Gs-coupling with IBMX had a significant effect in iPSC-derived microglia but not in human primaries. Yet, further investigations concerning the role of cAMP-sensitive effectors or the involvement of β-arrestin are still needed.

Given the major neuromodulatory role of norepinephrine in the CNS and recent evidence regarding the role of purinergic receptors in microglial functions (Bernier et al., 2019; Liu et al., 2019; Stowell et al., 2019; Yamashita et al., 2016), a better grasp of how the main adrenergic receptor in microglia, ADRB2, controls P2X7-dependent IL-1β release is key to understand how inflammatory states are triggered and regulated in the CNS, in both healthy and pathological conditions.

Figures and legends

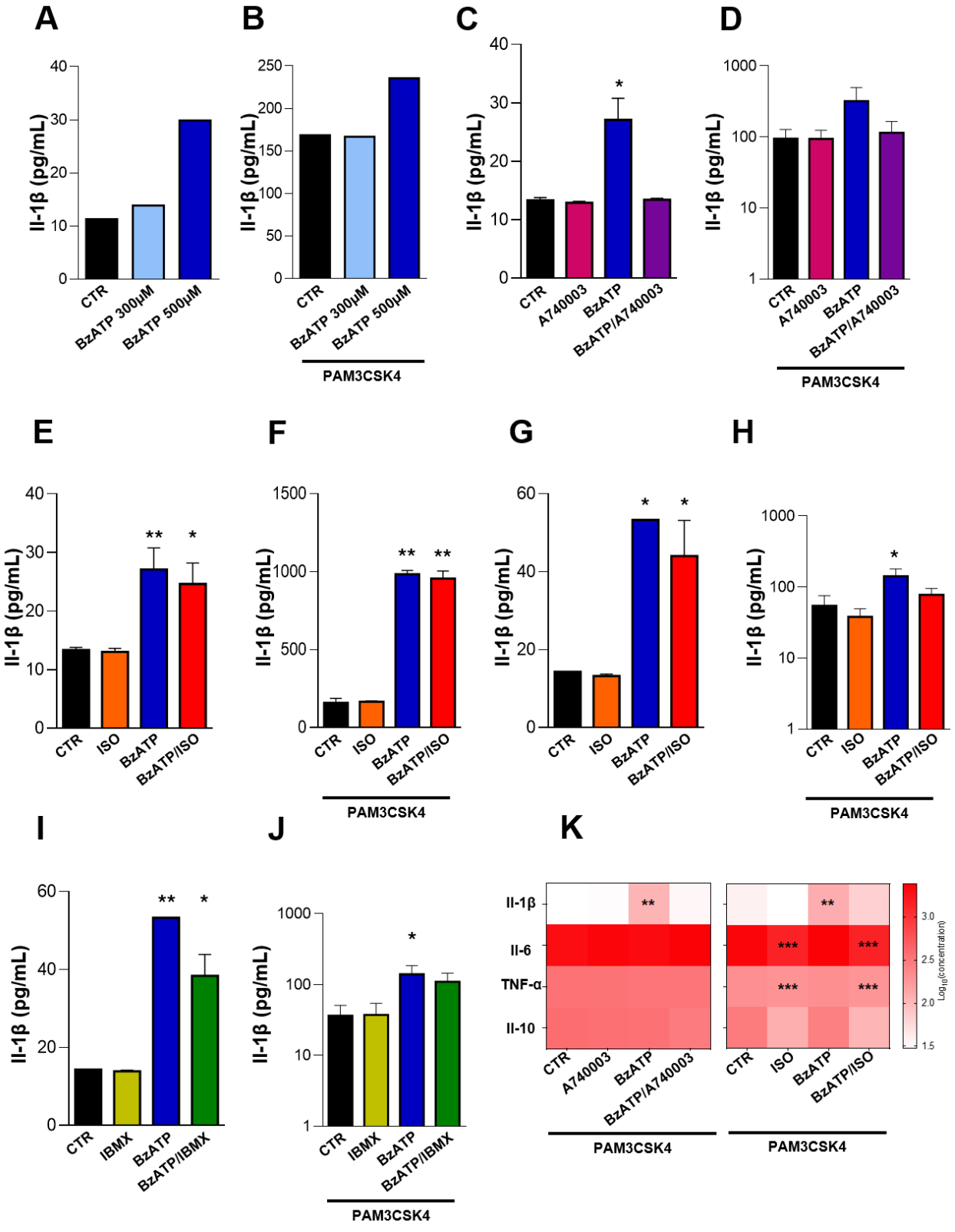


Figure 1. ADRB2 pre-activation reduces P2X7-dependent IL-1β release in human iPSC-derived microglia.

Microglia were treated for 24 h with vehicle, Pam_3CSK_4 (100 ng/ml), and/or isoproterenol (50 μM), and/or IBMX (100 μM). (**A**) IL-1β release from human iPSC-derived microglia (iMGL) induced by P2X7 agonist BzATP (n= 1). (**B**) IL-1β release from iMGL primed with Pam_3CSK_4 is induced by P2X7 agonist BzATP (n= 1). (**C**) IL-1β release from iMGL is induced by P2X7 agonist BzATP and suppressed by the selective P2X7 antagonist A740003 (10 μM). RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; $*P < 0.05$ (n= 3). (**D**) IL-1β release from iMGL primed with Pam_3CSK_4 is induced by P2X7 agonist BzATP and suppressed by the selective P2X7 antagonist A740003. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM (n= 5). (**E**) IL-1β release from iMGL is induced by P2X7 agonist BzATP and treated 3 hours with the ADRB2 agonist isoproterenol (50 μM). RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; $*P < 0.05$, $**P < 0.01$ (n= 3). (**F**) IL-1β release from iMGL primed with Pam_3CSK_4 is induced by P2X7 agonist BzATP and treated 3 hours with the ADRB2 agonist isoproterenol. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; $**P < 0.01$ (n= 2). (**G**) IL-1β release from iMGL pretreated with isoproterenol is induced by P2X7 agonist BzATP. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; $*P < 0.05$ (n= 2). (**H**) IL-1β release from iMGL primed with Pam_3CSK_4 is induced by P2X7 agonist BzATP and reduced by the pretreatment with isoproterenol. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; $*P < 0.05$ (n= 6). (**I**) IL-1β release from iMGL pretreated with IBMX (100 μM) is induced by P2X7 agonist BzATP. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ±

SEM; *$P < 0.05$, **$P < 0.01$ (n= 2). (**J**) IL-1β release from iMGL primed with Pam_3CSK_4 and pretreated with IBMX is induced by P2X7 agonist BzATP. RM one-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM; *$P < 0.05$ (n= 5). (**K**) Heatmap of cytokine levels (IL-1β, Il-6, TNF-α, and IL-10) released from iMGL induced by BzATP and treated with the selective P2X7 antagonist A740003. Heatmap of cytokine levels released from iMGL pretreated with isoproterenol and induced by BzATP. Two-way ANOVAs were performed followed by Dunnett's *post hoc* test. Mean ± SEM; **$P < 0.01$, ***$P < 0.001$ (n= 5-6). CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; TNF-α= tumor necrosis factor α.

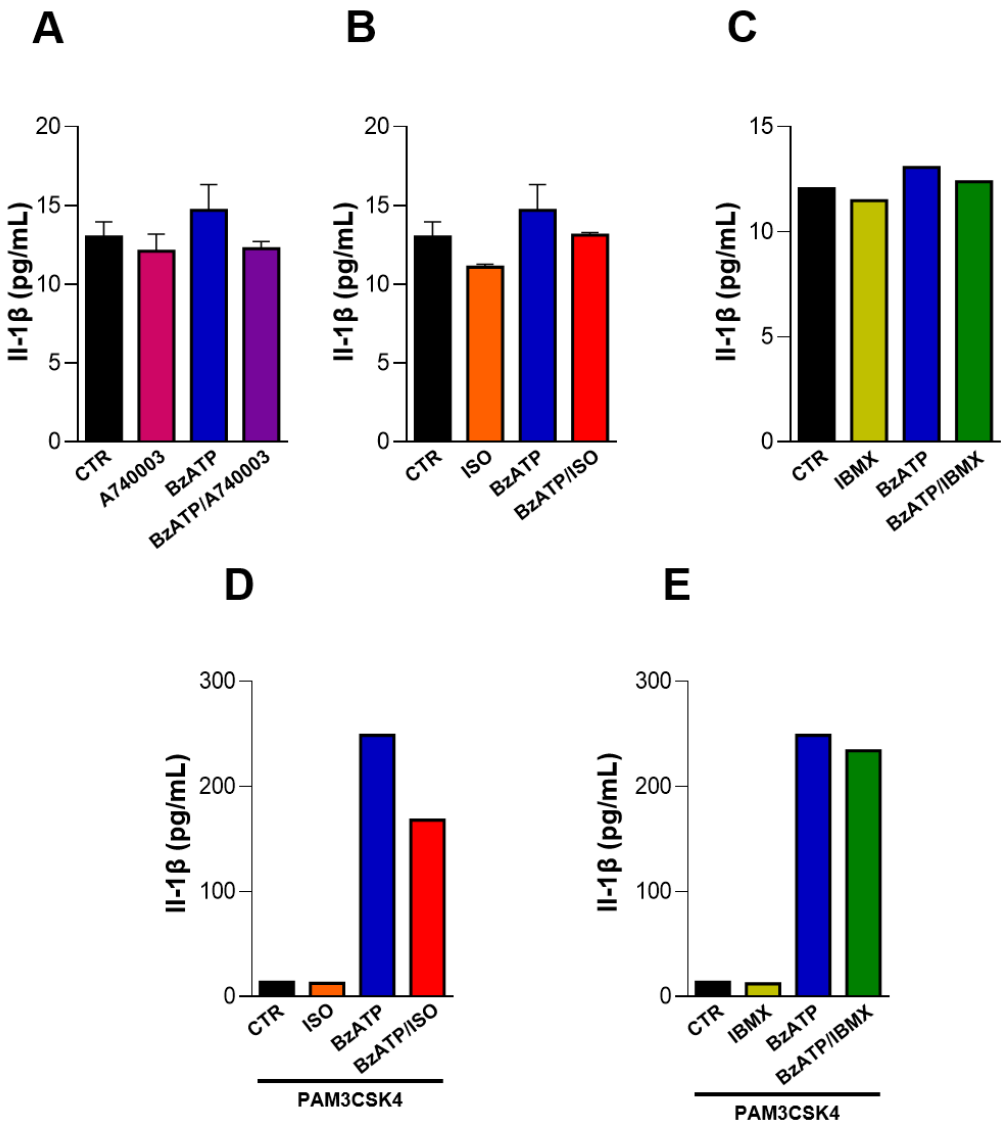


Figure 2. ADRB2 pre-activation reduces P2X7-dependent IL-1β release in human primary microglia.

Human primary microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (20 μM) and/or IBMX (100 μM). (**A**) IL-1β release from human primary microglia (hMGL) in presence of P2X7 agonist BzATP and selective antagonist A740003 (n=2). (**B**) IL-1β release from hMGL in presence of P2X7 agonist BzATP and the ADRB2 agonist isoproterenol (n=2). (**C**) IL-1β release from hMGL in presence of P2X7 agonist BzATP and IBMX (n=1). (**D**) IL-1β release from hMGL primed with Pam3CSK4 is

induced by P2X7 agonist BzATP and reduced by the pretreatment with isoproterenol (n=1). (**E**) IL-1β release from hMGL primed with Pam3CSK4 and pretreated with IBMX is induced by P2X7 agonist BzATP (n=1). CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-1β = interleukin-1β.

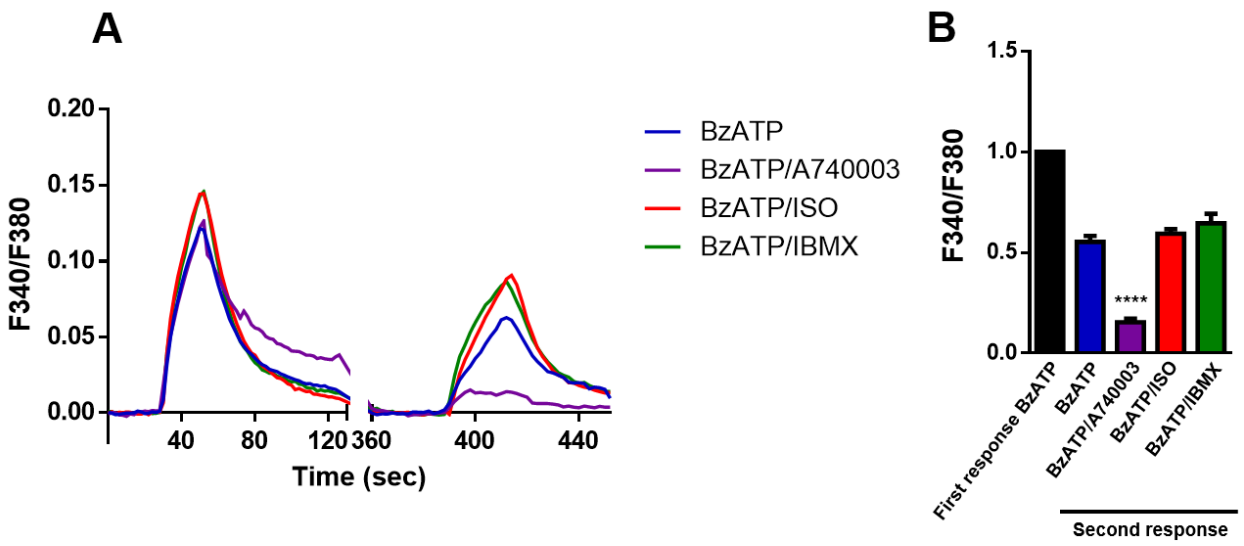


Figure 3. ADRB2 does not modulate P2X7 calcium transients in mouse primary microglia.

(**A**) Average traces showing calcium transients evoked by P2X7 activation in primary mouse microglia. The ratio 340/380 represents the intracellular calcium status of the cells. Traces on the left represent first stimulations, showing BzATP-evoked calcium response. Traces on the right side represent second stimulations with or without treatment (10 μM A740003, 10 μM isoproterenol, or 100 μM IBMX) applied in the interval of stimulations. (**B**) Quantitative comparison of second responses. One-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM of n = 123 for control (BzATP), n = 80 for BzATP + A740003, n = 103 for BzATP + ISO, n = 76 for BzATP + IBMX. ****P < 0.0001.

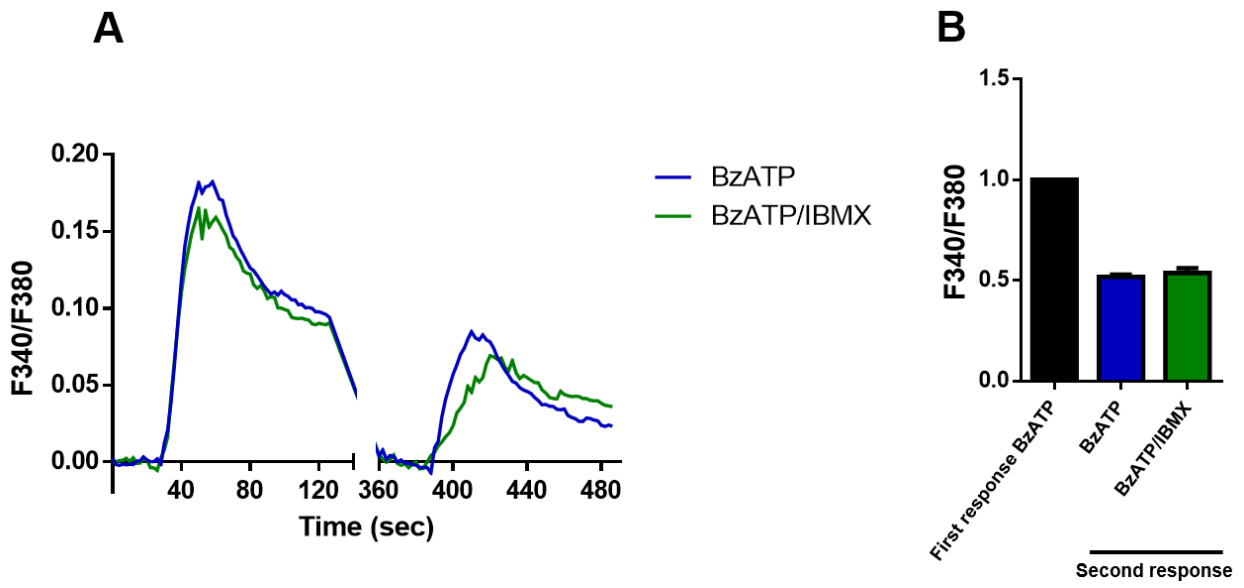


Figure 4. IBMX does not modulate P2X7 calcium transients in human primary microglia.

(**A**) Average traces showing calcium transients evoked by P2X7 activation in primary human microglia. The ratio 340/380 represents the intracellular calcium status of the cells. Traces on the left represent first stimulations, showing BzATP-evoked calcium response. Traces on the right side represent second stimulations with or without treatment with IBMX (100 µM) applied in the interval of stimulations. (**B**) Quantitative comparison of second responses. One-way ANOVA was performed followed by Dunnett's *post hoc* test. Mean ± SEM of *n* = 343 for control (BzATP), n = 295 for BzATP + IBMX.

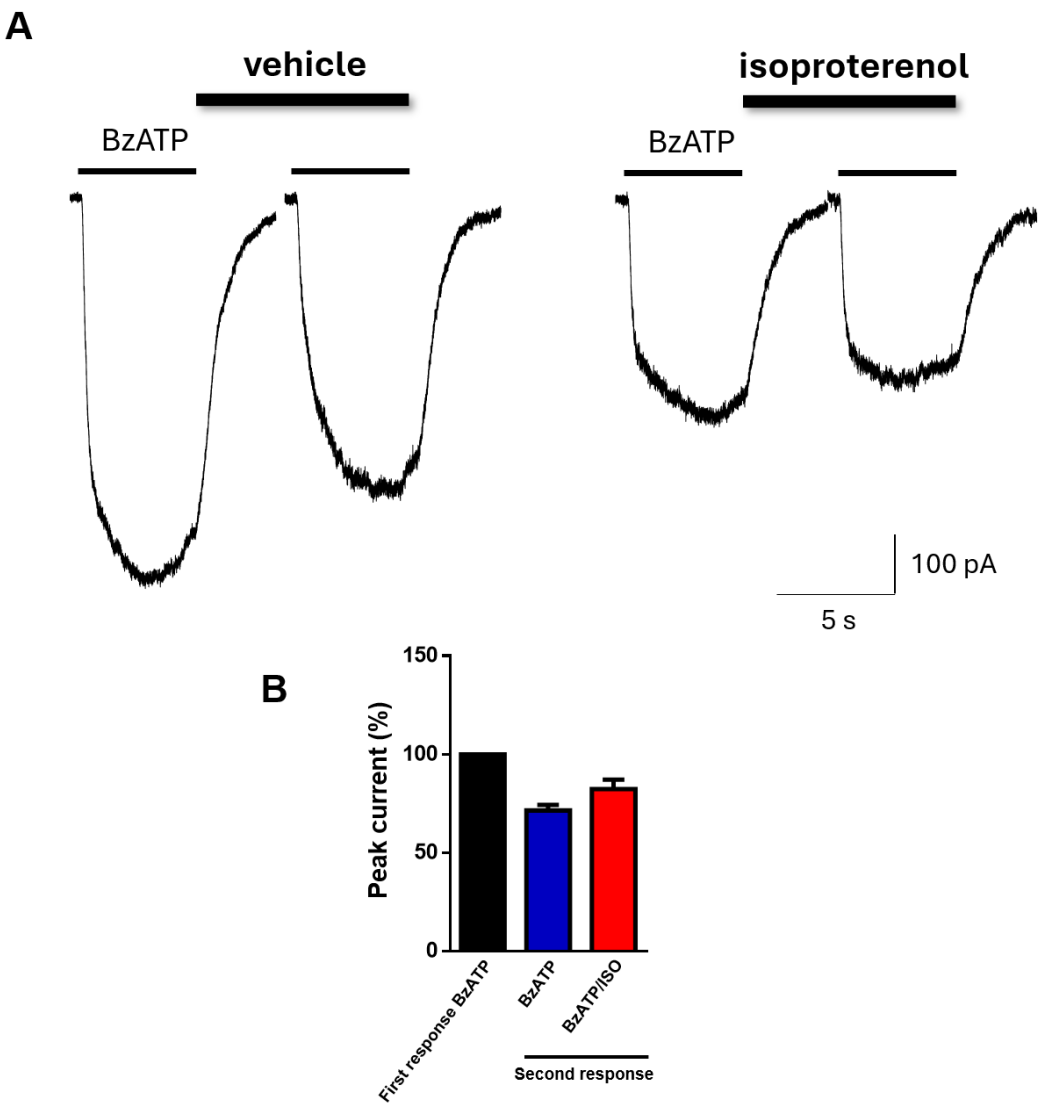


Figure 5. ADRB2 does not modulate P2X7 currents in mouse primary microglia.

(**A**) Typical traces in patch clamp recording of primary mouse microglia showing either a double stimulation of P2X7 with BzATP (100 µM) (left) or a double stimulation with BzATP including isoproterenol treatment applied in the interval of stimulations (right). (**B**) Quantitative comparison of second responses. One-way ANOVA was performed followed by Tukey's *post hoc* test. Mean ± SEM (n= 8 each).

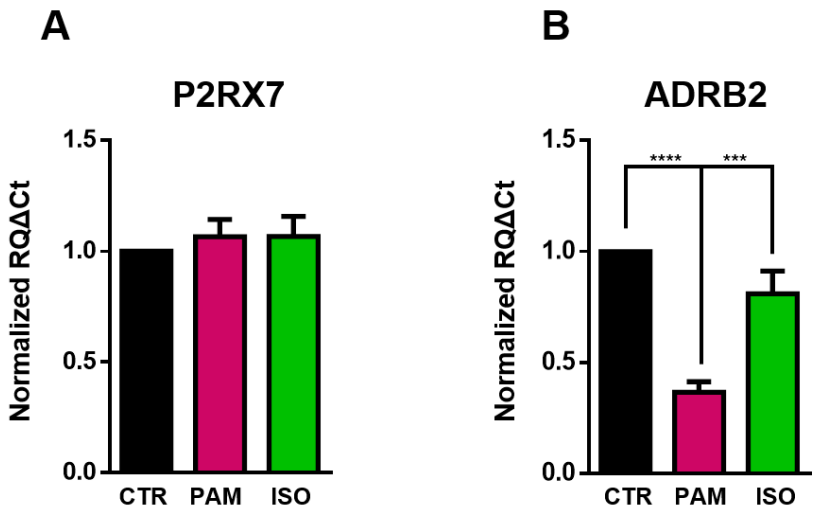
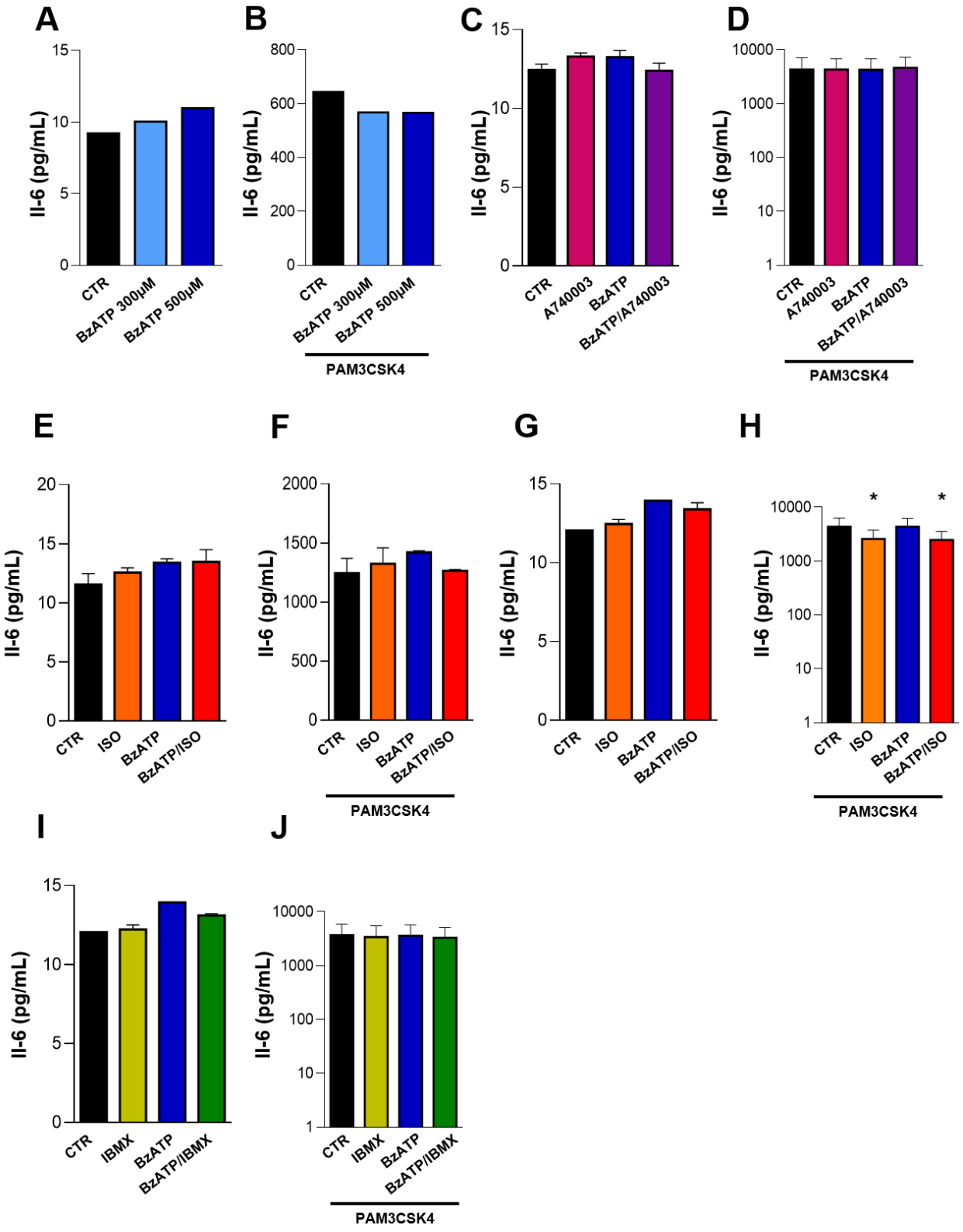


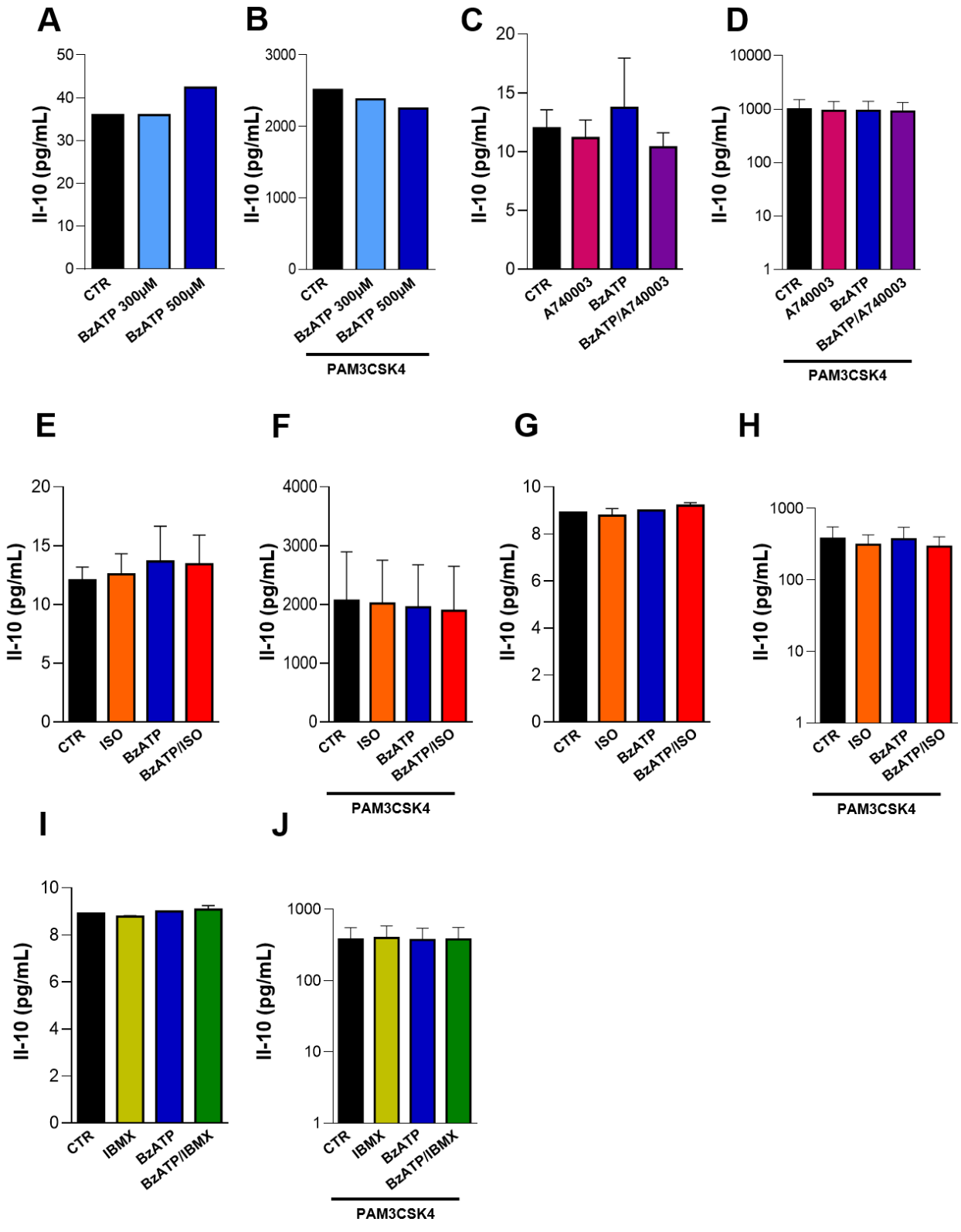
Figure 6. ADRB2 and P2X7 gene expression following activation of Pam3CSK4 or ADRB2 signaling in mouse primary microglia.

Real-time qPCR assessment of P2X7 (**A**) and ADRB2 (**B**) mRNA expression levels in primary mouse microglia treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), or isoproterenol (20 μM). One-way ANOVA was performed followed by Tukey's *post hoc* test. Mean ± SEM of $n = 7$; ***$P < 0.001$, ****$P < 0.0001$. CTR= control; PAM=Pam3CSK4; ISO=isoproterenol.


A
B
C
D
Il-6 (pg/mL)
CTR
BzATP 300µM
BzATP 500µM
A740003
BzATP
BzATP/A740003
PAM3CSK4
E
F
G
H
ISO
BzATP/ISO
*
I
J
IBMX
BzATP/IBMX

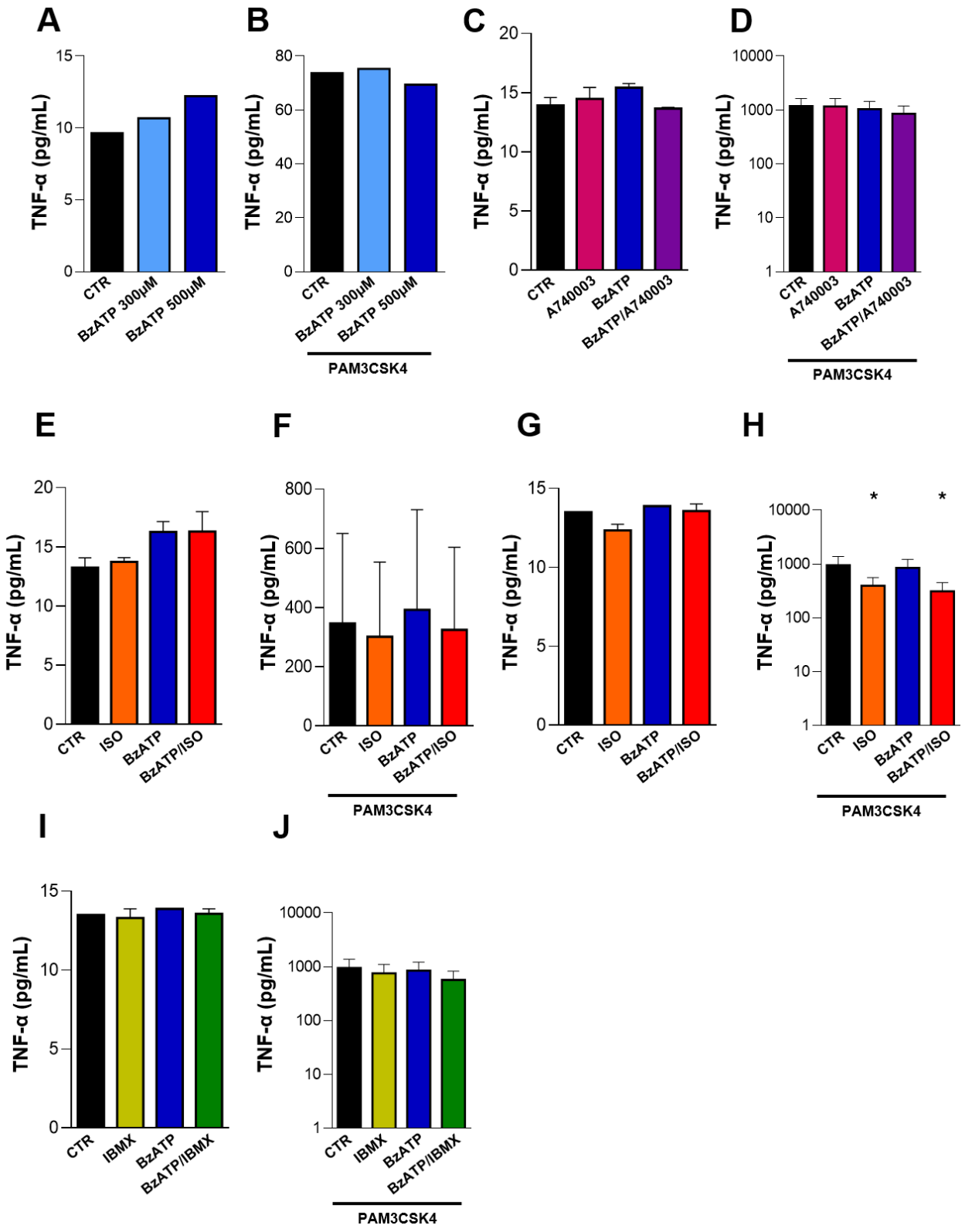
Supplemental Figure 1. ADRB2 pre-activation decreases IL-6 release in human iPSC-derived microglia.

Microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (50 μM), and/or IBMX (100 μM). IL-6 release from human iPSC-derived microglia (iMGL), in presence of the P2X7 agonist BZATP (**A**, n=1); primed with Pam3CSK4 and in presence of BzATP (**B**, n=1); in presence of BzATP and treated with the selective P2X7 antagonist A740003 (**C**, n=3); primed with Pam3CSK4, in presence of BzATP and A740003 (**D**, n=4); in presence of BzATP and treated with the ADRB2 agonist isoproterenol (**E**, n=4); primed with Pam3CSK4, in presence of BzATP and isoproterenol (**F**, n=2); pretreated with isoproterenol, in presence of BzATP (**G**, n=2); primed with Pam3CSK4, pretreated with isoproterenol, in presence of BzATP (**H**, $*P < 0.05$, n=5); pretreated with IBMX, in presence of BzATP (**I**, n=2); primed with Pam3CSK4, pretreated with IBMX, in presence of BzATP (**J**, n=5); RM one-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM=Pam3CSK4; ISO=isoproterenol; IL-6 = interleukin-6.


A
B
C
D
Il-10 (pg/mL)
CTR
BzATP 300µM
BzATP 500µM
PAM3CSK4
A740003
BzATP
BzATP/A740003
E
F
G
H
ISO
BzATP/ISO
I
J
IBMX
BzATP/IBMX

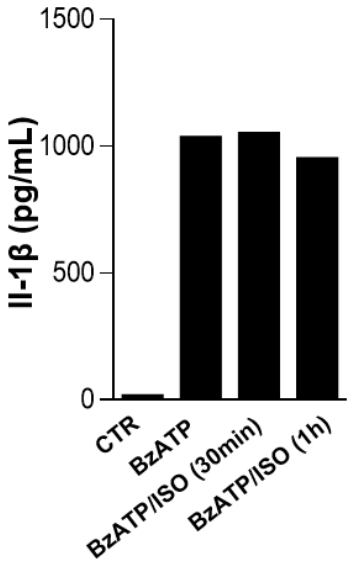
Supplemental Figure 2. No effect of ADRB2 pre-activation on IL-10 release in human iPSC-derived microglia.

Microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (50 μM), and/or IBMX (100 μM). IL-10 release from human iPSC-derived microglia (iMGL), in presence of the P2X7 agonist BZATP (**A**, n=1); primed with Pam3CSK4 and in presence of BzATP (**B**, n=1); in presence of BzATP and treated with the selective P2X7 antagonist A740003 (**C**, n=3); primed with Pam3CSK4, in presence of BzATP and A740003 (**D**, n=5); in presence of BzATP and treated with the ADRB2 agonist isoproterenol (**E**, n=4); primed with Pam3CSK4, in presence of BzATP and isoproterenol (**F**, n=2); pretreated with isoproterenol, in presence of BzATP (**G**, n=2); primed with Pam3CSK4, pretreated with isoproterenol, in presence of BzATP (**H**, n=6); pretreated with IBMX, in presence of BzATP (**I**, n=2); primed with Pam3CSK4, pretreated with IBMX, in presence of BzATP (**J**, n=6); RM one-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-10 = interleukin-10.


A
B
C
D
E
F
G
H
I
J
TNF-α (pg/mL)
CTR
BzATP 300μM
BzATP 500μM
A740003
BzATP
BzATP/A740003
ISO
BzATP/ISO
IBMX
BzATP/IBMX
PAM3CSK4
*
*

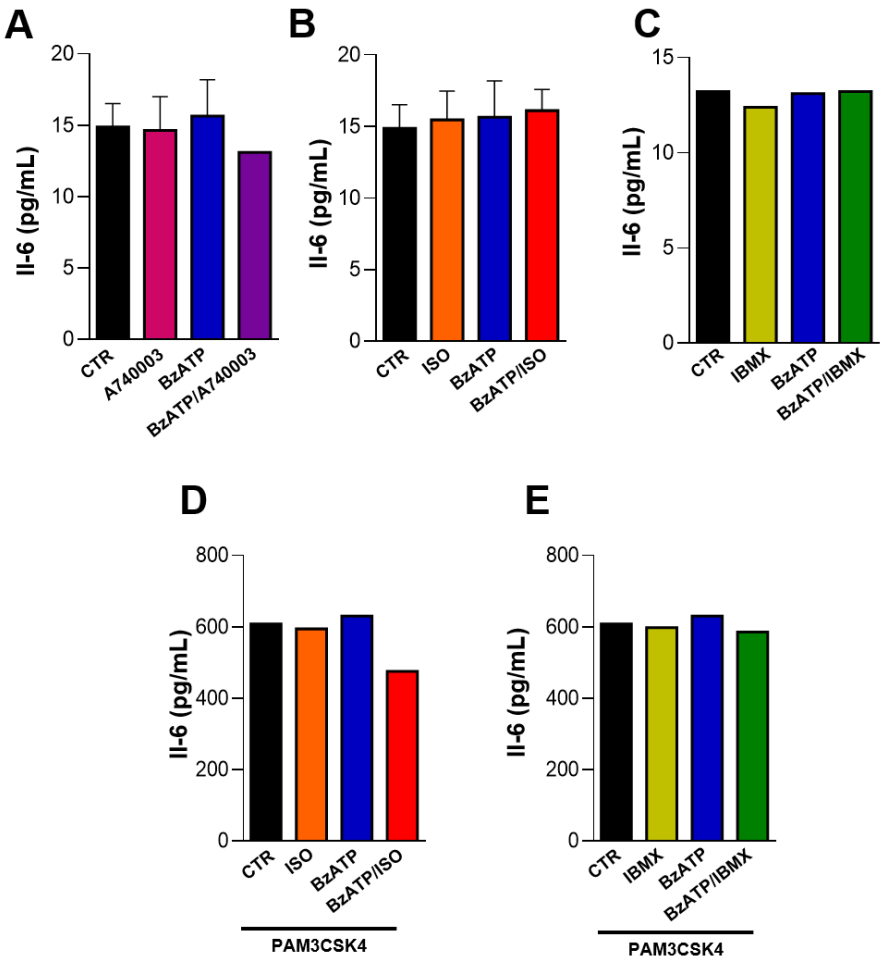
Supplemental Figure 3. ADRB2 pre-activation inhibits TNF-α release in human iPSC-derived microglia.

Microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (50 μM), and/or IBMX (100 μM). TNF-α release from human iPSC-derived microglia (iMGL), in presence of the P2X7 agonist BZATP (**A**, n=1); primed with Pam3CSK4 and in presence of BzATP (**B**, n=1); in presence of BzATP and treated with the selective P2X7 antagonist A740003 (**C**, n=3); primed with Pam3CSK4, in presence of BzATP and A740003 (**D**, n=5); in presence of BzATP and treated with the ADRB2 agonist isoproterenol (**E**, n=4); primed with Pam3CSK4, in presence of BzATP and isoproterenol (**F**, n=2); pretreated with isoproterenol, in presence of BzATP (**G**, n=2); primed with Pam3CSK4, pretreated with isoproterenol, in presence of BzATP (**H**, $*P < 0.05$, n=6); pretreated with IBMX, in presence of BzATP (**I**, n=2); primed with Pam3CSK4, pretreated with IBMX, in presence of BzATP (**J**, n=6); RM one-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; TNF-α = tumor necrosis factor α.



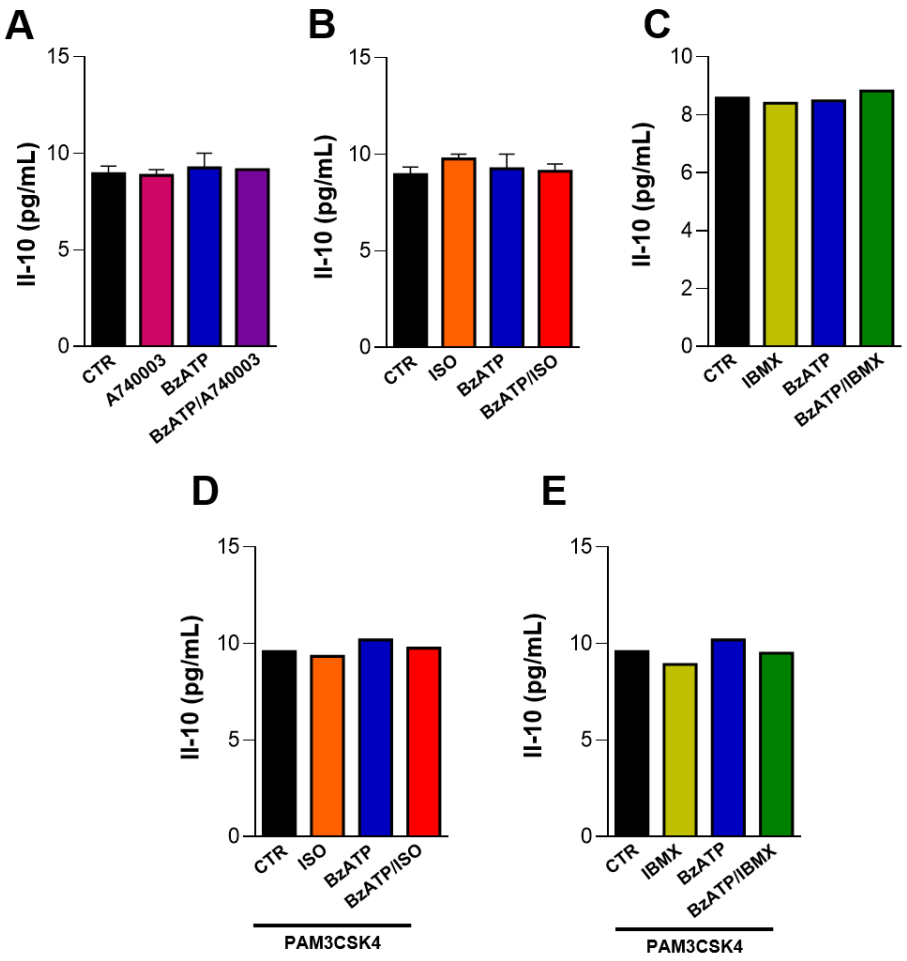
Supplemental Figure 4. No effect of ADRB2 acute pre-activation on P2X7-dependent IL-1β release in human iPSC-derived microglia.

Microglia were treated for 1 h or 30 min with vehicle or isoproterenol (50 μM). IL-1β release induced by P2X7 agonist BzATP was measured in human iPSC-derived microglia (n= 1).



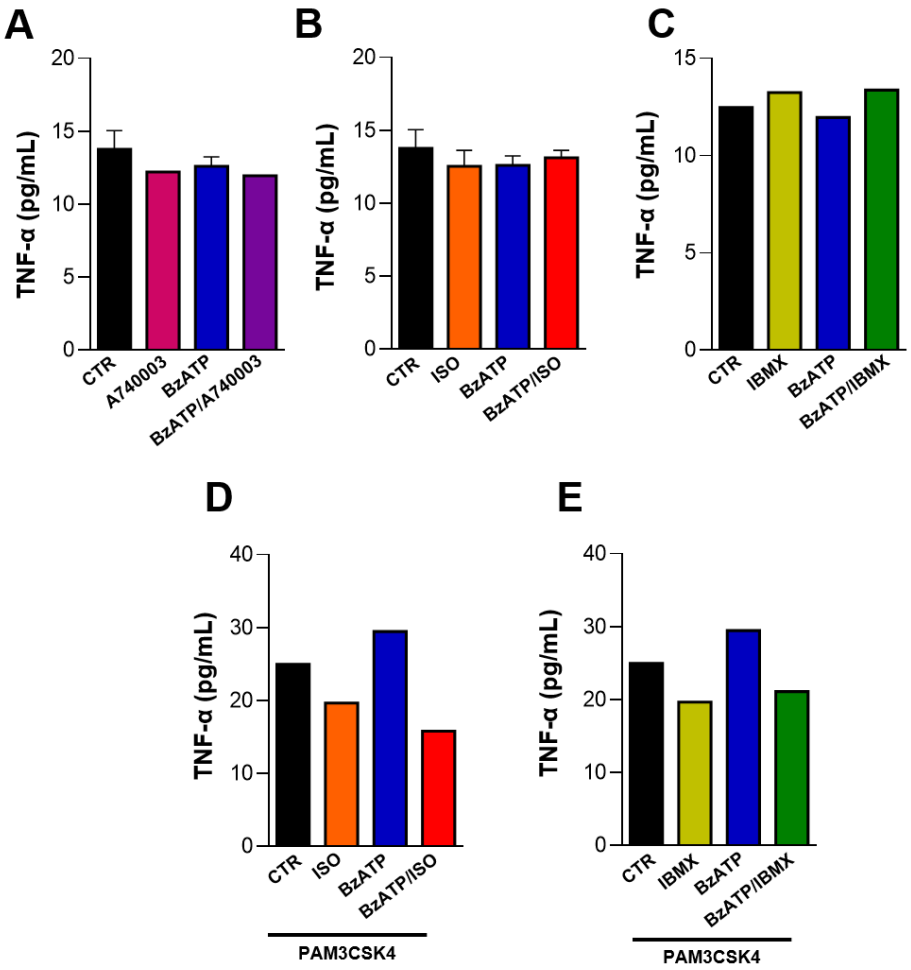
Supplemental Figure 5. ADRB2 pre-activation decreases IL-6 release in human primary microglia.

Human primary microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (20 μM) and/or IBMX (100 μM). IL-6 release from human primary microglia (hMGL), in presence of the P2X7 agonist BZATP and selective antagonist A740003 (**A**, n=2); in presence of BZATP and isoproterenol (**B**, n=2); in presence of BZATP and IBMX (**C**, n=1); primed with Pam3CSK4, pretreated with isoproterenol and in presence of BzATP (**D**, n=1); primed with Pam3CSK4, pretreated with IBMX and in presence of BzATP (**E**, n=1); One-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-6 = interleukin-6.



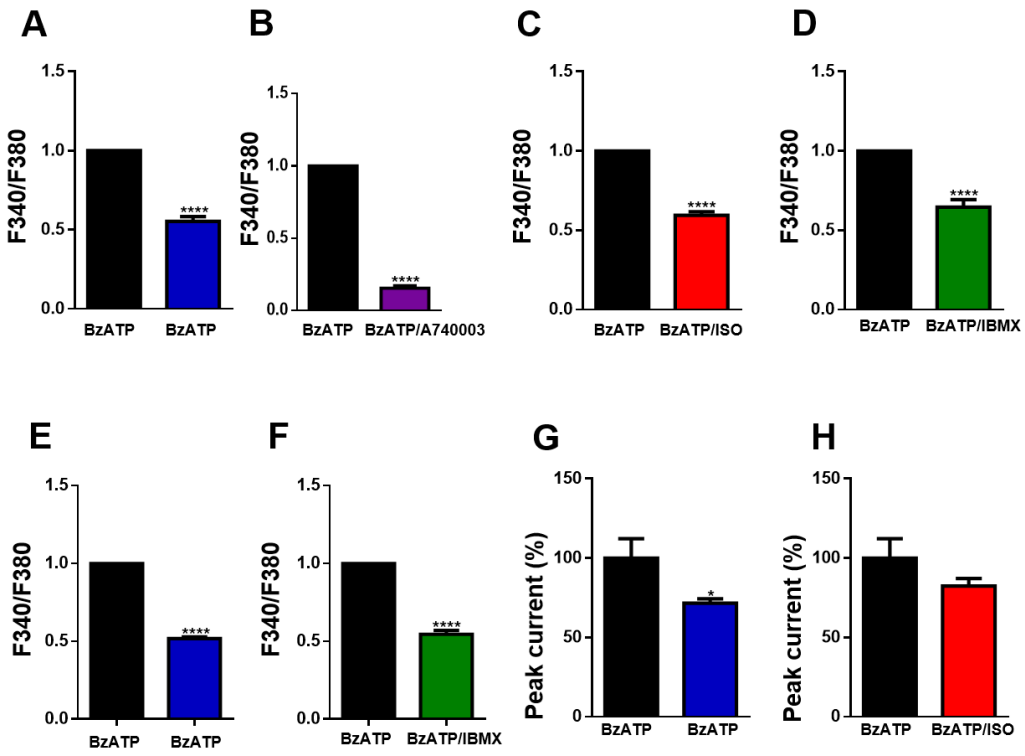
Supplemental Figure 6. No effect of ADRB2 pre-activation on IL-10 release in human primary microglia.

Human primary microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (20 μM) and/or IBMX (100 μM). IL-10 release from human primary microglia (hMGL), in presence of the P2X7 agonist BZATP and selective antagonist A740003 (**A**, n=2); in presence of BZATP and isoproterenol (**B**, n=2); in presence of BZATP and IBMX (**C**, n=1); primed with Pam3CSK4, pretreated with isoproterenol and in presence of BzATP (**D**, n=1); primed with Pam3CSK4, pretreated with IBMX and in presence of BzATP (**E**, n=1); One-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; IL-10 = interleukin-10.



Supplemental Figure 7. ADRB2 pre-activation decreases TNF-α release in human primary microglia.

Human primary microglia were treated for 24 h with vehicle, Pam3CSK4 (100 ng/ml), and/or isoproterenol (20 μM) and/or IBMX (100 μM). TNF-α release from human primary microglia (hMGL), in presence of the P2X7 agonist BZATP and selective antagonist A740003 (**A**, n=2); in presence of BZATP and isoproterenol (**B**, n=2); in presence of BZATP and IBMX (**C**, n=1); primed with Pam3CSK4, pretreated with isoproterenol and in presence of BzATP (**D**, n=1); primed with Pam3CSK4, pretreated with IBMX and in presence of BzATP (**E**, n=1); One-way ANOVAs were performed followed by Dunnett's *post hoc* test. CTR= control; PAM= Pam3CSK4; ISO= isoproterenol; TNF = tumor necrosis factor.



Supplemental Figure 8. Quantitative comparison of microglial P2X7 calcium transients and cationic currents using the double stimulation protocol.

Histogram representing the double stimulation of the calcium transients or calcium currents evoked by P2X7 activation with or without treatment inter-stimulations in mouse primary microglia (MPM) or human primary microglia (hMGL). Quantitative comparison of second responses normalized to first responses following vehicle in MPM of BzATP-evoked calcium transients (**A**, n=123); A740003 treatment in MPM of BzATP-evoked calcium transients (**B**, n=80); isoproterenol treatment in MPM of BzATP-evoked calcium transients (**C**, n=103); IBMX treatment in MPM of BzATP-evoked calcium transients (**D**, n=76); vehicle in hMGL of BzATP-evoked calcium transients (**E**, n=343); IBMX treatment in hMGL of BzATP-evoked calcium transients (**F**, n=295); vehicle in MPM of BzATP-evoked calcium currents (**G**, n=8); isoproterenol treatment in MPM of BzATP-evoked calcium currents (**H**, n=8). Unpaired t-test, **P* < 0.05, *****P* < 0.0001.

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Chapter 4. General Discussion and Conclusion

Summary of results

General discussion

4.1 Physiological roles of ADRB2-mediated inhibition in microglia

4.2 Potential mechanisms responsible for modulating microglial P2Y6 and P2X7

4.3 Therapeutic relevance of P2Y6 modulation

4.4 Therapeutic relevance of P2X7 modulation

Limitations

5.1 Origin of microglia

5.2 Microglia heterogeneity

5.3 In vitro/Ex vivo

5.4 Sex differences

Future Directions

Conclusion

Summary of results

The first objective for this dissertation was to identify a potential candidate as a modulator of microglial P2Y6, particularly to investigate whether the Gs-coupled β2 receptor is involved in the modulation of P2Y6-dependent phagocytosis in microglia. In Chapter 2, we show for the first time that the activation of ADRB2 decreases the calcium transients evoked by P2Y6 in mice and in humans. We demonstrate that ADRB2 modulation impact P2Y6-induced phagocytosis in mice, an inhibition that was further confirmed in humans. We did not find any influence of P2Y6 on the release of cytokines. Interestingly, our qPCR results suggest that ADRB2 affects the expression of microglial P2Y6. These results conclusively prove that ADRB2 influences microglial P2Y6-dependent phagocytosis in rodents and in humans.

The second objective of this thesis was to investigate whether ADRB2 could also be involved in the modulation of P2X7-dependent cytokine release. In Chapter 3, we report that only a 24-hour pretreatment with the ADRB2 agonist isoproterenol reduces the P2X7-dependent IL-1β release. To explore this modulation in more detail, we hypothesized that ADRB2 could modulate microglial P2X7 directly. Following a battery of assays, including calcium imaging, electrophysiology, and qPCR, we concluded that ADRB2 did not affect P2X7 calcium transients, P2X7 currents, or P2X7 expression in mice and/or in humans. These results establish that ADRB2 reduces microglial P2X7-dependent IL-1β cytokine release but does not act directly on the P2X7 receptor-channel.

General discussion

4.1 Physiological roles of ADRB2-mediated inhibition in microglia

Microglia are always in movement, constantly surveying their environment. However, depending on local cellular needs, microglia can become activated. There are various activation states of microglia with typical functions including phagocytosis and cytokine release.

Phagocytosis is a fundamental process characterized by the elimination of cellular debris, pathogens, dead cells, or synaptic pruning. Signaling molecules such as “eat-me” signals are generally released to indicate to professional phagocytes that the target releasing this signal needs to be cleared. In the CNS, microglia can sense cues, such as ADP or CX3CL1, to first move toward the target and then change from surveying mode to an active state to perform phagocytosis. UDP acts as an “eat-me” signal by activating microglial P2Y6 to induce phagocytosis.

Cytokine release is also a major function of microglia and is characterized by an inflammatory response depending on the needs of the environment. Similarly, microglia can detect danger signals such as ATP to switch from surveying mode to an active state to release cytokines. ATP acts as a DAMP by activating microglial P2X7 signaling to induce cytokine release.

As mentioned in the summary, in Chapters 2 and 3 we identified two microglial modulation mechanisms, where ADRB2 reduces P2Y6-dependent phagocytosis and P2X7-dependent IL-1β cytokine release. Interestingly, our qPCR results indicate that the expression of ADRB2 decreases in microglia activated by Pam3CSK4. This result

suggests that when microglia are surveying their environment, there is a high expression level of ADRB2. Indeed, they do not need to switch to an active state unless they find a signal, thus, ADRB2 inhibits the mechanisms of P2Y6 and P2X7 that are useful in active microglia. However, when microglia switch to an activated state, ADRB2 is downregulated, causing a disinhibition of either P2Y6-dependent phagocytosis or P2X7-dependent IL-1β cytokine release relying on the appropriate microglial state determined by the environment.

In conclusion, we postulate that ADRB2 acts as a negative neuromodulator of nucleotidic functions in microglia, by inhibiting P2Y6-dependent phagocytosis and P2X7-dependent IL-1β cytokine release in surveying microglia. In activated microglia, downregulation of ADRB2 facilitates these key microglial functions. We propose that low expression levels of ADRB2 in activated microglia is not sufficient to provide effective control, therefore relieving microglia from adrenergic inhibition and allowing them to perform the required homeostatic functions.

4.2 Potential mechanisms responsible for modulating microglial P2Y6 and P2X7

An interesting question is how ADRB2 impacts P2Y6 or P2X7 function.

In Chapter 2, we proved that P2Y6 calcium transients were reduced by the activation of ADRB2 in rodents and in humans. The specific mechanism that involves P2Y6 in the process of phagocytosis is not known. However, a recent study on a model of epileptogenesis demonstrated that intracellular calcium was necessary to induce phagocytosis through UDP/P2Y6 (Umpierre et al., 2024). Altogether, this suggests that ADRB2, by reducing the calcium transients of P2Y6, can decrease the level of calcium-

dependent phagocytosis evoked by P2Y6. Our qPCR results also indicate that, when microglia are treated with isoproterenol to activate ADRB2, the level of P2Y6 expression is significantly decreased. This result indicates that ADRB2 downregulates P2Y6, which could explain the lower activity of P2Y6-dependent phagocytosis in isoproterenol-treated microglia.

Altogether, our data suggest a double level of inhibition. In surveying microglia, the high expression of ADRB2 controls P2Y6 function by downregulating its expression as UDP/P2Y6-dependent phagocytosis is not needed during surveillance. However, over a shorter time course, we demonstrated that ADRB2 reduces P2Y6 calcium transients, providing a constitutive regulatory mechanism to control the level of phagocytosis.

As shown in Chapter 3, we did not observe changes in P2X7 calcium transients or P2X7 currents following ADRB2 activation. Our qPCR results indicate that ADRB2 activation has no effect on P2X7 expression. Therefore, while ADRB2 can reduce IL-1β release dependent of P2X7 activation, it does not directly modulate P2X7 receptor-channels. This suggests a modulatory mechanism downstream of P2X7 signaling.

4.3 Therapeutic relevance of P2Y6 modulation

P2Y6, as mentioned in previous sections, has been shown to be upregulated and involved in various neurodegenerative diseases and in neuropathic pain. Yet, the precise role of P2Y6 is not entirely clear as its engagement appears to have both beneficial and detrimental effects. Phagocytosis mediated by P2Y6 has been shown to clear neuronal debris in ischemic stroke and eliminate amyloid debris in AD (Anwar et al., 2020). Activation of P2Y6-dependent phagocytosis is beneficial in the onset of these pathologies by eliminating debris that contributes to neuroinflammation. However, phagocytosis

induced by P2Y6 appears to be detrimental in later stages. In PD, P2Y6 has been involved in the elimination of the dopaminergic neurons (Oliveira-Giacomelli et al., 2019). A recent paper proposed that P2Y6 can phagocytize stressed but viable neurons (Puigdellivol et al., 2021). In pathological contexts, P2Y6 might contribute to the elimination of viable neurons that should not be removed, thereby participating in neurodegeneration.

In addition, it is well known that the noradrenergic system progressively degenerates in the onset of AD and PD, preceding the primary symptoms. This suggests that a defective noradrenergic system may contribute to the initiation, progression, and severity of these diseases (Weinshenker, 2018). In Chapter 2, we showed that ADRB2 decreases P2Y6 calcium transients which might be important in the regulation of phagocytosis. The progressive loss of the noradrenergic control on P2Y6 in these diseases could explain the switch to the detrimental role of P2Y6. Indeed, the lack of noradrenergic control of P2Y6-dependent phagocytosis could lead to the elimination of viable neurons, perpetuating the process of degeneration.

In neuropathic pain, P2Y6 has been shown to contribute to mechanical allodynia and thermal hyperalgesia (Huang et al., 2018). Nevertheless, the role of P2Y6-dependent phagocytosis in neuropathic pain is not known. A recent interesting study demonstrated that, during the early stages of neuropathic pain, microglia selectively engulf spinal inhibitory synapses, participating in spinal hyperexcitability (Yousefpour et al., 2023). Moreover, microglial P2Y6 has been recently associated with synaptic pruning (Dundee, Puigdellivol, Butler, & Brown, 2023; Dundee, Puigdellivol, Butler, et al., 2023) and interfering with P2Y6 activity has been shown to reduce neuropathic pain (Huang et al.,

2018; Wang et al., 2019). One hypothesis is that P2Y6 participates in the pathological elimination of inhibitory synapses, thereby contributing to the development or maintenance of neuropathic pain through the modulation of synaptic connectivity and excitation/inhibition balance.

Furthermore, noradrenergic inputs have antinociceptive effects in the spinal cord. While the role of the α2 noradrenergic receptor has been well described, studies have also documented the antinociceptive role of the β2 noradrenergic receptor in various pain models (Arora et al., 2021; Damo et al., 2023; Uzkeser et al., 2012; Zhang et al., 2016). Stimulating the noradrenergic system (through α2 or β2 receptors) in the spinal cord may reduce neuroinflammation and hyperexcitability, contributing to pain relief.

A potential and additional role of ADRB2 in the mediation of antinociceptive effects, aside from dampening hyperexcitability and neuroinflammation, could be to inhibit P2Y6-dependent phagocytosis. This regulation could prevent the engulfment of spinal inhibitory interneurons, thus preserving the balance of excitation and inhibition in spinal circuits and potentially contributing to pain relief.

It is important to mention that P2Y6 has been associated with the release of the pro-inflammatory cytokine IL-6 via the activation of the JAK/STAT pathway (Bian et al., 2019). Here, we did not find evidence of P2Y6 involvement in cytokine release, yet we cannot exclude that P2Y6 may contribute to neuroinflammation by releasing other pro-inflammatory mediators. In this context, ADRB2 could reduce pro-inflammatory P2Y6 activity to achieve its antinociceptive effect.

Taken together, a selective activation of ADRB2 to limit P2Y6 detrimental effects might be a promising therapeutic strategy, yet a better discrimination between beneficial and detrimental effects of P2Y6 is still needed.

4.4 Therapeutic relevance of P2X7 modulation

P2X7, as mentioned in the previous sections, is upregulated and drives neuroinflammation in most neurodegenerative diseases and in neuropathic pain (Savio et al., 2018). As discussed in Chapter 3, the pro-inflammatory role of P2X7 signaling through recruitment of the NLRP3 inflammasome and IL-1β release is one of the major issues in pathological states. Silencing P2X7 activity produced promising results in reducing inflammation and degeneration in various diseases such as AD, PD, MS or neuropathic pain. Yet, several P2X7 antagonists (AZD9056, CE-224,535, EVT-401, GSK1482160) have failed in clinical trials (Territo & Zarrinmayeh, 2021). More selective antagonists (JNJ-55308942, JNJ-54175446) that only target P2X7 in the CNS are currently being tested in clinical trials (Territo & Zarrinmayeh, 2021). Another strategy is to use an indirect modulator to control microglial P2X7-dependent IL-1β release. Our results in Chapter 3 suggest that ADRB2 can reduce P2X7-dependent IL-1β release in microglia, and thus could represent a novel therapeutic avenue. However, a better understanding of this modulatory mechanism is needed to further consider clinical trials.

Limitations

5.1 Origin of microglia

The origin of microglia is determinant in the interpretation of our results. Significant differences have been identified between cell lines, primary cultures, and iPSC-derived microglia, as well as between rodents and humans (Warden et al., 2023).

Although mouse microglia are more readily available, significant differences in gene expression profiles, response to inflammation, and genes related to neurodegenerative diseases have been identified in comparison to human microglia (Warden et al., 2023). Evolutionary divergence, inbreeding, and housing conditions contribute to the distinction between mouse strains and human microglia (Smith & Dragunow, 2014). In this thesis we included results on human microglia as much as we could, based on available resources, i.e. iPSC-derived as well as primary microglia. It is important to note that human microglia also have limitations. The availability of human primary microglia is very limited and microglial phenotypes can be affected by ante mortem conditions, post-mortem delay, and transition to culture (Hasselmann et al., 2019; Timmerman et al., 2018). The iPSC-derived microglia have the advantage of being produced in large numbers, yet their phenotype is dependent on the method used for the differentiation and they may present transcriptional deficits (Hasselmann et al., 2019). We noticed differences between iPSC-derived and primary human microglia regarding the effective concentration of BzATP and the need to prime microglia with Pam3CSK4 to release IL-1β via P2X7, highlighting the importance of microglial origin in experimental outcomes.

5.2 Microglia heterogeneity

The heterogeneity of microglia is reflected by their transcriptional profile. There are different levels of cellular heterogeneity, including 1) microglial states depending on the environment and the physiological conditions (healthy or pathological), and 2) regional differences that are now recognized as a transcriptional continuum of the homeostatic gene signature (Sideris-Lampretsas & Malcangio, 2021). Indeed, it has been suggested that microglia, regardless of their anatomical location, share a common set of homeostatic genes, such as P2Y12 which expression is finely tuned by local environments. That is why, it is critical to identify the microglial states present in specific pathological conditions (such as DAM in AD) to better understand the mechanisms leading to dysregulation.

5.3 In vitro/ex vivo

One of the greatest limitations of this work is the lack of research conducted in vivo. All these results were obtained in cultured microglia (ex vivo) or iPSC-derived microglia (in vitro). In addition to origin and heterogeneity of microglia, our in vitro conditions represent an important limitation. Cell culture media combined with a variety of growth factors can influence the microglial profile. Furthermore, we investigated microglia in monoculture, without all the environmental cues and the potential interactions with other brain cell types (Timmerman et al., 2018).

Taken together, all these limitations could explain some of the divergent results from Chapters 2 and 3 compared to the literature. In Chapter 2, we did not find any involvement of microglial P2Y6 in the release of cytokines. P2Y6 has been associated with IL-6 release in a model of neuropathic pain (Bian et al., 2019) as well as TNF-α and IL-6 release in primary microglia primed by LPS (Yang et al., 2017). We used human

primary microglia whereas these studies used rodent (rat primary cells and murine BV2 cell line) microglia. Different species, culture conditions, as well as cellular heterogeneity could explain our results. In Chapter 3, we reported that the ADRB2 pathway reduces the release of IL-6 and TNF-α but not IL-1β, and P2X7 activation only induces the release of IL-1β but not IL-6 and TNF-α. ADRB2 has been shown to suppress microglial IL-6, IL-1β, and TNF-α release induced by LPS in mice (Heneka et al., 2010; Qian et al., 2011). A study in primary mouse microglia and in a mouse model of MS demonstrated that P2X7 was involved in TNF-α and IL-6 release (Sharp et al., 2008; Shieh et al., 2014). Similarly to Chapter 2, different species and heterogeneity could explain the divergent results.

5.4 Sex differences

Working with in vitro and ex vivo models, we could not address whether these modulations are sex-dependent. It is known that cell density, cell size, as well as genetic profile impact microglial functions that differ depending on sex, adding a degree of complexity to microglial heterogeneity (Masuda et al., 2020; Sideris-Lampretsas & Malcangio, 2021). Sex differences have been observed in chronic pain where women frequently report increased sensitivity to pain (Mogil, 2020). Abnormal P2X4 signaling in spinal microglia, contributing to hypersensitivity in chronic neuropathic pain, has been reported to be less prominent in females, including P2X4 upregulation and the effect of this pathway as well as the inhibitors used to improve the symptoms (Inoue & Tsuda, 2018).

Future Directions

Determine the effect of ADRB2 modulation of P2Y6 in pathological states

In Chapter 2, we identified that ADRB2 activation in microglia reduces P2Y6-dependent phagocytosis. Synaptic pruning through complement-dependent phagocytosis has been associated with the elimination of inhibitory interneurons in chronic neuropathic pain (Yousefpour et al., 2023). P2Y6 has been reported to be involved in synaptic pruning during development and aging (Dundee, Puigdellivol, Butler, & Brown, 2023; Dundee, Puigdellivol, Butler, et al., 2023). Therefore, it will be interesting to investigate whether microglial P2Y6-dependent phagocytosis plays a major role in neuropathic pain and, if so, whether ADRB2 modulation can be beneficial. This new modulatory role will also justify further investigations in disease models where P2Y6 has been shown to have critical roles, such as in AD and PD.

Intracellular calcium has been identified as a key element in activated microglia where its concentration significantly increases to perform major immune functions (Sharma & Ping, 2014). Abnormally elevated intracellular calcium concentrations have been linked to dysregulated signaling pathways in microglia (Tvrdik & Kalani, 2017). We show that ADRB2 exerts a negative modulation on P2Y6-dependent phagocytosis as well as P2Y6-evoked calcium transients. Recently, a group established the necessity of intracellular calcium to induce P2Y6-dependent phagocytosis (Umpierre et al., 2024). Therefore, it will be interesting to verify whether the effect of ADRB2 on P2Y6-evoked calcium responses is sufficient to inhibit P2Y6-dependent phagocytosis. In PD, as the noradrenergic system progressively degenerates, it may not be able to control the level

of microglial phagocytosis through P2Y6 due to its inability to control the level of UDP-evoked calcium transients.

Explore the mechanism underlying ADRB2 modulation of P2X7-dependent IL-1β release

In Chapter 3, we identified that ADRB2 activation in microglia reduces P2X7-dependent IL-1β release but does not directly act on P2X7. This modulation probably occurs downstream of P2X7 signaling; thus, it will be interesting to understand which molecular component is affected. The NLRP3 inflammasome, a tripartite molecule, composed of a nucleotide-binding NACHT domain with ATPase activity, an amino-terminal pyrin (PYRIN) domain and a carboxy-terminal leucine-rich repeat (LRR) domain that interacts with the pyrin domain of ASC to initiate inflammasome assembly, the two pore domain channels TWIK2 and THIK1, or caspase1 are all involved in IL-1β release downstream of P2X7 signaling and represent potential candidates.

Further experiments will establish which ADRB2-coupled intracellular pathway (canonical or non canonical) regulates P2X7-dependent IL-1β release. Interestingly, canonical pathways via cAMP/PKA or cAMP/EPAC as well as non-canonical pathways via β-arrestin have been shown to inhibit NF-κB activity, a transcription factor that induces the transcription of pro-inflammatory target genes (Kolmus et al., 2015). Considering that NF-κB can be activated by IL-1β (Pinteaux et al., 2002), it will be interesting to verify whether NF-κB is impacted by the negative modulation of ADRB2 on the P2X7-dependent IL-1β release.

ADRB2 modulation of other microglial purinergic receptors?

Another interesting line of research would be to investigate whether ADRB2 can influence other microglial P2 receptors, i.e. the ATP-gated channel P2X4 and/or the GPCRs P2Y12/13.

ADRB2 regulates at least one P2Y12/13 function (Bernier et al., 2019; Liu et al., 2019; Stowell et al., 2019), yet it remains to be tested whether the Gi-coupled P2Y12/13 receptors and the Gs-coupled ADRB2 receptor, with opposite signaling pathways, directly interact to influence chemotaxis.

Duloxetine, an inhibitor of norepinephrine reuptake, inhibits P2X4-mediated calcium transients (Yamashita et al., 2016), yet the direct involvement of ADRB2 in this inhibition is unknown. P2X4, mostly internalized in physiological states, is upregulated, and exerts toxic effects in pathological conditions (Duveau et al., 2020). The transgenic P2X4 knock-in mouse line that I created to induce P2X4 overexpression at the cell surface (Bertin et al., 2021) could be used to investigate the modulatory role of ADRB2 on abnormal P2X4 functions in microglia.

Conclusion

In conclusion, the goal of this dissertation is to provide evidence on the complexity of microglia as well as their critical role in healthy and pathological conditions. Among the variety of molecular elements participating in key microglial functions, we focused on a subset of P2 receptors known for their physiological relevance. This dissertation establishes a cell-autonomous modulatory role of the ADRB2 signaling pathway on P2Y6 and P2X7 receptors in microglia. We showed that ADRB2 reduces P2Y6-dependent phagocytosis and P2X7-dependent IL-1β release in rodents and in humans. Phagocytosis and cytokine release are typical functions of innate immune cells, thus, understanding the mechanisms underlying their regulation has tremendous biomedical relevance. We found that the norepinephrine receptor ADRB2 decreases expression levels and calcium transients of the UDP receptor P2Y6, whereas it did not directly affect calcium transients, currents, and expression of the ATP receptor P2X7. Although more research is required to determine the role of the P2Y6/ADRB2 crosstalk in pathological states, and to understand the molecular mechanism involved in the regulation of pro-inflammatory P2X7 functions through ADRB2, these findings represent a step forward in the understanding of complex mechanisms involved in many neurological diseases. These novel findings may contribute to the potential discovery of a viable therapeutic target to improve the quality of life of millions of patients around the world.

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