The role of purinergic receptors in the regulation of mRNA expression and release of inflammatory cytokines in cultured primary mouse glia

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Abstract

During neuroinflammation, glial cells are implicated as the source of diverse inflammatory mediators in the CNS. Available evidence has demonstrated that extracellular ATP induces production of various cytokines and chemokines through the P$_2$X$_7$ receptors in primary rat glia and murine microglia cell lines. However, the effects of purines on the cytokine synthesis and secretion in primary mouse glia remain unknown. In our study, we carried on a simultaneous examination for the effects of extracellular ATP on the production of six inflammatory cytokines (IL-6, IL-10, CCL2, IFN-γ, TNF-α, IL-12p70) in primary mouse microglia and astroglia. Our findings indicate that among these cytokines, the pro-inflammatory cytokines IL-6, TNF-α and chemokine CCL2 were up-regulated by the P$_2$X$_7$ activation in microglia, whereas the P$_2$X$_7$ activation provoked IL-6 and CCL2 production in astroglia. In microglia, the effects of BzATP on cytokine secretion were fully inhibited by non-selective P$_2$ receptor antagonists. Surprisingly, various selective P$_2$X$_7$ receptor antagonists blocked the BzATP-induced IL-6 and CCL2 release, but had no effects on TNF-α secretion. In P$_2$X$_7^{-/-}$ microglia, the production of IL-6, TNF-α, and CCL2 induced by ATP or BzATP was abolished. Together, these results implicate that the release of IL-6 and CCL2 is mainly mediated by the P$_2$X$_7$ receptors, while TNF-α is differently regulated. Accordingly, first we hypothesized that apart from the P$_2$X$_7$ receptors, BzATP can activate other P$_2$ receptors and induce the release of TNF-α. However, the data from other P$_2$ agonists and calcium microfluorometry have proven that P$_2$X$_7$ is the primary receptor stimulated by BzATP. Next, we further hypothesized that functional P$_2$X$_4$/P$_2$X$_7$ heterometric subunits might participate in TNF-α secretion by altering P$_2$X$_7$ pharmacological properties. Using P$_2$X$_4^{-/-}$ microglia we demonstrated that the P$_2$X$_4$ receptors did not affect the P$_2$X$_7$ pharmacology. It was also shown that
blocking the P₂X₇-mediated panx-1 activation did not alter the BzATP-evoked cytokine production. Furthermore, we found that in LPS-primed microglia, the P₂X₇ activation exerted suppressive actions on LPS-induced TNF-α release, whereas the release IL-6 and CCL2 was not influenced. Overall, the results of this study demonstrate the effects of P₂X₇ activation on IL-6, TNF-α and CCL2 induction in primary mouse glia. Most importantly, it is presented here that the production of microglial TNF-α is differentially regulated compared with IL-6 and CCL2.

**Keywords:** neuroinflammation, glia, cytokines, ATP, P₂X₇ receptors
**Abbreviations**

**A**

Aβ: amyloid β  
AC: adenylyl cyclase  
AD: Alzheimer’s disease  
AP-1: activating protein-1  
ADA: adenosine deaminase  
ADK: adenosine kinase  
ADP: adenosine 5’-diphosphate  
ALS: amyotrophic lateral sclerosis  
AMP: adenosine 5’-monophosphate  
APP: amyloid precursor protein  
ATP: adenosine 5’-triphosphate  
ACTB: actin β  
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid  
ADAMs: a desintegrins and metalloproteinases  
AdoHcy: S-adenosyl-homocysteine  

**B**

BBB: blood brain barrier  
BBG: brilliant blue G  
BCA: bicinechonic acid  
BMP: bone morphogenetic protein  
BSA: bovine serum albumin  
BDNF: brain-derived neurotrophic factor  

C
CBA: cytometric bead array
CBX: carbenoxolone
CGD: chronic granulomatous disease
CNS: central nervous system
CSF: cerebrospinal fluid
CSF-1R: colony stimulating factor-1 receptor
cAMP: 3', 5'-cyclic AMP
CCL2: CC motif ligand 2
CCL3: CC motif ligand 3
CCR2: CC chemokine receptor type 2
CNTF: ciliary neurotrophic factor
CNTs: connective nucleoside transporters
CXCL2: CXC motif ligand 2
CX3CL1: CX3C motif ligand 1
C/EBPβ: CCAAT/enhancer-binding protein β
D
DSM: Diagnostic and Statistical Manual of Mental Disorders
DTT: dithiothreitol
DMEM: Dulbecco’s modified Eagle medium
DMSO: dimethylsulfoxide
dNTPs: deoxynucleotide triphosphate
DPBS: Dulbecco’s phosphate-buffered saline
E
EAE: experimental autoimmune encephalomyelitis
ERK: extracellular signal receptor-activated kinase
ENTs: equilibrative nucleoside transporters
e-PDE: ecto-phosphodiesterase

F

FBS: fetal bovine serum

G

GAGs: glycosaminoglycans

GABA: \( \gamma \)-amino butyric acid

GDNF: glial-derived neurotrophic factor

GFAP: glial fibrillary acid protein

GPCRs: G protein-coupled receptors

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

H

HD: Huntington disease

HPA axis: hypothalamic-pituitary-adrenal axis

HIF-1\( \alpha \): hypoxia-inducible factor-1\( \alpha \)

HSCs: hematopoietic stem cells

I

IL-1\( \beta \): interleukin-1\( \beta \)

IL-1Ra: IL-1 receptor antagonist

IL-2: interleukin-2

IL-4: interleukin-4

IL-6: interleukin-6

IL-6R: interleukin-6 receptor

IL-10: interleukin-10

IL-12p70: interleukin-12p70

IL-18: interleukin-18

IFN-\( \gamma \): interferon-\( \gamma \)
IMDM: Iscove’s modified Dulbecco's Medium

J

JNK: c-Jun N-terminal kinase

L

LIF: leukemia inhibitory factor
LPS: lipopolysaccharide

M

MS: multiple sclerosis
MDD: major depressive disorder
MHC: major histocompatibility complex
MRI: magnetic resonance imaging
MCP-1: monocyte chemoattractant protein-1
MCAO: middle cerebral artery occlusion
M-MLV: moloney murine leukemia virus reverse transcriptase
MAPKs: mitogen-activated protein kinases

N

NO: nitric oxide
NGF: nerve growth factor
NTs: nucleoside transporters
3-NP: nitropropionic acid
5’-NT: 5’-nucleotidase
NECA: 5’-N-ethylcarboxamido-adenosine
NFAT: nuclear factor of activated T cells
NFκB: nuclear factor κB
NTPase: nucleoside triphosphatase

O
OSM: oncostatin M
6-OHDA: 6-hydroxydopamine

P
PD: Parkinson disease
PCR: polymerase chain reactions
PET: positron emission tomography
PLC: phospholipase C
PNI: peripheral nerve injury
PVDF: polyvinylidene difluoride
Panx-1: Pannexin-1
PPADs: pyridoxal-5'-phosphate-6-azo(phenyl-2',4'-disulfonic acid)
PPARγ: peroxisome proliferator-activated receptor γ

R
RA: rheumatoid arthritis
RNA: Ribonucleic acid
PARP: poly (ADP-ribose) polymerase
rt-PA: recombinant tissue-plasminogen activator

S
SNP: single nucleotide polymorphism
SOD1: superoxide dismutase-1
SAHH: S-adenosyl-homocysteine hydroxylase
STAT: signal transducers and activator of transcription

T
TBI: traumatic brain injury
TLR4: toll-like receptor 4
TDP-43: transverse response DNA- binding protein 43
TNF-α: tumor necrosis factor-α
TACE: TNF-α converting enzyme
TNFR: TNF-α receptor
U
UDP: uridine-5’-diphosphate
UTP: uridine 5’-triphosphate
W
WHO: World Health Organization
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1. Introduction

1.1 Neuroinflammation

Inflammation is generally defined as an active defense reaction against diverse insults such as injury and infection. Inflammatory reaction is designed to remove or inactivate noxious agents, to inhibit their deleterious effects and to maintain homeostasis. Within tissues outside the central nervous system (CNS), inflammation is characterized by the features of swelling, redness, heat and pain. Nowadays, more definitions of inflammation have been established. These definitions include invasion of circulating immune cells, and induction or activation of inflammatory mediators such as nitric oxide (NO), cytokines, and cyclooxygenase products. Inflammation often elicits the acute phase response, which can limit proliferation of invading pathogens. Acute phase response contains several features such as production of acute phase proteins, changes in cardiovascular function, altered neuroendocrine status, behavioral changes which lead to energy conservation, fever, and so on. The inflammatory responses activated in a regulated manner are beneficial in injury and infection. However, sustained or excessive inflammation can cause numerous diseases (Lucas 2006). For instance, recent studies have demonstrated that inflammatory responses are involved in the pathogenesis of atherosclerosis that was formerly viewed as a bland lipid storage disease (Libby 2002). It is reported that inflammation is associated with the formation of artherosclerotic plaques, and can influence the destabilization of plaques (Spagnoli 2007, Stoll 2006). Furthermore, in type 2 diabetes, there are apparent alterations in the immune system. These changes include altered levels of inflammatory mediators, changes in number and activation state of various leukocyte populations, and increased apoptosis. Therefore, these changes suggest that inflammation participates in the pathogenesis of type 2 diabetes (Donath 2011).
Inflammation in the CNS is also called neuroinflammation. Neuroinflammation represents the brain’s patterned response to insults with a number of immunomodulatory responses. Compared with other systems, the CNS was initially considered to be “immune privileged”. CNS immune privilege was interpreted as CNS isolation from the immune system by the blood brain barrier (BBB), the lack of lymphatic systems, and the absence of classical major histocompatibility complex (MHC)-positive professional antigen presenting cells (APCs) (Carson 2006, Wilson 2010). However, this viewpoint has been significantly revised over the last 20 years. In fact, peripheral immune cells can traverse the intact BBB after CNS injury (Engelhardt 2008). Microglia, the CNS resident macrophages, can serve as innate immune cells and direct the recruitment of leukocytes (Wilson 2010, Kleine 2006, Ransohoff 2012). In addtion, The CNS can recognize the extent of the immune responses taking place in peripheral tissues via receiving input signals (e.g. inflammatory mediators, vagal input) from inflamed, injured and infectious tissues (Hopkins 2007, Waldburger 2010).

It is undoubted that the CNS is different from other tissues in response to infection or inflammation. This is most evident in leukocyte recruitment, which is rapid in many organs, but delayed in the brain (Lawson 1995). Glial activation is one of the key features in neuroinflammation. While leukocyte invasion may be delayed in response to acute insults, activation of brain microglia and release of inflammatory mediators are rapid. In addition, although oedema is limited in the cranium, it can have detrimental effects by rising intracranial pressure (Lucas 2006).

Neuroinflammation has been implicated to be involved in many neurological disorders including stroke and cerebral ischemia, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), chronic neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson disease (PD), and even neuropsychiatric disorder like
MDD. In next section, I will discuss the functions of CNS cells under normal physiological conditions and their changes in response to inflammation.

1.2 Role of microglia and astroglia in the CNS

Microglia

The CNS is composed of two major types of cells, neurons and glia. Glia include macroglia (astroglia and oligodendroglia) and microglia. Microglia serve as resident macrophages in the CNS. They comprise approximately 13% of the cells in CNS white matter. In 1932, the migratory, phagocytic, non-activated and activated forms of microglia were first described by Pio del Rio Hortega. Unlike neurons and macroglia which are derived from neuroectoderm, now it is accepted that microglia are derived from primitive myeloid progenitors that arise from the extra-embryonic yolk sac before embryonic day 8 (E8.0). Recent evidence has demonstrated that microglia and macrophages derived from yolk sac are distinct from macrophages derived from definitive hematopoietic stem cells (HSCs) (e.g. monocytes). The development of microglia requires the factors which are dispensable for macrophages from HSCs (Ginhoux 2010, Kierdorf 2013, Schulz 2012). For instance, the differentiation of microglia strongly depends on the expression of colony stimulating factor-1 receptor (CSF-1R). Absence of CSF-1R greatly reduced the development of microglia, while the development of circulating monocytes is not affected (Ginhoux 2010). These data are contrasts to the studies which indicate that microglia arise from bone marrow precursors of the monocyte-macrophage lineage, and have ultrastructural, phenotypic and functional properties typical for cells of the monocyte-macrophage lineage. (Chan 2007, Cuadros 1998, Santambrogio 2001).

As surveillants of the CNS, microglia continuously monitor their microenvironment by using their mobile processes (Nimmerjahn 2005). In healthy
CNS, microglia can (1) assist in the development and remodeling of CNS to eliminate inappropriate synaptic connections by phagocytosis, (2) sense and react to CNS injury or infection (3) phagocytose the noxious stimuli and apoptotic debris (4) regulate neuronal activity (Tremblay 2011 (a), (b)). Under physiological conditions, microglia are in a “resting” or ramified state with morphology characterized by a small soma and multiple processes. However, under pathological conditions such as brain injury and ischemia, microglia are readily activated and undergo dramatic changes in their morphology to an enlarged soma with spike-like processes, showing proliferative responses and are migrating to injured sites. Activated microglia can rapidly enhance their surface expression of MHC antigens (Akiyama 1988, Bohatschek 2004, Moffett 1994) and drive the stimulation of T lymphocytes (Harms 2013, Jarry 2013). Activated microglia also increase the expression of immunomodulatory receptors which can facilitate not only phagocytosis (Koizumi 2007), but also the production pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) (Ferrari 1997, Friedle 2011, Hide 2000), chemokines such as CCL3, CXCL2 (Kataoka 2009, Shiratori 2010), and reactive oxygen species (Codocedo 2013, Ohtani 2000). These inflammatory mediators can augment the inflammatory responses by activating and recruiting other cells to the brain lesions. These functional properties of microglia are important for the maintenance of immune responses in CNS.

Activated microglia have both neuroprotective and neurotoxic functions, but the mechanisms involved in the determination of which of these functions activated microglia execute remains obscure. Recent study of Li et. al. suggested that the neuroprotective or neurotoxic function of microglia is determined by the equilibrium among factors released by activated microglia (Li 2007). It is shown that in CNS injury, dying/damaged neurons can trigger the activation of microglia. Microglial
activation can result in a decrease of neuronal damage and an increase in tissue repair (Dowding 1991). It is also demonstrated that in the rat model of cerebral ischemia, inhibition of microglial activation led to severe neurological deficits, a larger infarct volume, more neuronal loss, and decreased levels of neurotrophic factors (Yang 2012). These studies implicate the neuroprotective functions of microglial activation.

However, excessive activation of microglia may be detrimental and contribute to pathological conditions. Over-activation of microglia exposes the CNS to various cytokines, chemokines and reactive oxygen species. Excessive amount of these inflammatory mediators may have neurotoxic consequences and result in the continuous activation of microglia (Boje 1992). Furthermore, activated microglia phagocytose not only dying/damaged cells but also neighboring intact cells (Kim 2006). Activated microglia has been suggested to be associated with the pathogenesis of neurological disorders. Effective control of microglial activation may prevent the aggravation of the diseases.

Astroglia

Astroglia are the largest and most abundant glial cell type which constitute approximately 50% of the cell number in the adult CNS (Liu 2004, Moore 2011). They are widely distributed in gray and white matter, and provide neurons with structural support. Astroglia have been previously regarded just as passive housekeepers apt to maintain the optimal microenvironment for neuronal survival and function, however, recent acknowledgement has suggested the idea that astroglia contribute to the performance of the CNS.

Astroglia are highly branched cells with processes which contact most of the surfaces of neuronal dendrites and cell bodies, as well as some axonal surfaces in an ordered, non-overlapping manner. Processes of astroglia end in expansions which are
called “end-feet”. Astroglial end-feet join together to completely line the interfaces between the CNS and other tissues to form the molecular boundaries. During CNS development, the molecular boundaries formed by astroglia provide a pathway for neuronal migration. Astroglial end-feet are also cellular constituents for the formation of blood brain barrier (BBB). In healthy CNS, astroglia play essential roles in (1) regulation of blood flow, (2) provision of energy metabolites to neurons, (3) participation in synaptic function and plasticity, (4) production of cytokines and growth factors, and (5) maintenance of the homeostasis (balance of ions, neurotransmitters and fluids) of extracellular space. Due to the multiplicity and complexity of astroglial functions, the correct performance of astroglia is crucial for the neuronal survival (Brambilla 2013, Sofroniew 2010).

Although astroglia express sodium and potassium channels which can evoke inward currents, unlike neurons, astroglia do not propagate action potential. Due to this reason, astroglia have been long considered as non-excitable cells. In fact, astroglia display a particular form of excitability that is based on the regulated increases in intracellular calcium concentration ([Ca$^{2+}$]). The increases in [Ca$^{2+}$] are important in the intercellular communication between astroglia and astroglia, as well as astroglia and neurons (Pasti 1997). Elevations in astroglial [Ca$^{2+}$] can occur as intrinsic oscillations resulting from Ca$^{2+}$ release from intracellular stores, or be triggered by neurotransmitters such as glutamate and purines (Kim 1994, Stout 2002). The increase of astroglial [Ca$^{2+}$] elicits the release of glutamate, an excitatory neurotransmitter, from astroglia into extracellular space and thereby influence the activity of neurons (Pasti 2001). Therefore, the Ca$^{2+}$ signal conductance makes astroglia play a direct role in synaptic transmission (Sofroniew 2010).

Astroglia respond to all form of CNS insults by a process commonly referred to as reactive astrogliosis. Reactive astrogliosis results in (1) up-regulation of the
astroglial marker glial fibrillary acid protein (GFAP) and other genes (e.g. cytokines), (2) hypertrophy of astroglial cell body and processes, and (3) proliferation of astroglia beyond the previous domain of individual astroglia. The latter case causes substantive intermingling and overlapping of neighboring astroglial processes with blurring and disruption of individual astroglial domains. The long-lasting changes in tissue architecture will eventually lead to the formation of dense, narrow, and compact glial scars, which are commonly found in areas of severe lesions, infections or areas responding to chronic neurodegenerative stimuli (Sofroniew 2009, 2010).

It has been long considered that scars formed by reactive astrogliosis inhibit the regeneration of axon, and are the main impediment to functional recovery after CNS injury and diseases. However, now this point of view is revised. Recent findings indicate that reactive astrogliosis and glial scar formation play important roles in the regulation of CNS inflammation. First, astroglia respond to a number of important cytokines affecting the cellular state of the surrounding cells such as neurons and microglia as well as astroglia themselves (Jurič 2001, Katsuura 1989). Next, astroglia themselves are known to be the major sources of several important pro-inflammatory cytokines such as IL-1, IL-6 (Lampa 2012, Li 2009, Rubio 1993, van Wagoner 1999) and chemokines (van Neerven 2010) in response to pathological challenges. They also release many kinds of neuroprotective substances such as glutathione antioxidant, glial-derived neurotrophic factor (GDNF) (Sandhu 2009), brain-derived neurotrophic factor (BDNF) (Saha 2006) and nerve growth factor (NGF) (Toyomoto 2004). Furthermore, astroglia extensively interact with microglia in response to neuroinflammation and can modulate the activity of microglia (Farina 2007, Min 2006). Understanding the mechanisms underlying astroglial signaling and reactive astrogliosis has the potential to open doors to identify the molecule that might serve as a novel therapeutic target for neurological diseases.
1.3 Expression of cytokines in the CNS

In the CNS, cells such as microglia and astroglia can produce inflammatory mediators such as cytokines and chemokines in response to brain insults. Under physiological conditions, some cytokines and their cognate receptors are constitutively expressed throughout the CNS. The constitutive expression of these cytokines and their receptors suggests that they may contribute to normal physiological functions of the CNS. For instance, the constant presence of tumor necrosis factor-α (TNF-α) produced by glia is demonstrated to be essential for the modulation of synaptic scaling (Beattie 2002, Stellwagen 2006). In addition, during CNS development, the constitutive expression of chemokine CX₃C motif ligand 1 (CX₃CL1) can direct the migration of mesenchymal stem cells which can differentiate toward a neuronal phenotype (Ji 2004 (a), Woodbury 2000). In adult brain, the expression of CX3CL1 and its receptor CX3CR1 also plays an essential role in neuron-microglia communication (Noda 2011, Streit 2005).

Cytokines play an important role in neuroinflammation by enhancing the production of other inflammatory mediators, adhesion molecules, recruitment of immune cells into the CNS, and the activation of the glia. Cytokines can be simply classified as pro-inflammatory and anti-inflammatory. Proinflammatory cytokines are primarily responsible for the initiation of an effective defense against exogenous pathogens. However, over-production of the pro-inflammatory cytokines can be harmful and may ultimately lead to devastating consequences. In contrast, anti-inflammatory cytokines are crucial for down-regulating the exacerbated inflammatory process, and maintaining homoeostasis for proper functioning of vital organs. Excessive anti-inflammatory response may result in the suppression of body immune function (Ng 2003). Chemokines are the most numerous family of cytokines,
and are first noted for their ability to attract and activate leukocytes. Chemokines are also involved in innate immunity and pro-inflammatory responses (Feng 2000). In this section, the functions of two pro-inflammatory cytokines IL-6, TNF-α and chemokine CCL2 in the CNS is introduced.

**Interleukin-6 (IL-6)**

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis. IL-6 is originally identified as a B-cell differentiation factor (BSF-2), which serves as a factor that induces the maturation of B-cells into antibody producing cells (Hirano 1985). IL-6 is a crucial cytokine controlling the transition from innate to acquired immunity (Jones 2005). IL-6 has pro-inflammatory features to act as an endogenous pyrogen which is associated with fever (Dinarello 1991), and anti-inflammatory features to induce the synthesis of IL-1 receptor antagonist (IL-1Ra) as well as the release of soluble TNF-α receptors (Tilg 1994). IL-6 activities are shared by IL-6-related cytokines such as leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and oncostatin M (OSM). The pleiotropy and redundancy of IL-6 functions have been identified by characterizing a unique receptor system comprising two functional proteins: an 80 kDa IL-6 receptor (IL-6R) specific for IL-6, and a 130 kDa gp130 receptor, which is the common signal transducer of IL-6-related cytokines (Kishimoto 2010). Under physiological conditions, IL-6 expression in the brain is relatively low. IL-6 may influence neuronal functions by inducing the cholinergic phenotype of sympathetic neurons (Fann 1994, März 1998). During CNS development, the expression of IL-6 promotes the early vascular development (Fee 2000). In addition, IL-6 can act in concert with bone morphogenetic protein (BMP) to induce astroglia differentiation from neural stem cells (Taga 2005, Yanagisawa 2001). IL-6 also plays a role in
neurogenesis. The levels of IL-6 are enhanced in response to CNS insults, and this up-regulation of IL-6 leads to a diminished hippocampal neurogenesis (Vallières 2002). In addition, in vitro evidence clearly demonstrates that elevation in IL-6 levels suppresses the differentiation of neural stem/progenitor cells into neurons (Monje 2003, Nakanishi 2007). These results implicate a noxious role of IL-6 in neurogenesis.

**Tumor necrosis factor-α (TNF-α)**

TNF-α is originally discovered in the mouse serum during entotoxemia and recognized for its anti-tumor activity (Carswell 1975). TNF-α is synthesized as a 26 kDa membrane-bound polypeptide precursor that is then cleaved by proteolysis to release a 17 kDa subunit. The proteolysis is mediated by TNF-α converting enzyme (TACE) that belongs to the family of mammalian adamasins (or A Desintegrins And Metalloproteinases, ADAMs). The role of TNF-α in the CNS was not observed until microglia was found to produce TNF-α in 1987 (Frei 1987). Recent evidence has established that microglia and some populations of neurons can express TNF-α and its cognate receptors (Figiel 2007, Holmes 2004, Sakuma 2007, Veroni 2010, Welser 2012). TNF-α has been implicated in the pathogenesis of many neurological disorders, and is reported to exert both neurotoxic and neuroprotective action on neurons. These opposing effects may be explained by the presence of two distinct TNF-α surface receptors, TNFR1 (p55) and TNFR2 (p75) (MacEwan 2002, Wajant 2003).

TNFR1 is constitutively expressed in most tissues, while TNFR2 is highly regulated and typically found in cells of the immune system. In the vast majority of cells, TNFR1 appears to be the key mediator of TNF signaling, and only TNFR1 contains a cytoplasmic death domain and may directly induce apoptosis. TNFR2 seems to play a major role in the lymphoid system, and its role in the CNS is less
known (Wajant 2003). In the CNS, the distribution and expression levels of the TNFRs vary depending on the inflammatory regulation and apoptosis (Pan 2003, Wu 2005). The differential distribution of TNF-α receptors, their state of activation, and the down-stream signaling are implicated to play an important role in determining the harmful or beneficial action of TNF-α in the CNS.

Mice with deficiency in both TNFR1 and TNFR2 (TNFR1/2−/−) have provided ways to clarify the role of TNF-α in the CNS. Bruce et. al. have reported that TNFR1/2−/− mice displayed greater neuronal damage after ischemia or kainate-induced excitotoxic damage. The neuronal damage in TNFR1/2−/− mice is accompanied by elevated oxidative stress and reduced antioxidase levels (Bruce 1996). In vitro studies also showed that pretreatment of TNF-α in neurons or astrocytes treated with nitropropionic acid (3-NP) induced augmentation of antioxidase activity, and attenuated superoxide accumulation induced by 3-NP (Bruce-Keller 1999). These results indicate that TNF-α may exert protective action by stimulating antioxidant pathways. Study in mice lacking only one type of TNF-α receptor disclosed that TNFR1-mediated signaling is more important than TNFR2 in mediating neuroprotective action of TNF-α after brain insults (Gary 1998). It is reported that TNFR1 has deleterious but TNFR2 has protective effect on neurons (Fontaine 2002). The exact mechanisms underlying such distinct action of TNF-α receptors need further elucidation.

Although TNF-α is extensively characterized for its role in the immune system, it is distinct from other cytokines due to its essential role in synaptic plasticity, that is often referred to as synaptic scaling. TNF-α tightly regulates signaling molecules that directly induces trafficking of both glutaminergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and γ-amino butyric acid (GABA) receptors on neuronal synapses and influences synaptic
efficacy (Beattie 2002). Treatment with the soluble form of the TNFRI (sTNFR1), which serves as a TNF-α antagonist, leveled down AMPA receptor expression and caused a reduction in synaptic strength (Beattie 2002). It is also demonstrated that under normal physiological conditions, the constant presence of glial-derived TNF-α at low levels plays an essential role in the regulation of synaptic connectivity (Stellwagen 2006). Since TNF-α serves as an important pro-inflammatory cytokine and a modulator of synaptic transmission, elucidating of the mechanisms underlying its regulation may be crucial for the maintenance of CNS health.

**Chemokine CC motif ligand 2 (CCL2)**

Chemokines are composed of a family of chemoattractant cytokines which are subdivided into four families: CXC, CC, CX₃C, and C. This classification is based on the number and spacing of the conserved cysteine residues in the N-terminus of the proteins (Rollins 1997). Chemokines levels are up-regulated in response to inflammation, and they regulate the recruitment of monocytes, neutrophils, and lymphocytes. In addition, chemokines induce chemotaxis through the activation of G protein-coupled receptors (GPCRs), which also involve adhesion molecules and glycosaminoglycans (GAGs) (Gerard 2001, Hamel 2009, Moser 2004). CCL2, also called monocyte chemoattractant protein-1 (MCP-1), is a member of CC chemokine family and serves as a potent chemotactic factor for monocyte/macrophages. Many cell types can produce CCL2, but monocytes/macrophages are found to be the major source of CCL2 (Deshmane 2009, Semple 2009). In healthy CNS, CCL2 is expressed at low levels (Sheehan 2007, Yao 2010), and can be produced by both microglia and astroglia (Carrillo-de Sauvage 2012, Cho 2013, D'Mello 2009, Madrigal 2009). CCR2, the cognate receptor for CCL2, is demonstrated to be expressed on neurons and astroglia, but not microglia even after injury (Andjelkovic 2002, Foresti 2009, Gao
Like other cytokines, the levels of CCL2 are up-regulated in response to the brain insults. During CNS inflammation, the increased CCL2 can drive the subsequent infiltration of peripheral CCR2+ monocytes into the brain (Lim 2010, Mildner 2009). In addition, the CCR2 is demonstrated to be expressed on neural progenitor cells (Ji 2004 (b), Tran 2007). In response to neuroinflammation, binding of CCL2 on the CCR2 expressed on neural progenitor cells is suggested to involve in directing migration of neural progenitor cells to the lesion site (Belmadani 2006, Liu 2007, Yan 2006). Therefore, in addition to serving as a chemokine that attracts monocytes/macrophages, CCL2 may play an important role in neuronal regeneration after CNS injury.

1.4 Role of microglia, astroglia, and cytokines in neurological and neuropsychiatric disorders

As mention before, activated microglia and astroglia mediate the release a variety of cytokines in response to neuroinflammation, and are suggested to be involved in the pathogenesis of neurological disorders. In this section the role of microglia, astroglia and cytokines in neurological and neuropsychiatric disorders is discussed.

Stroke and cerebral ischemia

Stroke is the second leading cause of death worldwide and the leading cause of permanent disability. Over 80% of strokes are of the ischemia variety and are due to acute vascular occlusion. Recombinant tissue plasminogen activator (rt-PA) is currently the only acute therapy being used for stroke (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995), but its narrow therapeutic window and risk of haemorrhage limit its utilization. Recently a novel
potential target, the neuroinflammatory response in ischemia, has attracted the attention of many stroke researchers. An inflammatory response in ischemia is initiated in the metabolically active, but neurophysiologically silent region which surrounds the infarct core. This peri-infarct region is known as the ischemic penumbra. The inflammatory response triggered by ischemia is characterized by an increase in vascular permeability, influx of leukocytes, hyperthermia and activation of microglia (Weinstein 2010).

In the rat model of ischemia, after permanent middle cerebral artery occlusion (MCAO), microglia are activated and release a variety of inflammatory mediators. Activated microglia appear both in the infarct core and the ischemic penumbra followed by the later infiltration of neutrophils into the infarct core (Mabuchi 2000). Few days after the MCAO, microglia are observed to appear not only in ipsilateral cortex but also spread to the contralateral side. Moreover, there is a continuous increase in the number of activated microglia throughout the first week after MCAO (Jander 1998, Morioka 1993). In human stroke, evidence from positron emission tomography (PET) and magnetic resonance imaging (MRI) has confirmed the presence of activated microglia in the ischemia penumbra during the subacute phase of stroke (Price 2006). These data implicate that pharmacological agents controlling microglia activation may provide new therapeutic strategies for the treatment of ischemic stroke.

On the other hand, the role of reactive astrogliosis in ischemic brain lesions remains unclear. Recent studies indicate that during transient ischemia, reactive astroglia can provide essential metabolic support to neurons. Any failure of these supportive functions of astroglia will threaten the survival of neurons (Rossi 2007, Takano 2009). Additionally, transgenic mice which are devoid of reactive astrogliosis showed larger infarct volume and reduced glutamate uptake after MCAO (Li 2007).
This suggests a protective role of reactive astrogliosis in ischemia.

It is now clear that inflammatory cytokines have a direct involvement in ischemic injury. Patients with acute ischemic stroke had significant higher plasma IL-6 levels than normal controls, suggesting that IL-6 production is an inflammatory response to ischemia and may serve as a biomarker of ischemia (Cojocaru 2009). In mouse model of MCAO, blockade of IL-6 signaling had an increase in the number of apoptotic cells in the ischemic penumbra and enlargement of the infarct size. This suggests that endogenous IL-6 plays a crucial role in preventing neuronal damage from undergoing apoptosis in ischemia (Yamashita 2005).

Like IL-6, the levels of TNF-α are increased after ischemia. Transgenic rat over-expressing the murine TNF-α gene are more susceptible to apoptotic cell death after MCAO than non-transgenic animals (Pettigrew 2008). In contrast, pretreatment of TNF-α before MCAO induced protective effects against ischemia in mice (Nawashiro 1997). These data suggest dual neurotoxic and neuroprotective roles for TNF-α in ischemia.

In ischemia/reperfusion (I/R) injury, levels of CCL2 were found to elevate in ischemic area (Wang 1995). Blocking CCL2 significantly decreased I/R-induced enhancement of BBB permeability. CCL2 knock-out is demonstrated to be neuroprotective in mouse model of ischemia by reducing infarct size, accumulation of phagocytic macrophages, and hypertrophy of astroglia (Hughes 2002). Strategies targeting inflammatory cytokines may open doors for the development of pharmacological agents against stroke.

Multiple sclerosis (MS)

MS is an autoimmune inflammatory disorder which is initiated by breakdown of the BBB, followed by invasion of the T cells and the macrophages in CNS, and
eventually leads to myelin destruction. Focal demyelinated lesions in the white matter are the traditional hallmarks of MS. Because the injured areas of the CNS vary widely, MS can lead to a broad spectrum of clinical symptoms including fatigue, muscle weakness, areas of numbness and paralysis. This disease progresses in cycles of relapse, often associated with systemic infection and inflammation, and remission (Barnett 2004). Phagocytosis of myelin by activated microglia and blood-borne macrophages in actively demyelinating lesions is one of the key features of MS (Huizinga 2012). Politis and colleagues have detected the microglia activation in patients with various forms of MS, and found a direct correlation between cortical microglial activation and disease severity (Politis 2012).

In MS, reactive astrogliosis is not only a permanent feature. Reactive astroglia play a key role in pathogenic disease mechanisms underlying MS. For instance, in the most common (Charcot) type of MS, the demyelinated plaques are interspersed with and surrounded by reactive astroglia. Focal reactive astrogliosis is widespread throughout the white matter and in some regions of the gray matter. Unusual nuclear and cytological features called “Creutzfeldt astroglia” and “emperipolisis” may be observed in MS. “Creutzfeldt astroglia” are characterized by enlarged and multinucleated astroglia, while “emperipolisis” signifies the event that astroglia engulf one or more cells such as oligodendroglia and lymphocytes (Sofroniew 2010). In experimental autoimmune encephalomyelitis (EAE), which is an experimental model of MS (Constantinescu 2011), reactive astroglia form scars to surround inflammatory cells. Disruption of this astroglial scar formation is demonstrated to exacerbate the spread of inflammation, increase axonal degeneration, and worsen disease symptoms (Voskuhl 2009).

Inflammatory cytokines such as IL-2 and TNF-α are up-regulated in the serum and CSF of MS patients (Maimone 1991, Trotter 199). In the EAE model, inhibition
of TNF-α by selective soluble TNF-α blocker improves the clinical outcome by promoting axon preservation and remyelination (Brambilla 2011), suggesting that TNF-α can serve as a therapeutic target for MS.

**Amyotrophic lateral sclerosis (ALS)**

ALS is a progressive neurodegenerative disease characterized by selective death of upper and lower motor neurons of the brain and the spinal cord. The loss of neuronal synapses can result in paralysis and ultimately death. ALS is a disease of sporadic etiology with a plethora of aberrant physiological processes implicated in its pathogenesis. A hallmark of sporadic ALS is the presence of cytoplasmic ubiquitinated protein inclusions composed of TDP-43 (transverse response DNA-binding protein 43) in affected areas of CNS. A small fraction of cases termed familial ALS (fALS) are caused by various genetic mutations. About 20% of fALS is caused by mutations in superoxide dismutase-1 (SOD1) (Mackenzie 2007). Transgenic mice over-expressing mutant SOD1 (mSOD1) is taken as an animal model of ALS, and it develops a progressive motor neuron degeneration resembling ALS (Swarup 2011). It is demonstrated that in ALS patients, there is an increased number of activated glia. In experimental model of ALS, extensive proliferation of non-neuronal cells (microglia and astroglia) was reported to accompany the motor neuron loss. Microglia and astroglia may exert their neurotoxic functions in response to cytoplasmic mSOD1 by releasing neurotoxic substances. This viewpoint is supported by the *in vitro* study that mSOD1-containing microglia become activated more easily, and produce higher levels of NO than wild-type SOD1 expressing microglia (Weydt 2004). The selective knock-down of mSOD1 in microglia resulted in a significant increase in the survival of mSOD1 mice (Boillee 2006). Similarly, astroglia derived from both sporadic and familial ALS patients are toxic to motor neurons; knockdown of SOD1 significantly suppresses the astroglial-mediated toxicity
towards motor neurons (Haidet-Phillips 2011). These results indicate that in ALS, microglia and astroglia may influence the rate of progression of neurodegeneration. In addition, transgenic mice over-expressing mSOD1 show up-regulation of TNF-α (Hensley 2003). Together, these data implicate that glial-mediated neuroinflammation contributes to the progression of ALS.

**Alzheimer’s disease (AD)**

AD is the most common dementia that is characterized by the progressive decline in forming new memories and accessing existing ones, due to neuronal death in the hippocampus and frontal cortex. In post-mortem brain of AD patients, there is a notable presence of extracellular amyloid-β (Aβ) plaques derived from breakdown of amyloid precursor protein (APP), and intracellular neurofibrillary tangles which are composed of tau (τ) protein. Another fundamental event in AD pathogenesis is an inflammatory response which involves the gliosis. Activated microglia accumulated at perivascular sites of Aβ deposition and in senile plaques is one of the hallmarks of AD (Uchihara 1997). Once activated by Aβ, microglia initiate a vicious cycle of inflammatory events by synthesizing and releasing cytokines such as IL-1, IL-6, TNF-α and chemokines, leading to monocyte migration across the BBB. The elevation in TNF-α and IL-1 levels can lead to an increased expression of APP and Aβ peptides (Dash 1995, Goldgaber 1989, Yamamoto 2007). This indicates that microglia-mediated cytokine production participates in the augmentation of the disease state. In addition, recent evidence suggests that accumulation of mononuclear phagocytes in AD brain is dependent on CCL2. CCL2 and its receptor CCR2 regulate mononuclear phagocyte accumulation in a mouse model of AD. CCR2 deficiency (CCR2−/−) leads to lower mononuclear monocyte accumulation and higher Aβ levels (El Khoury 2007), suggesting that CCR2-dependent mononuclear phagocyte
accumulation can eliminate Aβ deposits, delay or stop the neurotoxic effects of Aβ, thus delay the disease progression of AD (Hickman 2010). Conversely, Kiyota et al. reported that CCL2 can enhance the expression of Aβ oligomer and exacerbate the neurocognitive dysfunction in AD (Kiyota 2009).

It has been demonstrated that the cytokine levels in both the autopsy specimens and the peripheral blood are enhanced in AD patients (Luterman 2000, Singh 1997). Studies also reveal that variants in cytokine genes may be risk factors of AD. It is reported that GC phenotype of G174C site in IL-6 gene is significantly higher in AD patients (Arosio 2004). Culpon et al. have demonstrated a protective role of TNF-α haplotype against AD (Culpan 2003). These studies suggest that inflammatory cytokines are likely to affect the susceptibility of AD.

Reactive astrogliosis is also a known feature of AD. Like activated microglia, reactive astroglia secrete various inflammatory mediators. Reactive astroglia can surround Aβ with their processes as if they form tiny scars around Aβ, perhaps act as protective barriers to isolate Aβ from healthy tissue. Reactive astroglia also take up and degrade extracellular deposits of Aβ (Wyss-Coray 2003). These data reveal that reactive astroglial functions and dysfunctions may play an important role in the progression of AD (Sofroniew 2010).

**Parkinson disease (PD)**

PD is the second most common neurodegenerative disorder after AD. It is characterized by slow and progressive loss of dopaminergic neurons in substantia nigra, and motor symptoms of tremor, muscle rigidity and bradykinesia. In addition to the neuronal loss, PD is pathologically characterized by the presence of abnormal protein inclusions such as Lewy bodies and Lewy neurites (Recchia 2004). Neuroinflammatory mechanisms probably also contribute to the neuronal
degeneration in PD. Post-mortem studies showed activation of microglia within the substantia nigra of PD patients (Croisier 2005, Zhang 2005). It has been demonstrated that agents which inhibit the activation of microglia provide neuroprotective effects in animal models of PD (Du 2001, Liu 2000).

Astrogliosis is another pathological characteristic of the substantia nigra in PD. In healthy individuals, astroglia are heterogeneously distributed in mesencephalon. The density of astroglia is low in substantia nigra pars compacta, which is a brain region severely affected in PD. Therefore, vulnerable neurons in substantia nigra pars compacta of PD have less surrounding astroglia which can release antioxidant glutathione and neuroprotective substances. This limited astroglial environment might be a susceptible factor for PD (Sofroniew 2010). These data suggest a protective role of astroglia in PD.

Studies of biological fluids in PD patients also support a role of neuroinflammatory processes in PD. The CSF levels of TNF-α and serum levels of IL-6 and TNF-α were found increased in PD patients (Dobbs 1999, LeWitt 2012, Mogi 1994). It is thought that higher plasma levels of IL-6 are associated with a greater risk of PD (Chen 2007). Evidence from genetic analyses indicate that heterozygous TNF-α polymorphism at position 308, as well as CC genotype of T1030C site in TNF-α promoter can increase risk of PD (Krueger 2000, Wu 2007).

**Major depressive disorder (MDD)**

Depression is a common mental disorder with unknown origin. According to the statistic data announced by World Health Organization (WHO) in 2012, depression is major contributor to the global burden of disease, and there are around 350 million people affected by depression (http://www.who.int/en/). There are different types of depression listed in Diagnostic and Statistical Manual of Mental Disorders (DSM).
MDD is one of the more serious kinds of depression, and its symptoms include depressed mood, loss of interest in activities once enjoyed, significant changes in sleep patterns and so on. These problems can become chronic and recurrent; at the worst, MDD can lead to suicide (DSM- V, 2013).

There are three major theories for the etiology of MDD: (1) monoamine neurotransmitter dysfunction (Carlsson 1957, Lingjaerde 1963), (2) dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Arborelius 1999, De Kloet 1988, Holsboer 1996), and (3) disturbed adaptive neuronal plasticity (Castrén 2013, Jun 2012). Based on several observations, a link between neuroinflammation and the pathophysiology of MDD has been hypothesized. First, there is evidence for a role of pro-inflammatory cytokines in the pathophysiology of MDD (Maes 1997, Tsao 2006). In MDD patients, there is a sustained elevation in blood levels of pro-inflammatory cytokines (e.g. IL-6, TNF-α) compared with healthy subjects, whereas antidepressant treatments can reduce the cytokine levels. Therefore, the pro-inflammatory cytokine levels may be taken as a predictor of antidepressant effects in MDD patients (Crnković 2012, Henje Blom 2012, Rethorst 2012). Second, under systemic infection or inflammation, the increased pro-inflammatory cytokines can trigger the “sickness behavior” that resembles certain somatic symptoms of MDD (Dantzer 2009). Furthermore, administration of cytokines can produce depressive-like behaviors. For instance, hepatitis C patients treated with interferon-α developed an induced MDD (Loftis 2013). Central administration of IL-6 and TNF-α is found to induce depressive-like phenotypes in mice (Kaster 2012, Sukoff Rizzo 2012).

As the immune cells of the CNS, microglia may be the mediator for the abnormal brain-immune interaction in MDD. Under physiological conditions, microglia can regulate the synaptic functions (Ji 2013) and the neurogenesis (Miyamoto 2013, Sierra 2010), which are both suggested in the pathogenesis of MDD.
Activation of microglia is observed in some animal models of MDD. Chronic psychological stress, which can induce the development of MDD, increased microglial activation in the prefrontal cortex of rats (Hinwood 2012). In another animal model of chronic stress, repeated social stress, number of the deramified Iba1+ microglia was enhanced in the medial amygdala, prefrontal cortex and hippocampus. Levels of the pro-inflammatory cytokines are also increased (Wohleb 2011 and 2012). Astroglia are also known to play an essential role in synaptic mechanisms. Dysfunction of astroglia may contribute to behavioural disorders (Halassa 2009). It is found that there is a layer-specific reduction in the density of astrocytes and GFAP immunoreactivity in the prefrontal cortex of patients with MDD (Johnston-Wilson 2000, Miguel-Hidalgo 2000, Si 2004).

**Other pathological conditions**

Many studies have also demonstrated the importance of neuroinflammation in the progression of other pathological conditions, such as traumatic brain injury (TBI) (Gentleman 2004, Myer 2006, Ramlackhansingh 2011), epilepsy (Choi 2008), neuropathic pain (Myers 2006) and Huntington disease (HD) (Möller 2010).

Taken together, a role of glial activation and cytokine production in the pathogenesis of neurological disorders is envisaged. Under pathological conditions, there is an elevation in the levels of purines released from dying/damaged cells. This increase in extracellular purines can activate the purinergic receptors expressed on glia and regulate many aspects of glial function. In next section, the role of purines in the CNS is discussed.

**1.5 Role of purines and purinergic receptors in the CNS**

It is now beyond dispute that purine compounds such as adenosine and adenosine
5’-triphosphate (ATP) exert their unequivocal neurotransmitter and neuromodulator actions, both inhibitory and excitatory, in mammalian CNS via the activation of a variety of purinergic receptors (Dale 2009). Purinergic receptors are divided into P₁ and P₂ receptors. P₁ receptors are primarily activated by adenosine, while ATP binds to P₂ receptors. The actions of adenosine and ATP at these extracellular receptors are critically dependent on the release of these purines from cells.

1.5.1 Adenosine and adenosine P₁ receptors

Adenosine

Adenosine is a ubiquitous chemical messenger present within and outside cells. In physiological conditions, the intracellular and extracellular concentrations of adenosine are in nanomolar range (Fredholm 2007). Adenosine can modulate many aspects of cellular activity in both physiological and pathophysiological conditions. Within cells, the metabolism of adenosine depends on the activity of adenosine kinase (ADK), which is a key enzyme of adenosine metabolism. ADK, together with ecto-nucleotidases, forms a substrate cycle between AMP and adenosine. Hydrolysis of S-adenosyl-homocysteine (AdoHcy) by S-adenosyl-homocysteine hydroxylase (SAHH) also contributes to the intracellular formation of adenosine (Figure 1) (Latini 2001, Wen 2012). Adenosine can be rapidly metabolized to inosine by adenosine deaminase (ADA). Bi-directional nucleoside transporters (NTs) such as connective nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) equilibrate the extra- and intracellular levels of adenosine (Molina-Arcas 2008, 2009). Because the relatively high activity of intracellular ADK, the intracellular concentrations of adenosine are normally low, therefore the net flux through nucleoside transporters is directed inwardly (Dunwiddie 2001). However, the levels of cytosolic adenosine can be enhanced by increasing cellular workload, and this
increase in cytosolic adenosine is associated with indices such as oxygen consumption (Fredholm 2007). When the cellular workload increases, there is an increase in the dephosphorylation of ATP to ADP and AMP. This can lead to the cytosolic accumulation of adenosine. In addition, under hypoxia, the activity of ADK can be inhibited by hypoxia-inducible factor-1α (HIF-1α). This inhibition in ADK results in a decreased conversion of adenosine to AMP, as well as a cytosolic accumulation of adenosine (Sitkovsky 2008). Therefore, in these situations the NTs can transport intracellular adenosine into extracellular space.

Extracellularly, adenosine can be produced by the metabolism of ATP and 3’, 5’-cyclic AMP (cAMP) via ectonucleotidases (Dunwiddie 2001, Gödecke 2008). In CNS, all cell types contribute to the accumulation of extracellular adenosine (Benarroch 2008). Recent findings indicate that under physiological conditions, the release of ATP from astroglia is the major source of synaptic adenosine (Pascual 2005). Furthermore, under pathological conditions, the ATP released from damaged/dying cells can lead to massive formation of extracellular adenosine (see section 1.5.3).

Figure 1: The metabolic cycle of adenosine. In healthy CNS, astroglia serve as the major source of extracellular ATP. The degradation of extracellular ATP and cAMP is
the main source of synaptic adenosine. After being released, ATP is hydrolyzed to adenosine by nucleoside triphosphatase (NTPase, CD39) and 5'-nucleotidase (5'-NT, CD73). Ecto-phosphodiesterase (e-PDE) and 5'-nucleotidase convert Camp to adenosine. Thereafter, adenosine is metabolized to inosine by ADA. Within the cells, metabolism of adenosine relies on the activity of adenosine kinase (ADK) and 5'-NT, which form a substrate cycle between AMP and adenosine. In addition, S-adenosyl-homocysteine hydroxylase (SAHH) also participates in the formation of adenosine. Because the activity of ADK is relatively high in cytosolic space, the extracellular adenosine is normally driven into the cells by bi-directional nucleoside transporters (NTs).

**P₁ receptors**

A large body of evidence supports the view that adenosine receptors govern cell function by coupling to G proteins. The signaling of adenosine receptors is traditionally thought to occur through inhibition or stimulation of adenylyl cyclase (AC) with a concomitant decrease or increase in intracellular cAMP concentrations. Based on their ability to decrease or increase intracellular cAMP concentrations, adenosine receptors were initially classified as inhibitory A₁ or stimulatory A₂ receptors (van Calker 1979). Subsequent studies have then divided the A₂ receptors into two groups: high-affinity A₂A receptors and low-affinity A₂B receptors (Jarvis 1989). The more recent discovery and characterization of the A₃ receptors have indicated that in addition to the A₁ receptors, the A₃ receptors direct certain cellular responses by decreasing intracellular AMP concentrations (Haskó 2008).

It is now apparent that other pathways, such as phospholipase C (PLC) and mitogen-activated protein kinases (MAPKs) are also involved in adenosine receptor-mediated signaling (Jacobson 2006). Briefly, activation of inhibitory A₁
receptors is not only linked to G\(_i\)-mediated inhibition of adenylyl cyclase (van Calker 1979), but can also result in increased activity of PLC (Rogel 2005, Tawfik 2004). In addition, A\(_1\) receptor activation can inwardly rectify K\(^+\) channels and inhibit Ca\(^{2+}\) channels (Fredholm 2011). The classical signaling pathways associated with A\(_3\) receptor activation consist of G\(_i\)-mediated inhibition of adenylyl cyclase and G\(_q\)-mediated stimulation of PLC (Gessi 2008). It was found that A\(_3\) receptor-dependent enhancement of chemokine CCL2 release in primary mouse astroglia was mediated by G\(_q\), but not pertussis toxin-sensitive G\(_i\) protein (Wittendorp 2004).

Activation of A\(_{2A}\) receptors increases the activity of adenylyl cyclase. In the peripheral systems, G\(_s\) is thought to be the major G protein associated with A\(_{2A}\) receptors. It is reported that in rat tail artery, activation of the A\(_{2A}\) receptors triggers the adenylyl cyclase and PLC pathways, and facilitates the release of adrenaline (Fresco 2004). In CNS, A\(_{2A}\) receptors are predominantly expressed in striatum, which is the major area of the basal ganglia (Svenningsson 1999). It is shown that striatal A\(_{2A}\) receptors mediate their effects through activation of G\(_{olf}\), which is similar to G\(_s\) and also couples to adenylyl cyclase (Kull 2000). The A\(_{2B}\) receptors are the least studied subtype of the adenosine receptor family. Stimulation of A\(_{2B}\) receptors can trigger adenylyl cyclase activation via G\(_s\) and PLC activation via G\(_q\). In human mast cells, cross-talk between these two pathways following A\(_{2B}\) receptor activation is essential for the production of interleukin 4 (IL-4) (Ryzhov 2006). The production of leukemia inhibitory factor (LIF) following A\(_{2B}\) receptor activation in primary mouse astroglia is dependent on ERK1/2- and p38-MAPK activation (Moidunny 2012).

1.5.2 Role the P\(_1\) receptors in neurological disorders

Adenosine is able to control CNS functions in both physiological and
pathophysiological conditions (Ribeiro 2010, Sims 2013). In response to pathological events, adenosine is largely generated at the lesion site by dying/damaged cells. The elevated extracellular adenosine can interact with four P1 receptor subtypes and control the following tissue damage. In this section the involvement of P1 receptor activation in the pathogenesis of CNS diseases is discussed.

Inhibitory neuromodulation by adenosine is mainly mediated by the A1 receptors (Fredholm 2005, Jacobson 2006) which result in a suppressed release of various excitatory neurotransmitters (Boison 2008). It has been demonstrated that adenosine is an endogenous anti-convulsant that elicits a profound anti-epileptic effect by stimulating the A1 receptors (Boison 2007). Additionally, activation of the A1 receptors was found to be beneficial in chronic pain (Heijne 2000, Wu 2005), and it protects against brain insults such as cerebral ischemia (Cunha 2005, Pearson 2006).

The expression levels of A3 receptors are low in all regions of the brain (Dixon 1996, Zhao 2002). Chronic administration of the A3 receptor agonist IB-MECA was demonstrated to be protective against global ischemia, whereas deletion of the A3 receptors has detrimental effects in a model of hypoxia (Lubitz 1994).

In contrast, inhibition of the A2A receptors is considered to exhibit profound neuroprotective (Pearson 2006) and anti-apoptotic effects (Silva 2007). The evidence from A2A receptor deficient (A2A−/−) mice has shown that inactivation of the A2A receptors prevents neuron death induced by ischemia (Chen 1999). In the chimeric mouse model which A2A receptor knock-out is combined with bone marrow transplantation, the neuroprotection against ischemic brain injury in global A2A+/− mice was abolished by selective reconstitution of the A2A receptors in bone marrow cells (Yu 2004). However, in contrast to adult mice, brain damage after hypoxic ischemia is aggravated in newborn A2A−/− mice. The paradoxical protective effects of both A2A receptor agonism and antagonism in experimental model of ischemia display the
complexity of the adenosine system. In neurodegenerative disease such as PD, blockade of the A2A receptors in striatopallidal neurons leads to an amelioration of the motor deficits (Fuxe 2007); antagonism of the A2A receptors has been indicated to have direct neuroprotective effects to slow down disease progression of PD (Schwarzschild 2006). In the experimental model of AD, selective antagonism of the A2A receptors prevents the cognitive deficits induced by Aβ (Dall'Igna 2007). These findings implicate that modulation of A2A receptors might be an intriguing therapeutic strategy for neurodegenerative diseases.

The examples listed above reveal that the main actions of extracellular adenosine are neuroprotective in pathological conditions. However, endogenous adenosine is an incomplete neuroprotective agent because stimulation of the adenosine receptors may further aggravate tissue damage under some situations. The deleterious effects of adenosine are suggested to be mainly mediated by activation of the A2A receptors.

1.5.3 ATP and ATP P2 receptors

ATP

In addition to act as a ubiquitous enzyme cofactor and the important source of the cellular energy, the purine nucleotide ATP also functions as a potent extracellular messenger exerting its effects via the activation of the P2 receptors. Under normal physiological conditions, the intracellular concentration of ATP is 3 to 10 mM, whereas the extracellular concentration of ATP is ~ 10 nM (Schwiebert 2003, Trautmann 2009). This low extracellular ATP concentration is maintained as a result of the activities of several enzymes which metabolize ATP to adenosine 5’-diphosphate (ADP), adenosine 5’-monophosphate, and adenosine (Figure 1) (Bours 2006, Zimmermann 2000). Due to these enzyme activities, there is a 10^6-fold gradient for ATP efflux. It is found that resting cells release ATP at basal rates (Lazarowski
The release of a very small fraction of cellular ATP is sufficient to activate some P$_2$ receptors (EC$_{50}$ 3-500 nM) (Fitz JG. 2007). The low concentration of extracellular ATP that exists in a “halo” surrounding resting cells can signal the presence of the neighboring living cells. In CNS, fully controlled, transient increases in extracellular ATP occur at purinergic synapses and are used for basic physiological signaling such as neurotransmission, neuron-glia communication, neurite growth and proliferation (Burnstock 2008, Trautmann 2009). However, in response to the pathological events such as stress, inflammation, hypoxia, cellular injury, or changes in the ionic environment which lead to rapid energy failure, oxygen deprivation and ionic imbalance, cells will be damaged or die and ATP can be powerfully released from the damaged plasma membrane (Gourine 2007, Melani 2005, Phillis 1993). In these cases, ATP serves as a potent immunomodulator that regulates the activation, migration, phagocytosis and release of inflammatory mediators in glial and immune cells.

**P$_2$ receptors**

The ATP P$_2$ receptors can be sub-divided into two distinct families: The P$_2$X ligand-gated ionotropic channel family that is involved in fast excitatory neurotransmission and the P$_2$Y metabotropic, heptahelic G protein-coupled receptor family (Burnstock 2000). Based upon the pharmacology and G-protein subunit coupling of the receptors, the P$_2$Y receptors have been further sub-divided into eight subclasses: P$_2$Y$_1$, P$_2$Y$_2$, P$_2$Y$_4$, P$_2$Y$_6$, P$_2$Y$_{11}$, P$_2$Y$_{12}$, P$_2$Y$_{13}$ and P$_2$Y$_{14}$. The different subclasses of P$_2$Y receptors are stimulated with varying potencies by ATP and ADP. Some P$_2$Y receptors, for instance, the P$_2$Y$_2$, P$_2$Y$_4$ and P$_2$Y$_6$ receptors can also be activated by UTP and UDP, leading some to term these receptors as “pyrimidinergic receptors” (Communi 1997). In the CNS, recent evidence indicates that UDP leaked
from damaged hippocampal neurons facilitates microglial phagocytosis through activation of the P2Y6 receptors (Inoue 2007). Following CNS injury, microglia can migrate, and extend processes toward sites of tissue damage which release nucleotides. It is reported that microglia from P2Y12 deficient (P2Y12-/-) mice exhibit normal basal motility, but are unable to polarize, migrate or extend processes toward nucleotides. This implicates that the G- coupled P2Y12 receptors may be the primary regulator to induce microglial chemotaxis at early stages of the response to CNS injury (Haynes 2006, Ohsawa 2007).

The seven P2X receptors (P2X1-7) bear two putative transmembrane domains connected by a large extracellular ligand-binding loop, and intracellular carboxyl- and amino-termini (Hansen 1997, Zemková 2008). Current evidence reveals that functional P2X receptors are composed of P2X subunits as trimeric homomers and heteromers (North 2000). The P2X receptors have been grouped into three classes based on agonist efficacy and desensitization characteristics. Group 1 includes the P2X1 and P2X3 receptors with high affinity for ATP (EC50 = 1 μM) which are rapidly activated and desensitized; group 2 includes the P2X2, P2X4, P2X5 and P2X6 receptors that have lower affinity for ATP (EC50 = 10 μM), and show slow desensitization and sustained depolarizing currents. Group 3 is represented by the P2X7 ligand-gated ion channel that has very low ATP affinity (EC50 = 300 - 400 μM) and shows little or no desensitization (Burnstock 2000). It is demonstrated that the P2X1-6 receptors are widely distributed in the CNS. The P2X2, P2X4 and P2X6 receptors are widespread in the brain and often form heteromultimers. The expression of the P2X1 and P2X5 receptors is found in cerebellum, and the P2X3 receptors are expressed in brain stem. (Burnstock 2010, 2012). Activation of the P2X receptors is involved in regulation of the CNS functions. For instance, the P2X2 and P2X5 receptors identified in cerebellar neural circuitry participate in motor learning and locomotor coordination (Brockhaus
2004, Kanjhan 1996). In addition, activation of the P₂X₄ receptors is known to play an important role in neuropathic pain. Recent studies indicate that after peripheral nerve injury (PNI), the expression of the P₂X₄ receptors on spinal microglia was up-regulated, and stimulation of microglial P₂X₄ receptors led to the release of neurotrophin brain-derived neurotrophic factor (BDNF) from activated microglia. P₂X₄ deficient (P₂X₄⁻/⁻) mice lack mechanical hyperalgesia induced by PNI, and is reported to display impaired BDNF signaling in spinal cord (Ulmann 2008). In addition, blockade of the P₂X₄ receptors in the spinal cord reverses tactile allodynia after PNI in rat (Nagata 2009). These results suggest that targeting the P₂X₄ receptors may provide strategies for neuropathic pain relief.

1.5.4 The P₂X₇ receptors and their unusually properties compared with other P₂X receptors

Among all subtypes of the P₂X receptors, the P₂X₇ (also known as P₂Z) receptors are well-distinguished because of their unique biological properties. Similar to other P₂X receptors, the P₂X₇ subunit is composed of two transmembrane-spanning domains (TM1 and TM2), a large extracellular loop with the ATP binding site containing 10 similarly spaced cysteines and glycosylation sites, and intracellular carboxyl- and amino-termini (Figure 2) (Skaper 2010). Available evidence has demonstrated that the minimum stoichiometric conformation of the functional P₂X₇ receptor channel is a trimer with three protein subunits arranged around a cation-permeable channel pore (North 2000, 2002).
Figure 2: Structure of the P₂X₇ receptor subunit. The P₂X₇ receptor subunit consists of two transmembrane-spanning domains TM1 and TM2, a large extracellular loop with ATP binding site, and intracellular carboxyl and amino termini.

Different from other P₂X receptors, the P₂X₇ receptors are only activated by high concentrations of ATP. Transient stimulation of the P₂X₇ receptors by ATP or its non-hydrolysable analogue BzATP results in a rapid and reversible channel opening that is permeable to Na⁺, K⁺, and Ca²⁺. However, pulsed or sustained activation of the P₂X₇ receptors leads to the opening of a non-selective pore which facilitates the uptake of the molecules up to 900 Da (Di Virgilio 1995). This pore is permeable to dyes such as ethidium (314 Da), YO-PRO-1 (376 Da), and lucifer yellow (457 Da) (Cankurtaran-Sayar 2009). In order to investigate whether the opening of the P₂X₇ ion channel is associated with the pore-forming property, Virginio and colleagues have used calmidazolium, a calmodulin antagonist, in HEK293 cells stably expressing rat P₂X₇ receptors. They found that BzATP-induced currents but not YO-PRO-1 uptake, is inhibited by calmidazolium, suggesting the channel and pore function of P₂X₇ receptors might be two separate molecular entities (Virginio 1999).

Under pathological conditions, the extracellular levels of ATP are dramatically increased. High extracellular concentrations of ATP can stimulate the P₂X₇ receptors and lead to an opening of the pore. With this pore-forming property, P₂X₇ receptor
activation can induce cell death by either apoptosis or necrosis. The P$_2$X$_7$ receptors are associated with activation of different caspases including caspase 1, 3, 8, and caspase substrates such poly (ADP-ribose) polymerase (PARP) and lamin B, which are required for apoptotic signaling (Ferrari 1999 (b)). In J744 mouse macrophages, extracellular ATP can also induce cell death by necrosis (Murgia 1992). Thus, P$_2$X$_7$ receptors may act as an essential switch for the death signaling in an apoptosis or necrosis fashion. These studies have suggested that pore formation by the P$_2$X$_7$ receptors is cytolytic, leading to the description of the P$_2$X$_7$ receptors as “death receptors”. The carboxyl-terminal cytoplasmic domain of P$_2$X$_7$ (AA 352-595) is longer than other P$_2$X subtypes, and is crucial for P$_2$X$_7$ pore formation, transduction and signaling (Buell 1997, Hu 1998, Surprenant 1996). Mutations in this domain have been identified to lead to loss of function of P$_2$X$_7$ pore both in human and mouse. For example, the only known nonsynonymous single nucleotide polymorphism (SNP) in this domain (rs48804829; T1352C) produces a proline to leucine change at amino acid 451 (P451L), is demonstrated to impair pore forming function of the P$_2$X$_7$ receptors (Sorge 2012). It has been suggested that P$_2$X$_7$ pore formation requires over 95 % of the cytoplasmic domain. Experiments performed with truncated P$_2$X$_7$ receptors expressed in HEK-293 cells and Xenopus oocytes reveal that truncation of the protein at residue 581 (of 595) allows only negligible influx of ethidium ion. Surprisingly, cells expressing a receptor truncated at position 582 give wild-type ethidium ion uptake. In contrast, formation of the ionic channel only needs a limited portion of the cytosolic domain (Smart 2002).

1.5.5 Role of the P$_2$X$_7$ receptors in neurological disorders and in production of the inflammatory mediators

The P$_2$X$_7$ receptors are expressed on cells of haemopoietic origin, including mast
cells, erythrocytes, monocytes, peripheral macrophages, dendritic cells, T and B lymphocytes, and epidermal Langerhans cells (North 2002, Surprenant 1996). Within the CNS, expression of functional P2X7 receptors is predominantly observed on microglia, but not on neurons and astroglia (Chu 2010, He 2012, Melani 2006, Sim 2004). However, in vitro evidence shows that the P2X7 receptors are not only expressed on cultured microglia but also on cultured neurons (Diaz-Hernandez 2008) and astroglia (Fang 2011, Gao 2011). As already described in previous sections many studies have suggested the involvement of activated microglia in the progression of several neurological disorders. Recent evidence has documented that increased microglia reactivity and increased P2X7 receptor expression are both seen in inflammatory settings such as MS and ALS (Yiangou 2006). In the animal model of ischemia after MCAO, expression of the P2X7 receptors on activated microglia in infarct core and penumbra was elevated (Melani 2006). Administration of the P2X7 antagonist brilliant blue G (BBG) produced a reduction in the extent of brain damage after ischemia (Arbeloa 2012).

In chronic neurodegenerative diseases, post-mortem studies have demonstrated that microglia obtained from AD patients showed stronger expression of P2X7 receptors than nondemented individuals. The human microglia exposed to fibrillar Aβ1-42 peptide showed elevated levels of P2X7 receptor mRNA compared with vehicle. (McLarnon 2006). Additionally, in a mouse model of AD, the P2X7 receptors were specifically up-regulated around the Aβ plaques (Parvathenani 2003), whereas antagonism of the P2X7 receptors reduces Aβ plaques (Diaz-Hernandez 2012). In the unilateral 6-hydroxydopamine (6-OHDA) rat model of PD, there was an increased immunoreactivity of P2X7 receptors protein in substantia nigra. Inhibition of the P2X7 receptors by A438079 prevented the 6-OHDA-induced depletion of striatal dopamine stores (Marcellino 2010).
Furthermore, in mouse and cell models of HD, increased levels of the P$_2$X$_7$ receptors and altered P$_2$X$_7$-mediated permeability were observed in somata and terminals of HD neurons. Cultured neurons expressing mutant huntingtin showed increased susceptibility to apoptosis induced by P$_2$X$_7$ receptor activation. Treatment of the P$_2$X$_7$ receptor antagonist BBG prevented neuron death and improve motor coordination (Diaz-Hernandez 2009). Overall, these studies suggest the tight relation between the P$_2$X$_7$ activity and progression of neurological disorders.

The role of the P$_2$X$_7$ receptors in neuropsychiatric disorder MDD remains unclear. Up to now, several studies have been undertaken to investigate the relation between the P$_2$X$_7$ receptors and MDD, showing that the P$_2$X$_7$ gene polymorphism is associated with the susceptibility of MDD (Hejjas 2009, Lucae 2006). In animal experiments, Basso and colleagues have profiled wild-type and P$_2$X$_7^{-/-}$ mice in behavioral models of depression-like behaviors (Basso 2009). They found that P$_2$X$_7^{-/-}$ mice displayed an antidepressant-like profile in tail suspension test (TST) and forced swimming test (FST).

Whether activated microglia increase their levels of P$_2$X$_7$ receptor expression or, conversely, P$_2$X$_7$ receptor over-expression induces microglial activation is still uncertain. Bianco et al. have indicated that the P$_2$X$_7$ receptors play an important role in microglial proliferation. Blockade of the P$_2$X$_7$ receptors by antagonists, or treatment of ATP-hydrolase apyrase strongly decreased microglial proliferation. This suggests a growth promoting role of the P$_2$X$_7$ receptors in microglia (Bianco 2006). However, it remains unclear whether the P$_2$X$_7$ channel or pore-forming property drives microglial proliferation. Monif and colleagues reported that in the absence of pathological insults, over-expression of the P$_2$X$_7$ receptors alone was sufficient to drive microglial activation and subsequent proliferation. By using a point mutant P$_2$X$_7$ receptor (P$_2$X$_7$RG345Y) which lacks pore-forming ability but has intact ion channel
conducantance, they demonstrated that the P$_2$X$_7$ receptor pore is responsible for the activation and proliferation of microglia (Monif 2009). The P$_2$X$_7$ receptors seem to play a pivotal role in microglia-mediated neuroinflammation.

Activated microglia are known to produce a variety of inflammatory mediators. Contributions of the P$_2$X$_7$ receptors to pro-inflammatory events such as synthesis and release of reactive oxygen species, cytokines, and chemokines in glia are well documented. Extracellular ATP induced the release of NO in rat microglia by activating the P$_2$X$_7$ receptors (Codocedo 2013, Ohtani 2000). In mouse microglia cell line N9 cells, activation of the P$_2$X$_7$ receptors by BzATP evoked the mRNA transcription of pro-inflammatory cytokine IL-6 (Friedle 2011). It is shown that in primary rat microglia extracellular ATP stimulated TNF-α secretion by activating the P$_2$X$_7$ receptors (Hide 2000). Besides, P$_2$X$_7$ receptor activation triggers the production of chemokines CCL2 in rat astroglia (Panenka 2001), CCL3 in microglial cell line MG-5 cells (Kataoka 2009), as well as CXCL2 in microglial cell line BV2 cells and primary rat microglia (Shiratori 2010).

The role of P$_2$X$_7$ activation in the release of two members of IL-1 family cytokines, IL-1β and IL-18, has been investigated in microglia (Ferrari 1997, Franchi 2007, Kahlenberg 2003, Rampe 2004). The production of IL-1β and IL-18 is known to rely on the activity of caspase-1, which is also called interleukin converting enzyme (ICE). Caspase-1 is synthesized as a low-activity zymogen and then cleaved to form the active caspase-1 (Dungan 2011, Raupach 2006). Briefly, IL-1β and IL-18 are produced as inactive precursors. The precursor can be accumulated in cytoplasm, or processed into mature, active form by caspase-1 and then secreted. Inflammasomes are nowadays regarded as the major determinant in the production of IL-1β and IL-18. Inflammasomes are multiprotein complex formed of NOD-like receptor proteins (NLRPs). Most importantly, inflammasomes can act as an activation platform for the
caspases required for cytokine processing (Franchi 2009, Sokolovska, 2013 Sollberger 2013, Stienstra 2010). Studies have indicated that activation of the P2X7 receptors mediates the activation of NLRP1 and NLRP3 inflammasomes which assemble caspase-1 to process the maturation and subsequent release of IL-1β and IL-18 (Deplano 2013, Divirgilio 2007). These data suggest an important role of P2X7-mediated inflammasome activation in the production of IL-1β and IL-18.
2. Aims of study

A large body of evidence has suggested that glia-mediated neuroinflammation is involved in the pathogenesis of CNS injuries and degenerative disorders. Glia such as microglia and astroglia can be activated rapidly and release a variety of inflammatory mediators under pathological conditions. Simultaneously, both ATP and adenosine are largely released from the dying/damaged cells, and serves as an immunomodulator that regulates the inflammatory responses including chemotaxis and cytokine production by activating the P$_2$ receptors. Expression of the P$_2$X$_7$ receptors is known to be enhanced in response to neuroinflammation. P$_2$X$_7$ receptors are expressed on microglia and astroglia in vitro, and several studies have demonstrated that activation of the P$_2$X$_7$ receptors increases the levels of several inflammatory cytokines in primary rat glia and murine microglia cell lines. However, the effects of purines on the cytokine regulation in primary mouse glia are less studied and still not understood up to now. Therefore, the aims of this study are:

(1) To investigate the effects of extracellular purines including adenosine and ATP in the regulation of inflammatory cytokines in primary mouse glia and

(2) to determine the purinergic receptors involved in cytokine production in primary mouse glia.
3. Materials and Methods

3.1 Buffers and solutions

Preparation of primary mouse glial cultures

Medium A was prepared by adding 6.5 mL D-(+)-glucose solution (45%, Sigma), 7.5 mL HEPES (1 M; PAA Laboratories GmbH) and 5 mL antibiotics (10000 U/mL penicillin/10 mg/mL streptomycin, PAA) into 481 mL Hank’s - buffered salt solution (HBSS, PAA). Trypsin medium was prepared by adding 13.5 mL medium A, 150 μL DNAse 1 (50 mg/mL; Roche, Mannheim, Germany) and 1.5 mL trypsin (2.5 %, Gibco) to make the final volume to 15 mL. Trypsin inhibitor medium was prepared by adding 12 mL medium A, 3mL fetal bovine serum with gold (FBS gold, PAA) 150 μL DNAse 1 and 150 μL trypsin inhibitor (from glycine max soybean, 10 mg/mL, Sigma) to make the final volume to 15 mL. Wash medium was prepared by adding 27 mL medium A, 3mL FBS gold, 300 μL DNAse 1 to make the final volume to 30 mL.

Ribonucleic acid (RNA) isolation

GTC buffer was prepared by adding 4 M Guanidinethiocyanate (236.32 g), 25 mM sodium citrate (12.5mL, 1M) to a final volume of 500 mL. Divide the buffer by 25 mL in 50 mL Falcon tube and store at 4 °C. Add 180 μL β-mercaptoethanol before use. For the preparation of sodium acetate solution, 16.4 g of sodium acetate was dissolved in 10 mL distilled water and 70 mL acetic acid. Adjust pH = 4.0 by using acetic acid carefully and make the final volume to 100 mL. Store the solution at 4 °C. The chloroform-isoamyl alcohol 49:1 solution was prepared by adding 49 mL chloroform and 1 mL isoamyl alcohol. Store the solution in glass container.

Protein extraction
The lysis buffer was prepared by adding 3.36 mL TRISMA (500 mM, pH = 6.8), 5.2 mL SDS (10 %), 2.6 mL glycerine (86 %), 40 μL orthovanadate (Na$_3$VO$_4$, 100mM) and 28.8 mL distilled water. Store at -20 °C.

Two reducing agents were used – dithiothreitol (DTT, used between 1-10 mM) and β-mercaptoethanol (used at a final concentration of 0.72 % (v/v)). They were added to reduce disulfide bonds and to prevent oxidation of free thiols.

Buffer for western blot gels

The 4 X stacking gel buffer was prepared by adding 60.55 g TRIZMA, 4 g SDS to a final volume 1 L (pH = 6.8). The 4X separating gel buffer was prepared by adding 181.65 g TRIZMA, 4 g SDS to a final volume 1 L (pH = 8.8). Ammonium per sulphate (APS) was prepared by dissolving 5 g APS in 30 mL of double distilled water and make a final volume up to 50mL. Store at 4 °C and use this solution within 2 months.

Electrophoresis

In DNA electrophoresis, we used Tris-acetate-EDTA (TAE) buffer, a 10X stock of which was prepared by adding 400 mM TRIZMA (96.8 g), 200 mM acetic acid (22.84 mL) and 10 mM EDTA (20mL, 500mM, pH 8.0) to a final volume of 2 L. In protein electrophoresis, we used a 10X denaturing Tris buffer that was prepared by adding 20 g sodium dodecylsulfate (SDS), 288 g glycine, and 60.6 g TRIZMA to 2 L of distilled water.

DNA loading buffer was prepared by adding 30 mg bromophenol blue, 5 mL glycerol, 20 μL EDTA (0.5M, pH = 8.0) and 5mL distilled water. Lammlui buffer 2X was prepared by adding 13 mL stacking gel buffer, 11.6 mL glycerine, 2 g SDS to make a final volume of 100 mL.
Western blot

Several kinds of buffers were used in western blot. In semi-wet transfer, two anode buffers and one cathode buffer were utilized in our system. Anode buffer 1 (5X) was prepared by adding 90.8 g TRIZMA (1.5 M) to a final volume of 500 mL (pH = 10.4). Anode buffer 2 (10X) was prepared by adding 15.15 g TRIZMA (0.25 M) to a final volume of 500 mL. Cathode buffer (10X) was prepared by adding 52.48 g 6-Aminocaproic acid, adjusting pH = 9.4 with 20 mL Anode 1 buffer to a final volume of 1 L.

As for the analysis of western blot, 10X Tris-buffered saline (TBS) was prepared by adding 121 g TRIZMA, 175.2 g sodium chloride to a final volume of 2 L (pH 7.5). The TBS-T wash buffer was prepared by adding 200 mL 10X TBS and 2 mL Tween-20 to a final volume of 2 L.
3.2 Compounds

All stimulants and inhibitors were dissolved in the solvents according to the supplier information. Ultra-pure water was purchased from Biochrom AG, and dimethylsulfoxide ((CH₃)₂SO, DMSO), was purchased from Applichem.

Lipopolysaccharide (LPS) from Salmonella typhimurium (Sigma, Cat. No. L9516) was resuspended in sterile PBS as 1 mg/mL stock at -20 °C and was used at a final concentration 100 ng/mL for cell stimulation in the experiments.

Adenosine deaminase (ADA) from calf intestine (5 mg/mL) was purchased from Roche (Mannheim, Germany).

Clodronate-liposomes and PBS-liposomes were obtained from ClodronateLiposomes.org (Amsterdam, Netherlands). The stock concentration of clodronate-liposomes is 5-7 mg/mL.

Adenosine 5’-triphosphate (ATP), a P₂ receptor agonist; 3’-O-(4-benzoylbenzoyl)-adenosine 5’-triphosphate (BzATP), a selective P₂X₇ receptor agonist; 2-(Methylthio)adenosine 5’-triphosphate tetrasodium salt hydrate (2-MeSATP), a potent P₂X receptor agonist; uridine 5’-triphosphate tris salt (UTP), a potent P₂Y receptor agonist; adenosine 5’-triphosphate-2’,3’-dialdehyde (oxATP), an irreversible P₂X₇ receptor antagonist; pyridoxal-5’-phosphate-6-azo(phenyl-2’,4’-disulfonic acid) tetrasodium salt hydrate (PPADs) and suramin sodium salt, P₂ receptor antagonists; brilliant blue G (BBG), a selective, non-competitive P₂X₇ receptor antagonist, were purchased from Sigma Chemical Co. (St. Louis, USA). Uridine-5’-diphosphate disodium salt (UDP), a potent P₂Y receptor agonist; 3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), a selective, competitive P₂X₇ receptor antagonist; (3β,20β)-3-(3-Carboxy-1-oxoproxy)-11-oxoolean-12-en-29-oic acid disodium (carbenoxolone, CBX), a pannexin-1 hemichannel inhibitor; ivermectin,
a positive allosteric modulator of the \( \text{P}_2\text{X}_4 \) receptors;  
5'-N-Ethylcarboxamidoadenosine (NECA), a non-selective agonist for \( \text{P}_1 \) receptors, were purchased from Tocris Bioscience (Bristol, UK).
Table 1: Compounds used in the experiment

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Chemical structure</th>
<th>Biological activity</th>
<th>Solvent and stock concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
<td>P2 receptor agonist; increases activity of Ca2+-activated K+ channels; substrate for ATP-dependent enzyme systems</td>
<td>Ultra-pure water, 100 mM stock</td>
</tr>
<tr>
<td>BzATP</td>
<td>3’-O-(4-benzoylbenzoyl)-adenosine 5’-triphosphate</td>
<td>Selective P2X purinergic agonist. It is more potent than ATP at homodimeric P2X7 receptors.</td>
<td>Ultra-pure water, 50 mM stock</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>2-(Methylthio)adenosine 5'-triphosphate</td>
<td>Potent P₂X and P₂Y receptor agonist (Tomé 2007)</td>
<td>Ultra-pure water, 10 mM stock</td>
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<tr>
<td>NECA</td>
<td>5'-N-Ethylcarboxamidoadenosine</td>
<td>Potent adenosine receptor agonist (Kᵢ values are 14, 20 and 6.2 nM for human A₁, A₂A and A₃ receptors respectively; EC₅₀ = 2.4 μM for human A₂B). Inhibits platelet aggregation and is centrally active in vivo.</td>
<td>DMSO, 10 mM stock</td>
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<tr>
<td><strong>UTP</strong></td>
<td>uridine 5’-triphosphate</td>
<td>Potent P&lt;sub&gt;2&lt;/sub&gt;Y receptor agonist</td>
<td>Ultra-pure water, 100 mM stock</td>
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<tr>
<td><strong>oxATP</strong></td>
<td>adenosine 5’-triphosphate-2’,3’-dialdehyde</td>
<td>Irreversible inhibitor of the P&lt;sub&gt;2&lt;/sub&gt;X&lt;sub&gt;7&lt;/sub&gt; receptors.</td>
<td>Ultra-pure water, 30 mM stock</td>
</tr>
<tr>
<td><strong>PPADs</strong></td>
<td>pyridoxal-5’-phosphate-6-azo(phenyl-2’,4’-disulfonic acid)</td>
<td>Selective P&lt;sub&gt;2&lt;/sub&gt; receptor antagonist which blocks responses at both pre- and post-junctional sites.</td>
<td>Ultra-pure water, 50 mM stock, keep in darkness</td>
</tr>
<tr>
<td><strong>Suramin</strong></td>
<td>8,8’-{Carbonylbis[imino-3,1-phenylene carbonylimino(4-methyl-3,1-phenylene) carbonylimino]}di(1,3,5-naphthalenetrisulfonic acid)</td>
<td>A polysulfonated naphthylurea anticancer agent that inhibits tumor cell proliferation. It inhibits the activity of</td>
<td>Ultra-pure water, 100 mM stock</td>
</tr>
</tbody>
</table>
topoisomerase II by blocking the binding of the enzyme to DNA. It's antiangiogenic activity may be related to its ability to bind to and inhibit the activity of several growth factors, including FGFα, FGFβ, and PGDF. It uncouples G-proteins from receptors. It is a broad spectrum antagonist at P₂X and
<table>
<thead>
<tr>
<th>BBG</th>
<th>P₂Y purinergic receptor antagonist. It has well documented antipROTOZOAL and ANTHELMINTIC activity.</th>
<th>Ultra-pure water, 30 mM stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP</td>
<td>uridine-5’-diphosphate</td>
<td>Endogenous P₂Y receptor agonist which preferentially activates P₂Y₆. Shown to be a competitive antagonist at P₂Y₁₄ receptors.</td>
</tr>
<tr>
<td>compound</td>
<td>chemical formula</td>
<td>activity</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>----------</td>
</tr>
<tr>
<td>A438079</td>
<td><img src="attachment.png" alt="image" /></td>
<td>Competitive P$_2$X$<em>7$ receptor antagonist (pIC$</em>{50} = 6.9$ for the inhibition of Ca$^{2+}$ influx in the human recombinant P$_2$X$<em>7$ cell line). Devoid of activity at other P2 receptors (IC$</em>{50} &gt;&gt; 10 , \mu$M). Possesses antinociceptive activity in models of neuropathic pain in vivo (McGaraughty, 2007).</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>(3β,20β)-3-(3-Carboxy-1-oxopropoxy)-1-1-oxoolean-12-en-29-oic acid disodium</td>
<td>Glucocorticoid that inhibits 11β-hydroxysteroid dehydrogenase (11-HSD) and blocks gap junction communication.</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>22,23-Dihydroavermectin B1</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive allosteric modulator of the α7 neuronal nicotinic acetylcholine receptor and the purinergic P₂X₄ receptor. Antihelmintic. Also modulates glutamate- and GABA-activated chloride channels. Potentiates glycine-gated currents at low concentrations (30 nM).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMSO, 30 mM stock</td>
<td></td>
</tr>
</tbody>
</table>
3.3 Equipments

Cell culture

All of the experiments for cell culture were manipulated in a Herasafe HS18 Laminar flow (Vertical) which is kept in sterile. For the preparation of primary cultures, mouse brains were also dissected in a HERAguard HPH18 Laminar flow which is kept in sterile, Both HS18 and HPH 18 were obtained from Heraeus Instruments (Hanau). The dissection of mouse brain was proceeded on a stereomicroscope (Leica MS5-MZ6, Leica Mikroskopie und Systeme GmbH, Wetzlar) connected to a KL1500 halogen lamp (15V/150W, Schott AG, Mainz). Surgical equipment for mouse brain dissection of and preparation of primary cell cultures was obtained from the following sources: Student Dumont #5 Forceps (tip shape: straight, tip: standard, tip dimension: 0.1 x 0.06 mm, length: 11 cm) for removing the meninges was purchased from Dumont. Micro-preparescissor was purchased from Hammacher instruments (110 mm, grade, sharpe, HWB 002-11), and sterile disposable scalpels from Feather Safety Razor Co. Ltd. (No. 10 and 11, Japan). Before the dissection, the equipment was sterilized by 70 % ethanol. Cell precipitation was done in centrifuge with swing bucket (Megafuge 3.0 RS, max. speed 3500 rpm) from Heraeus Instruments. Phase-contrast microscope (Model IMT-2, Olympus Optical Co. GmbH, Hamburg) was used to observe the cells. Primary cultures were maintained in a CO₂ water jacketed incubator (Model 3111, Forma Scientific Inc.) at 37 °C, 5 % CO₂, while cell lines were also maintained at 37 °C, 5 % CO₂ in a CO₂ incubator (D6450, Heraeus).

RNA isolation

RNA isolation was manipulated in cooled centrifuges (max. speed 13000 rpm, Biofuge fresco, Heraeus). General centrifugation was manipulated on desktop
Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were performed on a heated-lid TRIO Thermoblock (Biometra GmbH, Göttingen). It has a temperature range from 4-100 °C, a maximal heating rate of 1.5 °C/sec, and a maximal cooling rate of 1.0 °C/sec. PCR was also sometimes performed on a Techne Genius Thermal cycler (Model FGEN02TP/FGEN05TP). Detection of the PCR products was proceeded by running an agarose gel and then observed on a platform with ultra-violet (UV) light. The photos of the gels were taken by BioDoc Analyze from Biometra. The gels were run in the chambers from Pharmacia Biotech (GNA 200). The power suppliers for electrophoresis were also purchased from Pharmacia Biotech.

Other equipment includes – Distilled water was obtained from Millipore RiOs™ and Elix® water purification systems (Millipore GmbH), autoclave (Model Tech 120, Integra Biosciences GmbH, Fernwald) and -80 °C refrigerators -86 °C FREEZER (Forma Scientific Inc.).
3.4 Cell preparation

3.4.1 Cultured mouse primary mixed glia

Mixed glia were prepared from the cortices of neonatal C57/BL6 mouse pups (0-2 days). Mouse pups were obtained from Center for Biochemistry and Molecular Cell Research (ZBMZ, Albert-Ludwigs University of Freiburg). The pups were placed on sterile paper towels and killed by decapitation. Hold the head by sterile forceps, and an incision was made through the skin from the neck to the nose. Next, a cut was made through the foramen magnum towards the glabella to remove the skull. To remove the brain, a pair of sterile tweezers was slid under the brain. Lift the brain and cut the trigeminal nerve carefully. The brains were collected in a petri dish containing cold medium A and kept on ice. All procedures were carried on under the laminar flow.

Figure 3: Scheme of mouse brain dissection for the preparation of primary mixed glia. Mouse primary mixed glial cultures were prepared from the brain of 0-2 day old neonatal mice. Cultures were prepared from the cortical and midbrain areas which were dissected from other regions along the stippled lines.

The olfactory bulbs, cerebellum and brain stem were removed (Figure 3) by sterile scalpel (No. 10). Meninges were carefully removed from the cortical and subcortical zone by a pair of sterile tweezers. Then the brain tissue was minced by sterile scalpel (No. 11) and gently dissociated in medium A by 1 mL pipet. 
Afterwards, the tissue was trypsinized with trypsin medium for 20 min at 37 °C, and followed by 4 min trypsin inhibitor treatment with trypsin inhibitor medium at room temperature. After two wash steps, tissue was tritutrated gently by fire-polished Pasteur pipets in 1 mL Dulbecco’s modified Eagle medium (DMEM, with 4.5 g/L glucose and L-glutamine; PAA Laboratories GmbH, Germany) containing 10 % fetal bovine serum with gold (FBS gold; PAA), 1 % sodium pyruvate (1 mM, PAA), 1 % antibiotics (100 U/mL penicillin/100 µg/mL streptomycin, PAA), and then filtered gently through a cell strainer (70 mm Ø; BD Falcon) into 24 mL DMEM. After the centrifugation (960 rpm, 12 min at 12 °C), cell viability was determined by trypan blue and 4-7×10^5 living cells were seeded in culture flasks (75 cm^2, Greiner). Cultures were maintained in DMEM containing 10 % FBS gold in a humidified atmosphere (5 % CO₂) at 37 °C. Culture medium was changed the second day after the preparation and every 6-7 days thereafter.

3.4.2 Cultured mouse primary microglia

Fourteen days after the preparation of mixed glia, once the cells reached confluence, microglia obtained as floating cells were collected by gentle shaking (150 rpm, 15 min), and transferred to appropriate culture plates. For experimentation, primary microglia were plated at 7.5-10×10^4 cells/well in 24-well plates for gene expression and cytokine release analyses, at 7.5-10×10^4 cells/well on a coverslip (13 mm Ø) coated with poly-L-lysine in 24-well plates for immunofluorescent detection, at 1.5-2×10^5 cells/well in 6-well plates for western blot, or at 3-4×10^5 cells/well on a coverslip (30 mm Ø) coated with poly-L-lysine in 6-well plates for calcium microfluorometry.

3.4.3 Cultured mouse primary astroglia and microglia depletion
Four weeks after the preparation, mixed glia were passaged by trypsinization and replated into 24-well plates (3-5x10^5 cells/well). To obtain purified astroglia, mixed glia were treated by clodronate-liposomes (ClodronateLiposomes.org, Amsterdam, Netherlands) 100-140 µg/ml for 4 hr at 37°C to eliminate the co-cultured microglia. PBS-liposomes solution was served as a control. Clodronate and liposomes are not toxic. Free clodronate does not pass phospholipid bilayers of liposomes and cell membranes, but liposomes are easily swallowed by phagocytic cells. Once clodronate is delivered into phagocytic cells using liposomes as vehicle, it will not escape from the cells. After disrupting the phospholipid bilayers of the liposomes under the influence of the lysosomal phospholipases in the phagocytic cells, the clodronate which is dissolved in the aqueous compartments between the liposomal bilayers will be released into the cells. Then the clodronate is excessively accumulated in the intracellular space, and the cells are irreversibly damaged and die by apoptosis. After the clodronate-liposomes or PBS-liposomes treatment, cells were gently washed twice by warm Dulbecco’s phosphate-buffered saline (DPBS, PAA), and maintained in DMEM containing 10 % FBS gold at 37 °C. Forty-eight hours after microglia depletion, the expression of microglial marker CD11b and astroglial marker GFAP were detected by primers in polymerase chain reaction (PCR). Primer sequences used in PCR are listed in Table 2.

3.4.4 Immortalized murine microglia cell lines BV-2 and N9 cells

In this study, we used BV-2 and N9 cells within 3 weeks after the thaw. BV-2 cells were maintained in DMEM (with glucose 1 g/L, sodium pyruvate, and L-glutamine; PAA) containing 10 % FBS (Biochrom AG), 1% antibiotics, whereas N9 cells were maintained in Iscove’s modified Dulbecco's Medium (IMDM, with L-glutamine and 25 mM HEPES; Gibco) containing 5% FBS, 1% antibiotics in a
humidified atmosphere (5 % CO₂) at 37 °C. For experimentation, BV-2 and N9 cells were passaged by trypsinization and seeded at 7.5-10×10⁴ cells/well into 24-well plates for gene expression analysis, at 1.5-2×10⁵ cells/well in 6-well plates for western blot, or at 3-4×10⁵ cells on a coverslip (30 mm Ø) coated with poly-L-lysine in 6-well plates for calcium microfluorometry.

3.5 Stimulation of the cells

**Cultured mouse primary microglia**

Expression of cytokine mRNA:

Culture medium change and stay 1hr at 37 °C with 5 % CO₂ -> 2 hr stimulation by agonists -> lyse the cells with GTC/2-mercaptoethanol buffer

Cytokine release analysis

Culture medium change and stay 1hr at 37 °C with 5 % CO₂ -> (1hr pre-incubation of antagonists) -> 24 hr stimulation by agonists -> collect the culture supernatants

**BV-2 and N9 cells**

Expression of cytokine mRNA:

Culture medium change and stay 1hr at 37 °C with 5 % CO₂ -> 2 hr stimulation by agonists -> lyse the cells with GTC/2-mercaptoethanol buffer

**Cultured mouse primary astroglia**

Expression of cytokine mRNA:

Culture medium change and stay 1hr at 37 °C with 5 % CO₂ -> 3 hr stimulation by agonists -> lyse the cells with GTC/2-mercaptoethanol buffer

Cytokine release analysis

Culture medium change and stay 1hr at 37 °C with 5 % CO₂ -> (1hr pre-incubation of
antagonists) -> 24 hr stimulation by agonists -> collect the culture supernatants

**Cultured mouse primary mixed glia**

Expression of cytokine mRNA:

Culture medium change and stay 1 hr at 37 °C with 5 % CO\(_2\) -> 3 hr stimulation by agonists -> lyse the cells with GTC/2-mercaptoethanol buffer

### 3.6 Total RNA isolation

Cells were lysed by 4 M guanidine thiocyanate (GTC)/2-mercaptoethanol buffer and total RNA was extracted with the sodium acetate/phenol/chloroform-isoamyl alcohol step. After the stimulation, 500 μL GTC/2-mercaptoethanol buffer was added into the well and then the cell lysates were collected in 2 mL eppendorf tubes. The cell lysates were mixed thoroughly with 2 M sodium acetate/phenol/chloroform-isoamyl alcohol (49:1) by vortexing, and then stayed on ice for 15 min. Thereafter, the samples were centrifuged at 13000 rpm, 4 °C for 15 min, and then isopropanol precipitated at -20 °C overnight. After being centrifuged at 13000 rpm, 4 °C for 15 min, the isopropanol was discarded and the RNA pellets were washed twice by 70% ethanol. The pellets were air-dried 15-20 min at 37 °C on a thermoblock then dissolved in sterilized, RNase-free Tris-HCl buffer (10 mM, pH = 7.0). Keep the samples at 65 °C for 5 min on a thermoblock and then put them on ice for 10 min. The total RNA concentrations (μg/μL) were determined by spectrophotometer (BioPhotometer, Eppendorf). An \(A_{260}/A_{280}\) ratio between 1.8 and 2.0 was considered a pure preparation for RNA.
3.7 Reverse transcription polymerase chain reaction

Total RNA 0.5 µg was subjected and mixed with random hexamer oligonucleotides (0.5 µg/µL, biomer.net) in a 16 µL reaction volume. The samples were denatured at 70 °C for 10 min to melt secondary structures within the RNA. The samples were immediately cooled on ice and centrifuged. Reverse transcription was performed in the master mix comprising of M-MLV 5 X reaction buffer (50 mM Tris-HCl, pH 8.3 at 25 °C, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT, Promega), 5 mM deoxynucleotide triphosphates (dNTP mix, Invitex), 2.8 U/µl recombinant RNasin, (Promega) and 20 units of moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Madison, USA). The master mix was added to make the reaction volume of 25 µL and then incubated at 22 °C for 10 min followed by 37 °C for 60 min. The RNasin is a 50 kDa protein which exerts it inhibitory effects by non-covalently binding to RNases at a 1:1 ratio. At the end of the reaction, the temperature was elevated to 95 °C for 5 min to deactivate the enzyme.

The quality of the cDNA was controlled by actin-β (ACTB) primers and potential genomic DNA contamination was checked by running the reactions without reverse transcriptase in PCR amplifications. Primer sequences for the murine genes are listed in Table 2.

**Master mix for random hexamer treatment:**

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>End con.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>0.5 µg/RNA con.</td>
</tr>
<tr>
<td>Random hexamer</td>
<td>1</td>
</tr>
<tr>
<td>H₂O, ultra-pure</td>
<td>15-(0.5 µg/RNA con.)</td>
</tr>
<tr>
<td>Total volume</td>
<td>16</td>
</tr>
</tbody>
</table>

One microliter was subjected to PCR reaction without reverse transcription.

**Reaction:** 70 °C, 10 min -> 4 °C, 5 min

**Master mix for reverse transcription:**

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>End con.</th>
</tr>
</thead>
</table>

78
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MLV reverse transcriptase 5X buffer</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>50X dNTPs master (12.5 mM/each)</td>
<td>1</td>
<td>1.25 mM/each</td>
</tr>
<tr>
<td>Recombinant RNasin Ribonuclease inhibitor (40 U/μL)</td>
<td>0.7</td>
<td>2.8 U/μL</td>
</tr>
<tr>
<td>M-MLV reverse transcriptase (200 U/μL)</td>
<td>1</td>
<td>20 U/μL</td>
</tr>
<tr>
<td>H₂O, ultra-pure</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

**Reaction:** 22 °C, 10 min -> 37 °C, 60 min -> 95 °C, 5 min -> 4 °C, 5 min

### 3.8 Primer design and synthesis

The primer pairs used in this study were synthesised by biomers.net GmbH ([http://www.biomers.net/de/index.html](http://www.biomers.net/de/index.html)). Primers were designed by using Primer-Blast ([http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome](http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)). The nucleotide sequences for the design of primers were obtained from National Institute for Biological Information (NCBI, Bethesda, USA) ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) or Ensembl Genome Browser ([http://www.ensembl.org/index.html](http://www.ensembl.org/index.html)). The specificity of the primer pairs for the detected genes were also checked by Primer-Blast. The primer pair that only specifically annealed to the detected gene was selected. The optimal length of the primer is 18-25 bp, with 40-60 % GC content, and a melting temperature (T<sub>melting</sub>) between 55-65 °C, with negligible secondary structures. The annealing temperature (T<sub>annealing</sub>) was usually kept within 5 °C lower than the T<sub>melting</sub>. The formulae used to calculate the annealing temperature are listed below:

Primers < 20 bps: Ta = [4(G + C) + 2(G + C)] - 5 °C

Primers > 20 bps: 62.3 °C + 0.41 °C (% GC) - 500/length - 5 °C
3.9 Polymerase chain reaction (PCR)

After the reverse transcription, the expression of the detected genes was investigated by PCR. This reaction was carried out in the 0.5 mL tubes (Biozym) on a thermocycler. One microliter cDNA obtained from the reverse transcription was subjected into the reaction. The master mix containing a final concentration of 0.2 μM specific primers, 2 mM MgCl₂, 0.25 mM dNTP and 0.03 U/μl of DNA polymerase from *Thermus aquaticus* (Taq) in a 10X supplied reaction buffer (100 mM Tris-HCl, pH 9.0 at 25 °C, 500 mM KCl and 1 % Triton X-100).

After the reaction, the PCR products were examined by running electrophoresis on agarose (PeqGold Universal-Agarose, Peqlab Biotechnology GmbH) gels. The 2 % agarose gels were made in 1X TAE buffer. After being heated in a microwave, the gel solution was cooled to 60 °C and ethidium bromide (EtBr, end concentration 0.5 μg/mL, Bio-rad) was added in to the solution. PCR product 10 μL were mixed with 6X loading buffer and the gel was run. For the estimation of the product size, one well on the gel was also loaded with a 100 bp marker (GeneRuler 100 bp DNA ladder, Fermentas). The gel was observed on a platform with UV light and the photos were taken by the BioDoc Analyze (Biometra).

<table>
<thead>
<tr>
<th><strong>Master mix for PCR</strong></th>
<th><strong>Volume (μL)</strong></th>
<th><strong>End con.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer without MgCl₂</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>1.6</td>
<td>2 mM</td>
</tr>
<tr>
<td>50X dNTPs master (12.5 mM/each)</td>
<td>0.4</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>Forward primer (5’ -&gt; 3’, 5 μM)</td>
<td>0.8</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Backward primer (3’ -&gt; 5’, 5 μM)</td>
<td>0.8</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Taq-polymerase (5 U/μL; Genaxxon bioscience)</td>
<td>0.12</td>
<td>0.03 U/μL</td>
</tr>
<tr>
<td>H₂O, ultra-pure</td>
<td>13.28</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Reaction: 95 °C, 5 min -> 95 °C, 30 sec -> T_{annealing}, 45 sec -> 72 °C, 45 sec -> 72 °C, 10 min -> 4°C, 5 min x 35 cycles (ACTB x 25 cycles)

Annealing temperature of the primer pair for detected genes was listed in Table 2.

**Table 2: Sequences of primer pairs for the detection of reference gene ACTB, P2 receptors and glial markers in PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>T_{annealing} °C</th>
<th>PCR product bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>GCCGCTTAGATGAGTGGATAGGATGCCAAGCCGGGCCTGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>363</td>
</tr>
<tr>
<td>P2X1</td>
<td>GGTTCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>299</td>
</tr>
<tr>
<td>P2X2</td>
<td>ATGGACGTGGTGGGCTATGGGCTAGAGGCCTATTATAGGAGGGCTG</td>
<td>58</td>
<td>373</td>
</tr>
<tr>
<td>P2X3</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>361</td>
</tr>
<tr>
<td>P2X4</td>
<td>GGTTCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>292</td>
</tr>
<tr>
<td>P2X5</td>
<td>GGTTCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>258</td>
</tr>
<tr>
<td>P2X6</td>
<td>ATGGACGTGGTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>283</td>
</tr>
<tr>
<td>P2X7</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>274</td>
</tr>
<tr>
<td>P2Y1</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>412</td>
</tr>
<tr>
<td>P2Y2</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>247</td>
</tr>
<tr>
<td>P2Y3</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>320</td>
</tr>
<tr>
<td>P2Y4</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>384</td>
</tr>
<tr>
<td>P2Y5</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>238</td>
</tr>
<tr>
<td>P2Y6</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>265</td>
</tr>
<tr>
<td>CD 11b</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>219</td>
</tr>
<tr>
<td>GFAP</td>
<td>GCCGCTTTGGGCTATGGGCTAGAGGCCTTTAAGGAGGGCTG</td>
<td>58</td>
<td>278</td>
</tr>
</tbody>
</table>
3.10 Real time-quantitative PCR (real time-qPCR) analysis

The levels of cytokines (IL-6, CCL2, TNF-α) mRNA transcription were quantified by real time-qPCR. The real time-qPCR analysis for cytokines was performed by using hydrolysis probes purchased from Universal ProbeLibrary (UPL, Roche, Mannheim, Germany). The probes were labelled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye. The primer pairs for cytokines detection were synthesized by biomers.net GmbH. The sequences of primer pairs used in real time-qPCR are listed in Table 3. First, the efficiency of the amplification for each gene was determined by running a serial dilution of cDNA standards for each gene in real-time qPCR. BV-2 cells treated with LPS were utilized as the control for cytokine gene induction. The cDNA samples obtained from BV-2 were serially diluted (stock, 5X, 25X, 125X, 625X). After the dilution, 3 μL cDNA from each diluted standard was subjected into the 96-well PCR plates (Hard-Shell Thin-Wall 96-Well Skirted PCR Plates, Bio-rad) to mix with the master mix consisting a final concentration of 0.1 μM specific probes and 0.5 μM specific primers, H2O grade (Roche) in the 2X LightCycler® 480 Probes Master (Roche) that contains FastStart Taq DNA Polymerase for hot start PCR, which significantly improves the specificity and sensitivity of qPCR by minimizing the formation of nonspecific amplification products. The total volume for the reaction is 20 μL. The efficiency of amplification between 90-110% was regarded eligible. For the quantification of samples, the cDNA samples were diluted 3X and then added 3μL in to the reaction. Data were obtained using C1000™ Thermal Cycler (CFX96 real-time PCR system, Bio-Rad). Reaction conditions for qPCR were as following: 45 cycles of amplification by denaturing to 95°C for 10 sec and extending at 60°C for 30 sec. It is found that the stimulants we used in our study did not cause a fluctuation of the reference gene (actin-β (ACTB) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)) expression. Therefore,
the cytokines mRNA levels were normalized to the levels of both ACTB and GAPDH by CFX Manager™ software (Bio-Rad).

**Master mix for real time qPCR**

2X LightCycler Probes Master  10
Probes (10 μM)  0.2  0.1 μM
Forward primer (5’ -> 3’, 20 μM)  0.5  0.5 μM
Backward primer (3’ -> 5’, 5 μM)  0.5  0.5 μM
H₂O, grade  5.8
cDNA  3

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>20</td>
</tr>
</tbody>
</table>

**Reaction:**

1. 95 °C, 5 min (slow remp rate to 4.4 °C/sec)
2. 95 °C, 10 sec (slow remp rate to 4.4 °C/sec)
3. 60 °C, 30 sec (slow remp rate to 2.2 °C/sec) x 44
4. 72 °C, 1 sec + plate read (slow remp rate to 4.4 °C/sec), go to step 2.
5. 40 °C, 10 sec (slow remp rate to 1.5 °C/sec)

**Table 3: Sequences of primer pairs for the reference genes and cytokines measured in real-time qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>CTAAGGCAACCCGTGAAAAG  ACCAGAGGCATACAAGGGACA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGTCGGTGCGATCTGAC   CCTGCTTCACCACCCCTTGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGATGGATGCTACCAACTGG  TTCAIIGTACTCCAGGTAGCTATGG</td>
</tr>
<tr>
<td>CCL2</td>
<td>CATCCACGTGTGGCTCA    GAICAICTGGCTGGTGAATGAGT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TCTTCTCATTCTGGTTGTG  GGTCGGGCAIAGAATGAGT</td>
</tr>
</tbody>
</table>
3.11 Cytometric bead array (CBA)

The principle of CBA assays is capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead has been conjugated with a specific antibody for analyte. The detection reagent is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, the sandwich complexes (capture bead + analyte + detection reagent) are formed (Figure 4). These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of the bead and the detector. In this experiment, the CBA mouse inflammation kit was purchased from Becton Dickinson Biosciences (BD Biosciences, Heidelberg, Germany). After the stimulation, culture supernatants were collected and the secretion of six inflammatory cytokines (IL-6, IL-10, CCL2, IFN-γ, TNF-α, IL-12p70) was measured according to the manufacturer's guidelines. First, the lyophilized recombinant standard spheres were transferred to a 15 mL conical, polypropylene tube and reconstituted with 2 mL assay diluent. Equilibrate at room temperature at least for 15 min, mix gently and then perform a serial dilution (Figure 5). Briefly, six bead populations with distinct fluorescence intensities coated with capture antibodies specific for IL-6, IL-10, CCL2, IFN-γ, TNF-α and IL-12p70 (50 μL) were mixed with PE-conjugated detection antibodies (50 μL) and 50 μL recombinant standard or assay diluent (as a negative control), or test samples then incubated together 2 hr at room temperature in the darkness to form sandwich complexes. Afterwards, the test samples were washed once by 1 mL wash buffer provided in the kit, and then centrifuged at 200 g, room temperature for 5 min. Remove the supernatants, add 300 μL wash buffer to resuspend the bead pellets then start the acquisition using
FACSCalibur (BD Biosciences). The sandwich complexes formed from each cytokine is resolved in a red channel of the flow cytometer (Figure 6). The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine. After acquisition of sample data, the cytokine concentrations were calculated using the proprietary FCAP™ Software v1.0.1 (Soft Flow Inc., New Brighton, USA).

Figure 4: The formation of sandwich complexes in CBA. CBA assays are detection methods to capture a soluble analyte or set of analytes with beads, making it possible to detect analytes using flow cytometry. Each capture bead has been conjugated with a specific antibody for analyte. The detection reagent is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, capture bead, analyte, detection reagent will form the sandwich complexes.

Figure 5: Serial dilution of the reconstituted standard in CBA.
Figure 6: Analysis of the fluorescence intensities of sandwich complexes in CBA. Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-6, IL-10, CCL2, IFN-γ, TNF-α, and IL-12p70 proteins. The six bead populations are mixed together with detected cytokines to form the sandwich complexes, which are resolved in a red channel of a flow cytometer.
3.12 Determination of total protein concentrations

In this study, the total protein content from the cell lysates was determined by the BCA (bicinchoninic acid) method (Smith 1985). BCA method is known as a copper-based protein assay which is dependent on the “biuret reaction”. In an alkaline environment containing sodium potassium tartrate, the cupric ions (Cu$^{2+}$) form a colored chelate complex with the amino acid residues of proteins. This is known as the biuret reaction because it is chemically similar to a complex that forms with the organic compound biuret (NH2-CO-NH-CO-NH2) and Cu$^{2+}$. The BCA protein assay combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation (Ca$^{+}$) by BCA. Therefore, two steps are involved in this assay. First is the biuret reaction, whose faint blue color results from the reduction of Cu$^{2+}$ to Cu$^{+}$ (Figure 7). Second is the chelation of BCA with Cu$^{+}$, leading to an intense purple color. The BCA/ Cu$^{+}$ complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. In the determination of protein content, the bovine serum albumin (BSA, 1-40 µg/µL, Sigma) was used as a standard, and the standard curve was generated for each experiment. The reaction was performed in a 96-well plate at 95°C for 30 min on a thermoblock. Plates were measured at 570 nm wavelength by MRX microplate reader (Magellen Bioscience) and analysed by DYNEX Revelation Version 4.25.
Figure 7: The biuret reaction by reducing the copper ion from Cu^{2+} to Cu^{+} in BCA assay. BCA method contains two steps of reaction. First is the biuret reaction, whose faint blue color results from the reduction of Cu^{2+} to Cu^{+}. Second is the chelation of BCA with Cu^{+}, leading to an intense purple color.

3.13 Western blot

Wild-type, P_{2X7}{^/-}, and P_{2X4}{^/-} primary microglia were harvested and transferred to 6-well plates. BV-2 and N9 cells were passaged and seeded into the 6-well plates. After the incubation at 37 °C overnight, the cells were lysed by scraping the cells with 1 mL tips in lysis buffer. The cell lysates were then transferred into 1.5 mL eppendorf tubes. The total protein content in the samples was measured by BCA Protein Assay Kit (Pierce) as mentioned above. Add 1 μL dithiothritol (DTT) buffer for 100 μL of protein, and the samples were heated at 95 °C for 5 min on a thermoblock. Five microgram of total protein from the cell lysates of primary microglia, while 20 μg of total protein from the cell lysates of BV-2 and N9 cells was subjected and resolved in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride (PVDF, 0.45μm, Millipore) membranes. The membranes were blocked for 1 h in Tris-buffered saline with 0.1% Tween 20 (TBS–T) containing 5% non-fat dried milk (Blotting-Grade Blocker, Bio-rad). Then, the membranes were washed once in TBS-T and incubated with rabbit anti-P_{2X7} or rabbit anti-P_{2X4} antibody (1:500, Alomone Labs, Jerusalem, Israel) in TBS–T containing 1% bovine serum albumin (BSA), overnight, at 4°C. After washing three times with TBS–T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:25,000 in TBS–T containing 1% BSA, GE Healthscience, GE Healthcare) for 1 h at room temperature. The membranes were washed three times again. The immunoreactive bands were visualized using an ECL western blot detection system.
(GE Healthscience, GE Healthcare) and analyzed using Fusion Image Acquisition System (Peqlab, Erlangen, Germany). To confirm protein loading, membrane were stripped for 30 min, blocked for 1hr and then probed with antibody recognizing actin (rabbit anti-actin antibody 1:5,000, Sigma). The western blot data shown are representative for at least three independent experiments.

3.14 Calcium microfluorometry

Calcium microfluorometry is a ubiquitous technique that uses fluorescent indicator molecules to monitor the changes of intracellular free calcium concentrations in single cells. It is employed to investigate electrically excitable tissues such as muscles, neurons, and glia, where membrane-related electrical activity is tightly coupled to significant calcium movements into or within cells (Neubauer 2010). Several molecular probes, namely fluorescent dyes, are capable of sensing the local calcium concentration with high selectivity. In our study, we used fura-2 acetoxymethylester (fura-2AM, Teflabs.com, USA), which is a dual excitation ratiometric and sensitive indicator dye, to detect the changes of intracellular calcium. Fura-2 can be excited successively at two different wavelengths (340 and 380 nm, Figure 7). The emitted light (510 nm) increases with increasing intracellular calcium at one excitation wavelength, and decreases at a second wavelength. Once the levels of intracellular free calcium increases, Fura-2 binds to the calcium and emitted more intense fluorescence under the excitation at 340 nm (representative for fura-2-bound calcium), whereas the fluorescence emitted under the excitation at 380 nm (representative for free calcium) attenuates. Generally, the concentration of fura-2 used in the experiment is between 0.5-5 µM. In this study, the intracellular calcium changes after the stimulation of P2 receptor agonists or antagonists in microglial cells were investigated. The murine N9 microglial cells and primary mouse microglia were
cultured on glass cover-slips (30 mm Ø) coated by poly-L-lysine (Sigma) at a density of 3-4×10⁵ cells overnight in culture medium. Next, the cover-slips were rinsed shortly in Ringer-Bic buffer, containing 1mM probenecid, 145 mM NaCl, 0,4 mM KH₂PO₄, 1,6 mM K₂HPO₄·3H₂O, 5 mM D-glucose, 1 mM MgCl₂·6H₂O and 1,3 mM Ca-Gluconate·H₂O (pH~7.4) or in calcium-free Ringer-Bic buffer containing 145 mM NaCl, 0,4 mM KH₂PO₄, 1,6 mM K₂HPO₄·3H₂O, 5 mM D-glucose, 1 mM MgCl₂·6H₂O and 5 mM ethylene glyco-bis (2-aminoethylether) -N,N,N’,N’-tetraacetic acid (EGTA). Cells were loaded with 2 µM fura-2 together with 0.4 % pluronic acid F-127 (Molecular Probes, Grand Island, NY, USA) in 1 mL Ringer-Bic buffer for 20-30 min at 37°C. After an additional washing step of 5 min in plain Ringer-Bic buffer, the cover-slips were fixed in a plastic perfusion chamber (37°C) and attached to an inverted microscope (Axio Observer, Zeiss, ZBSA, University Freiburg) equipped with a monochromator (Till Photonics), and 2 cooled CCD cameras (Axiocam Rev.3, 1300x1000, and Axiocam 640x480) supported by Axiovision software (Zeiss). Using a Fluar 40x/1.3 Oil Ph3 M27 objective digital images were taken at an emission wavelength of 510 nm using paired exposure at 340 nm and 380 nm excitation wavelengths at a frequency of 2 Hz. Changes in intracellular calcium levels were expressed as the ratio of the 340 and 380 nm excitation wavelengths (Δ340/380) in time (seconds). During recording experiments, compounds were administered directly to the Ringer-Bic buffer. Triton X-100, a non-ionic detergent, can enhance the permeability of the plasma membrane then increase the calcium influx from extracellular milieu. We used 1 % triton X-100 as a positive control for calcium influx. Cells non-responsive to triton were excluded from the experiments.
Figure 8: Excitation spectra of fura-2 for the indicated values of the free Ca\(^{2+}\) concentration (0-39.8 µM).

3.15 Immunofluorescent (IF) detection

Cultured mouse primary microglia were cultured on glass cover slips (13 mm Ø) in 24-well plates and reached confluence the following day. To determine the intracellular expression of IL-6, TNF-α and CCL2, the cells were stimulated with 1 mM ATP, 500 µM BzATP or 100 ng/mL LPS for 4 hr. In order to block the cytokine secretion, cells were treated by Brefeldin A (BFA, Ready Made solution from Penicillium brefeldianum, Sigma) 10µg/mL at the last 30 min of the stimulation. After fixation in 4% paraformaldehyde for 1 hr and washing in DPBS, cells were blocked in DPBS containing 10% horse serum (PAA) and 0.5% triton X-100 (Carl Roth GmbH & Co., Germany) for 1hr. The cells were then incubated overnight with a 1:100 dilution of primary goat-anti-mouse IL-6 (R&D systems Inc., Minneapolis, MN, USA), rabbit anti-mouse CCL2 (Santa Cruz Biotechnology, Inc., CA, USA) and rat anti-mouse TNF-α (BioLegend Inc., San Diego, USA) antibodies. After washing 3 times with DPBS and incubated in Alexa Fluor 568-conjugated donkey anti-goat, 488-conjugated donkey anti-rabbit and 594-conjugated donkey anti-rat immunoglobulin G (IgG) (Invitrogen Corp, Carlsbad, USA) overnight. Finally, the cells were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI;
Invitrogen). Fixed cells probed only with Alexa Fluor 568-conjugated donkey anti-goat, 488-conjugated donkey anti-rabbit and 594-conjugated donkey anti-rat immunoglobulin G (IgG) secondary antibodies were served as negative controls for non-specific staining. All staining procedures were performed at room temperature. Representative fluorescence photographs were taken by using a spectral confocal microscope (LSM 510 META, Zeiss, ZBSA, University Freiburg). The photographs were analyzed by ZEN 2009 light edition software (Zeiss).

3.16 Data analysis and statistics

All results are expressed as the mean ± SEM of three independent experiments. The statistical analyses were performed by Statistical Product and Service Solutions (SPSS) using an unpaired t-test, one-way ANOVA pre hoc test followed by Scheffe’s post hoc test and two-way ANOVA followed by simple main effect test.
4. Results

4.1 Role of purine nucleotides ATP and the ATP P₂ receptors in the production of cytokines in cultured mouse primary microglia

4.1.1 ATP P₂ receptors expressed on primary microglia

To examine the P₂ receptors expressed on primary microglia, the total RNA of unstimulated cells was extracted, and reverse-transcribed to cDNA. The expression of the P₂ receptors on mouse primary microglia was investigated by PCR (Figure 9). It shows that the P₂X₄, P₂X₇, P₂Y₆, P₂Y₁₂ receptors are predominantly expressed on unstimulated primary microglia. Expression of the P₂X₁, P₂Y₁, P₂Y₂, P₂Y₁₃ and P₂Y₁₄ on microglia is weak.

Figure 9: Detection of the ATP P₂ receptors expressed on primary microglia by PCR.

We found that unstimulated microglial cells predominantly express the P₂X₄, P₂X₇, P₂Y₆, P₂Y₁₂ receptors, whereas expression of the P₂X₁, P₂Y₁, P₂Y₂, P₂Y₁₃ and P₂Y₁₄ receptors is relatively weak. Experiments were repeated three times.
4.1.2 Dose-dependent effects of ATP on microglial cytokine release

The effects of ATP on microglial cytokine release were investigated. Primary microglia were stimulated 24 hr with different concentrations of ATP (1 μM, 10 μM, 100 μM, 1 mM). Culture supernatants were collected, and the levels of IL-6, IL-10, CCL2, IFN-γ, TNF-α and IL-12p70 were measured by CBA. We found that among the concentrations of ATP we used, 1 mM ATP evoked the most robust cytokine release in primary microglia (Figure 10). Among the detected cytokines, only CCL2 and TNF-α were induced by 1 mM ATP. Accordingly, we treated the cells with 1 mM in the following experiments.

Figure 10: Dose-dependent effects of ATP in primary microglia. ATP dose-dependently increased the release of CCL2 and TNF-α in primary microglia, whereas the levels of IL-6, IL-10, IFN-γ and IL-12p70 were not changed. The Values represent the mean ± SEM of two independent experiments, and N=1 in each experiment. Volume of culture medium: 600 μL/well.
4.1.3 ATP at 1 mM significantly increased CCL2 and TNF-α release in primary microglia

According to the data shown in Figure 10, we treated the primary microglia 24 hr in the presence of control vehicle or 1 mM ATP. The levels of inflammatory cytokines in supernatants were measured by CBA. Figure 11 shows that the release of CCL2 and TNF-α was significantly increased by 1 mM ATP treatment. Notably, there is a continuous basal release of CCL and TNF-α from unstimulated microglia.

Figure 11: Effects of 1 mM ATP on microglial cytokine induction. ATP at 1 mM significantly enhanced the secretion of CCL2 and TNF-α. Values represent the mean ± SEM of three independent experiments. Unpaired t-test, **p < 0.01, *p < 0.05 vs. vehicle. Volume of culture medium: 600 μL/well.
4.1.4 Significant induction of microglial CCL2 and TNF-α mRNA expression after 1 mM ATP stimulation

To address the mechanisms of CCL2 and TNF-α release induced by ATP in microglia, cells were incubated in the presence of control vehicle or 1 mM ATP for 2 hr. The expression of CCL2 and TNF-α mRNA was detected by PCR and real-time qPCR. The PCR and qPCR results in Figure 12 revealed that 1 mM ATP led to a marked increase in the levels of CCL2 and TNF-α mRNA, indicating that 1 mM ATP induces mRNA expression, de novo synthesis and subsequent release of CCL2 and TNF-α.

![Image of PCR and qPCR results showing increase in CCL2 and TNF-α mRNA expression after 1 mM ATP stimulation.](image)

Figure 12: Effects of 1mM ATP on the mRNA transcription of CCL2 and TNF-α. CCL2 and TNF-α mRNA expression in microglia was detected by PCR (upper) and real-time qPCR (lower) after 1 mM ATP stimulation. The upper figure indicates that unstimulated primary microglia constitutively express CCL2 and TNF-α mRNA. The...
lower figure shows the results as a ratio of CCL2 and TNF-α mRNA to the reference genes actin-β and GAPDH. Data were normalized by control vehicle. Values represent the mean ± SEM of three independent experiments. Unpaired t-test, *** p < 0.001, ** p < 0.01 vs. vehicle. Volume of culture medium: 600 μL/well.
4.1.5 Dose-dependent effects of BzATP on microglial cytokine release

The dose-dependent curve of ATP (Figure 10) shows that 1 mM ATP evoked the release of CCL2 and TNF-α, whereas other concentrations of ATP had no tendencies to stimulate cytokine release. Therefore we supposed that the receptor mediates CCL2 and TNF-α release might be the P₂X₇ receptors. To determine the role of the P₂X₇ receptors in microglial cytokine regulation, we examined the effects of a non-hydrolysable, more potent P₂X₇ receptor agonist 3’-O-(4-benzoylebenzoyl)-adenosine 5’-triphosphate (BzATP) on cytokine release. It shows that BzATP dose-dependently increased the IL-6, IL-10, CCL2 and TNF-α release in primary microglia (Figure 12). However, the increase in IL-10 levels induced by BzATP was quite low. Compared with 1 mM ATP, BzATP induced a more pronounced cytokine production.

Figure 13: Dose-dependent effects of BzATP on cytokine production in primary microglia. Microglia were treated 24 hr by 100, 200 and 500 μM BzATP. BzATP at 500 μM stimulated a significant increase in IL-6, IL-10, CCL2, and TNF-α secretion. Values represent the mean ± SEM of four independent experiments. One-way ANOVA and Scheffe’s post hoc test, "p < 0.001 vs. vehicle. Volume of culture medium: 200 µL/well.
4.1.6 BzATP dose-dependently enhanced the levels of microglial cytokine mRNA

Microglia were treated 2 hr in the presence of vehicle or BzATP and the expression of cytokine mRNA was detected by real-time qPCR. Significant effects of BzATP on IL-6, CCL2, TNF-α release (Figure 13) and mRNA expression (Figure 14) were observed at the concentration of 500 µM.

![Figure 14: BzATP dose-dependently increased the levels of microglial cytokine mRNA. Microglia were stimulated with 200 and 500 µM BzATP for 2 hr. BzATP at 500 µM significantly enhanced the mRNA expression of IL-6, CCL2 and TNF-α, while 200 µM BzATP only increased the expression of TNF-α. Data were normalized by control vehicle. Values represent the mean ± SEM of three independent experiment. One-way ANOVA and Scheffe’s post hoc test, *** p < 0.001, ** p < 0.001, * p < 0.001 vs. vehicle. Volume of culture medium: 200 µL/well.](image-url)
4.1.7 Intracellular expression of cytokine in primary microglia

In order to confirm the ATP- or BzATP-stimulated cytokine production, microglia were incubated 4 hr in the presence or absence of ATP and BzATP. The intracellular expression of IL-6, CCL2 and TNF-α was detected by immunofluorescent (IF) staining. Bacterial endotoxin lipopolysaccharide (LPS) 100 ng/ml was utilized as a positive control for cytokine induction. To enhance the fluorescent intensity, cells were treated with 10 μg/ml brefeldin A (BFA) 30 min before the end of stimulation. BFA is a compound that blocks the release of proteins from the Golgi - trans Golgi network (TGN), and leads to the intracellular accumulation of proteins (Dinter and Berger 1998). After the stimulation, cells were washed by DPBS and fixed 1 hr by 4% paraformaldehyde (PFA). The subsequent immunocytochemical analysis showed that intracellular IL-6 (Figure 15.1B) and CCL2 (Figure 15.2B) staining in unstimulated microglia, whereas intracellular TNF-α was hardly detectable in unstimulated cells even in the presence of BFA. BzATP stimulation did not influence staining for IL-6 and CCL2 but induced the intracellular TNF-α staining (Figure 15.3D, arrows indicate the positive staining of TNF-α). Stimulation with LPS 100 ng/ml caused a remarkable increase of intracellular IL-6 and TNF-α (Figure 15.1E and 15.3E). Negative controls without anti-IL-6, anti-CCL2 and anti-TNF-α primary antibodies (Figure 15.1A, 15.2A, 15.3A) confirmed the specificity of the IL-6, CCL2, and TNF-α staining in cultured mouse primary microglia.
Figure 15.1: Intracellular IL-6 expression in primary microglia. A) No signals were detected in negative control group, in which only secondary antibody but no anti-IL-6 primary antibody was added to the cells. B) Unstimulated microglia constitutively express intracellular IL-6. C-D) The increase in intracellular IL-6 caused by 1mM ATP and 500 μM BzATP was not observed by IF-staining. E) LPS at 100 ng/mL stimulated a significant increase in intracellular IL-6. Scale bar: 20 μM.
Figure 15.2: Intracellular CCL2 expression in primary microglia. A) No signals were detected in negative control group, in which only secondary antibody but no anti-CCL2 primary antibody was added to the cells. B) Like IL-6, unstimulated microglia express intracellular CCL2. C-E) The increase in intracellular CCL2 caused by 1 mM ATP, 500 μM BzATP and 100 ng/mL LPS was not observed by IF-staining. Scale bar: 20 μM
Figure 15.3: Intracellular TNF-α expression in microglia. A) No signals were detected in negative control group, in which only secondary antibody but no anti-TNF-α primary antibody was added to the cells. B) The expression of intracellular TNF-α in unstimulated microglia was not observed by IF staining. C) The alteration in intracellular TNF-α caused by 1 mM ATP was not seen, however, D-E) BzATP at 500 μM and 100 ng/mL LPS enhanced the levels of intracellular TNF-α. Scale bar: 20 μM.
4.1.8 Non-selective P₂ antagonists inhibited BzATP-induced cytokine release

To determine whether the BzATP-induced cytokine release is mediated via P₂ receptor activation, the effects of non-selective P₂ antagonists PPADs and suramin on BzATP-evoked cytokine induction were tested. Cells were preincubated 1 hr in the presence or absence of PPADs or suramin at 1 mM, and then stimulated 24 hr with 500 µM BzATP. After the stimulation, culture supernatants were collected and the levels of cytokines were measured by CBA. It is found that 1mM PPADs and suramin almost completely repressed the release of cytokines induced by BzATP (Figure 16).

![Figure 16: Inhibitory effects of non-selective P₂ receptor antagonists on BzATP-induced cytokine release. One milimolar PPADs and suramin completely blocked the IL-6, CCL2 and TNF-α release induced by 500 µM BzATP. Values represent the mean ± SEM of three independent experiment. Two-way ANOVA and simple main effect, *** p < 0.001 vs. vehicle; ###,&&& p < 0.001 vs. 500 µM BzATP. Volume of culture medium: 200 µL/well.]
4.1.9 Effects of the selective P$_2$X$_7$ antagonists on BzATP-induced cytokine release

Effects of the selective P$_2$X$_7$ antagonists oxATP, BBG, and A438079 on cytokine induction triggered by BzATP were examined. Microglia were pre-incubated in the presence or absence of antagonists for 1 hr, and then stimulated 24 hr by 500 μM BzATP. After the stimulation, culture supernatants were collected and the levels of cytokines were measured by CBA. The irreversible P$_2$X$_7$ antagonist oxATP at 300 μM significantly inhibited the IL-6 and CCL2 release induced by BzATP. Intriguingly, 300 μM oxATP alone induced the release of TNF-α, although this induction is not statistically significant (Figure 17.1). Treatment of the non-competitive P$_2$X$_7$ antagonist BBG at 50 μM (Figure 17.2), and the competitive P$_2$X$_7$ antagonist A438079 at 10 μM (Figure 17.3A) led to a significant inhibition of IL-6 and CCL2 release. Surprisingly, the TNF-α release stimulated by BzATP was not suppressed by the selective P$_2$X$_7$ antagonists at the concentrations as mentioned. We increased the concentrations of A438079 from 10 to 50 μM and found that 50 μM A438079 significantly inhibited the IL-6, CCL2 and TNF-α release stimulated by BzATP (Figure 17.3B).

Figure 17.1: Effects of oxATP on BzATP-induced cytokine release. The BzATP-induced IL-6, CCL2 but not TNF-α release was significantly inhibited by 300 μM oxATP.
μM oxATP. Interestingly, oxATP per se increased the release of TNF-α. Values represent the mean ± SEM of three independent experiments. Two-way ANOVA and simple main effect, **p < 0.01, *p < 0.05 vs. vehicle; ## p < 0.01 vs. 500 μM BzATP. Volume of culture medium: 200 μL/well.

Figure 17.2: Effects of BBG on BzATP-induced cytokine release. The release of IL-6 and CCL2 induced by BzATP were significantly suppressed by 50 μM BBG. The levels of TNF-α were not altered by BBG. Values represent the mean ± SEM of three independent experiments. Two-way ANOVA and simple main effect, *** p < 0.001, ** p < 0.01 vs. vehicle; ### p < 0.001, ## p < 0.01 vs. 500 μM BzATP. Volume of culture medium: 200 μL/well.
Figure 17.3: Effects of A438079 A) 10 μM and B) 50 μM on BzATP-induced cytokine release. The production of IL-6, CCL2, was repressed by 10 μM A438079. However, A438079 at 50 μM significantly suppressed the IL-6, CCL2, and TNF-α secretion induced by 500 μM BzATP. Values represent the mean ± SEM of three independent experiments. Two-way ANOVA and simple main effect, *** p < 0.001 vs. vehicle; ### p < 0.001, ## p < 0.01 vs. 500 μM BzATP. Volume of culture medium: 200 μL/well.
4.1.10 Expression of the P₂X₇ receptor protein on wild-type (WT) and P₂X₇ receptor knock-out (KO) microglia

We examined the expression of P₂X₇ receptor protein on WT and P₂X₇⁻/⁻ microglia. Primary microglia harvested from WT and P₂X₇⁻/⁻ mixed glial cultures were seeded in 6-well plates, and the cell lysates were subjected to the western blot. It is shown that P₂X₇⁻/⁻ microglia do express truncated, but not functional P₂X₇ receptor protein versus WT microglia (Figure 18).

![Western Blot](image)

Figure 18: The P₂X₇ receptors expressed on WT and P₂X₇⁻/⁻ microglia. Expression of reference protein actin was also detected. Experiments were repeated three times.
4.1.11 Effects of ATP and BzATP on cytokine induction in $P_2X_7$ microglia

To further elucidate the role of the $P_2X_7$ receptors in ATP- and BzATP-induced cytokine production, we examined the effects of ATP and BzATP on the mRNA expression and the release of cytokines in $P_2X_7$ microglia. In $P_2X_7$ microglia, neither 1 mM ATP nor 500 μM BzATP induced the cytokine release and the mRNA expression (Figure 19.1 and 19.2). The levels of cytokine production induced by the positive control LPS in wild-type and $P_2X_7$ microglia are shown in Figure 19.3.

Figure 19.1: Cytokine release in $P_2X_7$ microglia. Cells were stimulated 24 hr with 1 mM ATP and 500 μM BzATP. The supernatants were collected and the release of cytokines was measured by CBA. It shows that the cytokine production induced by ATP and BzATP was abolished in $P_2X_7$ microglia. Value represent the mean ± SEM of 5 independent experiments. Volume of culture medium: 600 μL/well.
Figure 19.2: Expression of cytokine mRNA in P2X7−/− microglia. Cells were stimulated 2 hr with 1 mM ATP and 500 μM BzATP. The levels of cytokine mRNA were quantified by real-time qPCR. ATP and BzATP did not increase the levels of cytokine mRNA in P2X7−/− microglia. Data were normalized by control vehicle. Values represent the mean ± SEM of three independent experiments. Notice: the Cq values of the IL-6 expression in vehicle were not detectable. Volume of culture medium: 600 μL/well.
Figure 19.3: Effects of LPS on cytokine production in WT and P2X7−/− microglia. LPS at 100 ng/mL significantly stimulated cytokine induction in A) WT and B) P2X7−/− microglia. Values represent the mean ± SEM of three independent experiments. Unpaired t-test, *** p < 0.001, ** p < 0.01, * p < 0.05 vs. vehicle. Volume of culture medium: 600 μL/well.
4.1.12 Effects of oxATP on the cytokine production in P<sub>2</sub>X<sub>7</sub><sup>−/−</sup> microglia

To determine whether the TNF-α release induced by oxATP is mediated by the P<sub>2</sub>X<sub>7</sub> signaling, P<sub>2</sub>X<sub>7</sub><sup>−/−</sup> microglia were treated by 300 µM oxATP. We found that 300 µM oxATP per se significantly induced TNF-α, but not IL-6 or CCL2 release in P2X<sub>7</sub><sup>−/−</sup> microglia after 24 hr stimulation (Figure 20).

![Figure 20](image)

Figure 20: Effects of the irreversible P<sub>2</sub>X<sub>7</sub> antagonist oxATP 300 µM on cytokine release in P<sub>2</sub>X<sub>7</sub><sup>−/−</sup> microglia. The release of TNF-α was increased by oxATP 300 µM, whereas the levels of IL-6 and CCL2 were not changed. Values represent the mean ± SEM of five experiments. Unpaired t-test, *p < 0.05 vs. vehicle. Volume of culture medium: 600 µL/well.
4.1.13 Effects of ATP and BzATP in LPS-primed primary microglia

We further investigated the effects of ATP and BzATP on the cytokine production induced by LPS at 100 ng/mL. The cells were treated with vehicle, LPS and LPS combines ATP or BzATP for 24 hr. Culture supernatants were collected and the levels of cytokines were measured (Figure 21).

![Figure 21: Effects of ATP and BzATP on LPS-induced cytokine production. A) LPS at 100 ng/mL increased the release of IL-6, CCL2 and TNF-α. ATP at 1mM significantly suppressed the TNF-α secretion evoked by LPS. Values represent the mean ± SEM of three independent experiments. B) Compared with ATP, BzATP at 500 μM had tendencies to decrease the levels of TNF-α induced by LPS. Values represent the mean ± SEM of two independent experiments. One-way ANOVA and](image-url)
Scheffe’s post hoc test, *** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \) vs. vehicle; ## \( p < 0.01 \), # \( p < 0.05 \) vs. 100 ng/mL LPS. Volume of culture medium: 200 μL/well.
4.1.14 Pannexin-1 inhibitor CBX did not change BzATP-induced cytokine release

To determine whether the P₂X₇-mediated pannexin-1 (Panx-1) activation is involved in BzATP-evoked cytokine induction, we investigated the effects of Panx-1 inhibitor carbenoxolone (CBX) on BzATP-induced cytokine production. Cells were pre-incubated 1 hr in the presence or absence of 10 or 30 μM CBX, and then stimulated 24 hr with 500 μM BzATP. Figure 22 shows that CBX at 10 and 30 μM did not change the levels of cytokine release induced by BzATP.

![Figure 22: Effects of the Panx-1 inhibitor CBX on BzATP-induced cytokine release. CBX at 10 and 30 μM did not alter the BzATP-induced cytokine release. Values represent the mean ± SEM of one independent experiment. Two-way ANOVA and simple main effect, ***p<0.001, *p<0.05 vs. vehicle. Volume of culture medium: 600 μL/well.](image-url)
4.1.15 \( P_2Y \) receptor agonists UTP, UDP and 2-MeSATP did not increase the levels of cytokine production in microglia

Microglia were stimulated with the potent \( P_2X \) agonist 2-MeSATP, the potent \( P_2Y \) agonists UTP and UDP at 100 \( \mu \text{M} \). These compounds had no tendencies to enhance the levels of cytokine production 24 hr after application (Figure 23).

![Figure 23: Effects of UTP, UDP and 2-MeSATP on cytokine release. These \( P_2 \) agonists at 100 \( \mu \text{M} \) did not induce cytokine production in primary microglia after 24 hr treatment. Values represent the mean ± SEM of three independent experiments. Volume of culture medium: 600 \( \mu \text{L} \)/well.](image-url)
4.1.16 Involvement of the P2X4 receptors in P2X7-mediated cytokine production

Due to the disability of the selective P2X7 antagonists to inhibit the release of TNF-α, we supposed that the P2X4 and P2X7 receptors might form the heteromeric subunit in microglia thus alter the pharmacological properties of the P2X7 receptors. First, to exclude the involvement of the P2X4 receptors in microglial cytokine induction, microglia were treated 24 hr with control vehicle or low concentration of ATP (50 μM) in the presence or absence of 3 μM ivermectin, which is a positive allosteric modulator of the P2X4 receptors. Figure 24A shows that neither 50 μM ATP alone nor co-stimulation of 50 μM ATP and 3 μM ivermectin stimulated cytokine production in microglia. Next, we examined the effects of BzATP, P2X7 receptor antagonists BBG and A438079 in P2X4 receptor-deficient (P2X4−/−) microglia. Wild-type microglia were taken as control (data not shown). Cells were pre-treated 1 hr with 50 μM BBG or 10 μM A438079, and then stimulated 24 hr by 500 μM BzATP. Culture supernatants were collected and the levels of cytokines were detected by CBA. It shows that the effects of BzATP and the P2X7 antagonists on microglial cytokine production were not altered by knockout of the P2X4 receptors (Figure 24B). To confirm that the functional P2X4 receptor protein is knocked-out, and the expression of functional P2X7 receptor protein is normal on P2X4−/− microglia, the P2X7, P2X4 receptors and actin in wild type, P2X7+/− and P2X4−/− microglia was detected by western blot. Figure 24C shows that P2X7+/− microglia express functional P2X4 receptors, while P2X4−/− microglia express functional P2X7 receptors.
Figure 24: Role of the P2X4 receptors in microglial cytokine regulation. A) Effects of
50 μM ATP and 3 μM ivermectin on microglial cytokine release. Cells were treated 24 hr with vehicle or 50 μM ATP in the presence or absence of 3 μM ivermectin. The cytokine release was not altered by ATP alone or co-treatment of ATP and ivermectin. B) The production of cytokines in P2X<sub>4</sub>−/− microglia. The effects of BzATP and the P2X<sub>7</sub> antagonists on cytokine release P2X<sub>4</sub>−/− microglia were examined. The P2X<sub>4</sub> knockout did not influence the actions of BzATP and the selective P2X<sub>7</sub> antagonists in P2X<sub>4</sub>−/− microglia. C) The expression of functional P2X<sub>4</sub>, P2X<sub>7</sub> receptor proteins and actin of wild-type, P2X<sub>7</sub>−/− and P2X<sub>4</sub>−/− microglia were investigated by western blot. Values represent the mean ± SEM of two independent experiments. Two-way ANOVA and simple main effect, **p<0.01 vs. vehicle; #p<0.01 vs. 500 μM BzATP. Volume of culture medium: 600 μL/well.
4.2 Intracellular calcium changes evoked by ATP P₂ receptor activation in cultured primary mouse microglia

4.2.1 Calcium response induced by ATP, UTP and BzATP

To gain more knowledge about the pharmacology of the used P₂ receptors antagonists ATP, UTP and BzATP induced, intracellular Ca²⁺ signalling in microglia was investigated. The non-selective P₂ receptor agonist ATP stimulated a biphasic Ca²⁺ response consisting of an early (25 sec after stimulation) peak which was followed by a second more broader peak (35 sec later than the early peak) before Ca²⁺ levels returned to basal line (Figure 25A). As a P₂Y receptor agonist, UTP triggered a biphasic Ca²⁺ response consisting of a sharp rise to a peak value (25 sec after stimulation), followed by a rapid decline to an elevated plateau level (Figure 25B). By contrast, BzATP elicited a sustained increase (20 seconds after stimulation) in intracellular Ca²⁺ levels that reached the peak value 65 seconds after stimulation, and was maintained for more than 50 sec (Figure 25C).

To determine whether ATP, UTP and BzATP mobilized intracellular or extracellular sources of Ca²⁺ or both, further experiments were carried out in Ca²⁺-free buffer containing the Ca²⁺ chelator ethylene glyco-bis (2-aminoethylether) -N,N,N’,N’- tetraacetic acid (EGTA) 5 mM. In the absence of extracellular Ca²⁺, ATP triggered a monophasic (25 sec after stimulation), transient rise of intracellular Ca²⁺, (Figure 25D), thus the second peak seen in Ca²⁺ containing buffer was absent. UTP evoked a single, but later and attenuated peak (35 sec after stimulation) compared to that in Ca²⁺ containing buffer (Figure 25E). These data indicate that ATP- and UTP-dependent Ca²⁺ signals in microglia are due to Ca²⁺ release from intracellular stores and influx of extracellular Ca²⁺. In contrast, the BzATP response was almost completely blocked in Ca²⁺ free buffer, indicating that BzATP was only mobilizing the influx of extracellular Ca²⁺ (Figure 25F).
Figure 25: Intracellular free Ca\(^{2+}\) changes of primary microglia treated with ATP, UTP, and BzATP in the presence or absence of extracellular Ca\(^{2+}\). Microglia were plated onto 30 mm Ø coverslips and stimulated 130 sec after beginning the experiment with non-selective P\(_2\) receptor agonist ATP 1 mM, P\(_2\)Y receptor agonist UTP 100 μM, or potent P\(_2\)X\(_7\) receptor agonist BzATP 500 μM in Ringer-Bic buffer with or without Ca\(^{2+}\). The activation of P\(_2\)Y receptors is relied on PLC-dependent pathway that is responsible for Ca\(^{2+}\) release predominantly from intracellular stores, while P\(_2\)X receptors are responsible for Ca\(^{2+}\) influx from extracellular sources. Here it shows that BzATP is a selective agonist at the P\(_2\)X receptors and does not bind P\(_2\)Y receptors, while ATP and UTP evoked the Ca\(^{2+}\) response by activating both P\(_2\)X and P\(_2\)Y
receptors. The response was measured by an inverted microscope. The data represent the means of 9-11 single cells.
4.2.2 Effects of the non-selective P₂ antagonists on ATP- and BzATP-induced calcium response

To determine whether the non-selective P₂ antagonists affected ATP- or BzATP-induced Ca²⁺ response, the intracellular Ca²⁺ changes were measured in microglia pre-treated with PPADs. Ninety seconds after beginning the experiment, the cells were pre-treated by Ringer-Bic buffer with or without 500 µM PPADs, and then treated with ATP or BzATP in the presence or absence of 500 µM PPADs 50 sec later. The Ca²⁺ response stimulated by ATP was observed between 155-240 sec, while that induced by BzATP was observed between 160-240 sec. Triton X-100 was added 260 sec after beginning the experiment and used as an inducer for Ca²⁺ influx. Figure 26A-B show that both ATP-induced intracellular Ca²⁺ release and Ca²⁺ influx from extracellular sources were suppressed by PPADs. In addition, PPADs blocked the BzATP-elicited Ca²⁺ influx to near basal levels (Figure 26C-D).
Figure 26: Inhibitory effects of the non-selective P₂ antagonists PPADs on ATP- and BzATP-evoked Ca²⁺ response. Primary mouse microglia were plated onto 30 mm Ø coverslips and pre-treated 90 sec after beginning the experiment with or without the non-selective P₂ receptor antagonist PPADs at 500 μM in Ringer-Bic buffer with Ca²⁺. Both ATP- and BzATP-induced Ca²⁺ changes were inhibited by PPADs. The values of Δratio were determined by the ratio changes induced by ATP (155-240 sec) or BzATP (160-240 sec) in the presence or absence of antagonists to the mean of basal levels from 0-90 sec. The response was measured by using an inverted microscope. The data represent the means of 9-13 single cells. Unpaired t-test, *** p<0.001 vs. 1 mM ATP or 500 μM BzATP.
4.2.3 Effects of the selective P₂X₇ antagonists on ATP-and BzATP-induced calcium response

To examine whether the ATP- or BzATP-induced Ca²⁺ response are influenced by the selective P₂X₇ antagonists, the intracellular Ca²⁺ changes were measured in microglia pre-treated with or without 50 µM BBG or 10 µM A438079. Ninety seconds after beginning the experiment, the cells were pre-treated by Ringer-Bic buffer or the selective P₂X₇ antagonists, and then treated with ATP or BzATP in the presence or absence of the selective P₂X₇ antagonists 50 sec later. Interestingly, the Ca²⁺ response stimulated by BBG or A438079 itself was seen 30 sec after the application. As for the ATP-induced Ca²⁺ response, we determined that the intracellular Ca²⁺ increase caused by P₂Y receptor activation was between 155-179 sec, whereas the Ca²⁺ influx induced by P₂X receptors was between 180-240 sec. The Ca²⁺ changes evoked by BzATP was observed between 160-240 sec. BBG or A438079 significantly attenuated roughly 25 % of the ATP-triggered Ca²⁺ influx from extracellular sources (Figure 27A-D) and the Ca²⁺ influx caused by BzATP stimulation (Figure 27E-H, BBG ~ 30 % and A438079 ~ 60 % attenuation).
Figure 27: Inhibitory effects of the selective P$_2$X$_7$ antagonists on ATP and BzATP-evoked Ca$^{2+}$ response. A-C) Effects of BBG and A438079 on ATP-evoked intracellular free Ca$^{2+}$ changes in primary microglia. Cells were plated onto 30 mm Ø coverslips and pre-treated 90 sec after beginning the experiment by the selective
P₂Y₇ receptor antagonists BBG 50 μM and A438079 10 μM in Ringer-Bic buffer with Ca²⁺. BBG and A438079 led to a significant inhibition of the Ca²⁺ response stimulated by P₂X receptor activation (180-240 sec). D) The values of Δratio were determined by the ratio changes induced by ATP in the presence or absence of antagonists (180-240 sec) to the mean of basal levels from 0-90 sec. E-G) The Ca²⁺ influx induced by BzATP was significantly suppressed by BBG and A438079. It shows that BzATP is a selective agonist at the P₂X₇ receptor in primary microglia, and therefore the selective P₂X₇ receptor antagonists can repress the Ca²⁺ response evoked by BzATP. H) The values of Δratio were determined by the ratio changes induced by BzATP in the presence or absence of antagonists (160-240 sec) to the mean of basal levels from 0-90 sec. The response was measured by using an inverted microscope. The data represent the means of 7-9 single cells. Unpaired t-test, ***p<0.001, **p<0.01, *p<0.05 vs. 1 mM ATP or 500 μM BzATP.
4.3 Role of adenosine and adenosine P₁ receptors in P₂X₇-mediated cytokine production in cultured primary mouse microglia

4.3.1 Effects of endogenous adenosine in P₂X₇-mediated microglial cytokine production

To determine the possible involvement of endogenous adenosine in P₂X₇-mediated cytokine regulation, microglia were pre-incubated 1 hr in the presence or absence of 1 U/mL adenosine deaminase (ADA) to eliminate the endogenous adenosine, and followed by 24 hr of 1 mM ATP stimulation. The ADA was present in culture supernatants during the stimulation. Thereafter, culture supernatants were collected for CBA measurement. We found that ADA pre-treatment itself increased the TNF-α release, and enhanced the TNF-α increase stimulated by 1 mM ATP as well (Figure 28A). The release of CCL2 was not influenced by ADA. In addition, we also detected the effects of ADA pre-treatment on the expression of cytokines mRNA. Cells were pre-incubated 1hr in the presence or absence of ADA and then treated 2hr with vehicles or 1 mM ATP. It is found that pre-treatment of ADA elevated the TNF-α mRNA expression (Figure 28B). To determine action of the P₂X₇ receptors on cytokine induction, we utilized P₂X₇ receptor agonist 500 μM BzATP for cell stimulation. Pre-incubation of ADA also increased the TNF-α, but not CCL2 release triggered by 500 μM BzATP (Figure 28C).
Figure 28: Effects of adenosine deaminase (ADA) on ATP-or BzATP-induced cytokine production. A) Microglia were pre-incubated 1 hr in the presence or absence of 1 U/mL ADA to eliminate the endogenous adenosine in culture medium, follow by 24 hr treatment of control vehicle or 1 mM ATP. After the treatment, culture
supernatants were collected and detected by CBA. It is shown that ADA pre-treatment per se enhanced the TNF-α release, and the ATP-induced TNF-α secretion was also increased by ADA. B) For cytokine mRNA detection, cells were pre-incubated 1 hr in the presence or absence of 1 U/mL ADA, and then stimulated 2 hr by 1 mM ATP. Total RNA was extracted and the mRNA expression of cytokines was measured by real-time qPCR. The expression of TNF-α mRNA was increased by ADA pre-incubation. C) Effects of ADA on cytokine release elicited by BzATP. Microglia were pre-incubated 1 hr in the presence or absence of 1 U/mL ADA, and then treated by vehicle or 500 µM BzATP for 24 hr. The levels of BzATP-induced TNF-α release was significantly elevated by ADA. Values represent the mean ± SEM of three independent experiments. Two-way ANOVA and simple main effect, *** p<0.001, ** p<0.01, * p<0.05 vs. vehicle; ### p<0.001, ## p<0.01, # p<0.05 vs. 1 mM ATP or 500 µM BzATP. Volume of culture medium: 600 µL/well.
4.3.2 Involvement of adenosine P\textsubscript{1} receptor activation on P\textsubscript{2}X\textsubscript{7}-mediated microglial cytokine release

To investigate the role of the P\textsubscript{1} receptors in the cytokine release induced by P\textsubscript{2}X\textsubscript{7} receptor activation, microglia were co-stimulated 24 hr by the non-selective P\textsubscript{1} agonist 5'-N-ethylcarboxamido-adenosine (NECA) at 1 µM in the presence or absence of 1 mM ATP or 500 µM BzATP. One hour before the co-stimulation, cells were incubated with 1U/mL ADA to diminish the endogenous adenosine in culture medium. We found that 1 µM NECA mildly reduced the ATP-induced CCL2 and TNF-α release, but the changes of TNF-α release resulted from the NECA and ATP co-stimulation was not significant (Figure 29A). In contrast to ATP, the BzATP-induced TNF-α release was slightly, but significantly inhibited by co-stimulation, whereas the levels of CCL2 secretion were not altered (Figure 29B).
Figure 29: Effects of NECA on ATP and BzATP-induced cytokine release. A) Co-stimulation of ATP and non-selective P₁ receptor agonist NECA in microglia. Cells were pre-treated with 1 U/mL ADA for 1 hr, and then co-stimulated 24 hr with 1 mM ATP and 1 μM NECA. NECA decreased the CCL2 release induced by ATP. B) Effects of NECA on BzATP-induced cytokine release. Cells were pre-treated 1 hr with 1U/mL ADA and co-treated with 500 μM BzATP and 1μM NECA for 24 hr. The BzATP-induced TNF-α release was attenuated by NECA, but the release of CCL2 was not changed. Values represent the mean ± SEM of three independent experiments. Two-way ANOVA and simple main effect, *** p<0.001, ** p<0.01 vs. vehicle; # p<0.05 vs. 1 mM ATP or 500 μM BzATP. Volume of culture medium: 600 μL/well.
4.4 Effects of purines on cytokine production and calcium response in immortalized murine microglia BV2 and N9 cells

4.4.1 Expression of functional P₂X₇ receptor protein on BV2 and N9 cells

BV2 and N9 cells were seeded in 6-well plates and incubated overnight at 37°C. Cells were lysed and the concentrations of total protein in cell lysates were determined. Twenty microgram of total protein was subjected and the expression of P₂X₇ receptor protein was examined by western blot (Figure 30).

![Image of Western Blot]

Figure 30: The P₂X₇ receptor proteins expressed on BV2 and N9 microglia. BV2 and N9 cells express both functional and truncated P₂X₇ receptors. Experiments were repeated three times.
4.4.2 Purines did not stimulate the increase in cytokine mRNA in BV2 and N9 cells

To compare the effects of purines on cytokine production in mouse primary microglia and immortalized murine microglial cell lines, BV2 and N9 cells were stimulated by different concentrations of ATP and the P2X7 agonist BzATP. Cells were incubated 2 hr in the presence or absences of agonists, and the total RNA was extracted. The expression of IL-6, CCL2 and TNF-α mRNA was detected by real-time qPCR. The expression of IL-6 in untreated and agonist-treated BV2 and N9 cells was not detectable by real-time qPCR. Figure 31 shows that in BV2 and N9 cells, ATP and BzATP did not evoke any increase in the levels of cytokine mRNA, whereas LPS 100 ng/mL triggered the CCL2 and TNF-α expression.
Figure 31: Effects of ATP and BzATP on cytokine mRNA expression in A) BV2 and B) N9 cells. Cells were treated with different concentrations of ATP (100 μM, 500 μM, 1mM), BzATP at 500 μM, and LPS at 100 ng/mL. ATP at different concentrations and BzATP did not induce the mRNA expression of cytokines, but LPS increased the levels of cytokine mRNA in BV2 and N9 cells. Values represent the mean ± SEM of two independent experiments. Volume of culture medium: 600 μL/well.
4.4.3 Calcium response induced by 1 mM ATP and the effects of P₂X₇ receptor antagonists on 1 mM ATP-induced intracellular calcium changes in N9 cells

The effects of 1 mM ATP on intracellular Ca²⁺ changes in N9 microglia was investigated. Hundred thirty seconds after beginning the experiment, the cells were stimulated by 1 mM ATP. Like mouse primary micorglia, ATP at 1 mM stimulated a biphasic Ca²⁺ response. It consists of an early (23 sec after stimulation) peak which was followed by a second much weaker, but more broader peak (32 sec later than the early peak) before Ca²⁺ levels returned to basal line (Figure 32A). Triton X-100 1% was added 240 sec after beginning the experiment and used as an inducer for Ca²⁺ influx. Additionally, to examine whether the ATP-induced Ca²⁺ response is affected by selective P₂X₇ receptor antagonists, the intracellular Ca²⁺ changes were measured in N9 cells stimulated by 1 mM ATP in the presence or absence of BBG or A438079. For ATP-induced Ca²⁺ response, we determined that the intracellular Ca²⁺ increase caused by P₂Y receptor activation was between 145-159 sec, whereas the Ca²⁺ influx induced by P₂X receptors was between 160-200 sec. Figure 32B-D show that the P₂X₇ receptor antagonists did not inhibit the intracellular Ca²⁺ changes evoked by P₂X receptor.
Figure 32: Effects of the selective P2X7 antagonists on ATP-stimulated Ca2+ response in N9 cells. A-C) Effects of BBG and A438079 on ATP-evoked intracellular free Ca2+ changes in N9 microglia. Cells were plated onto 30 mm Ø coverslips and treated 130 sec after beginning the experiment by 1mM ATP in the presence or absence of the selective P2Y7 receptor antagonists BBG 50 μM and A438079 10 μM in Ringer-Bic buffer with Ca2+. BBG and A438079 did enhance the Ca2+ response evoked by P2X receptor activation (160-200 sec). D) The values of Δratio were determined by the ratio changes induced by ATP in the presence or absence of antagonists (160-200 sec) to the mean of basal level from 0-130 sec. The response was measured by using an inverted microscope. The data represent the means of 7-9 single cells. Unpaired t-test, ***p<0.001 vs. 1 mM ATP.
4.5 Role of purine nucleotides ATP and the P$_2$X$_7$ receptor in the production of cytokines in cultured mouse primary mixed glia and astroglia

4.5.1 Effects of ATP and BzATP on the cytokine mRNA expression in primary mixed glia

To compare with the results from pure microglia and purified primary astroglia, the mixed glia without clodronate-liposomes treatment were stimulated with ATP and BzATP for 3 hr. Total RNA was extracted and the transcription of cytokine genes was measured by real-time qPCR. The figures show the results as a ratio of CCL2 and TNF-α mRNA to the reference genes actin-β or GAPDH. It is found that ATP at 1 mM and BzATP at 200 μM significantly stimulated the gene expression of IL-6, CCL2, and TNF-α in primary mixed glia (Figure 33). These results show that the selection of reference genes did not influence the results of cytokine gene expression.
Figure 33: Effects of ATP and BzATP on the cytokine gene expression in primary mixed glia. Cells were treated with 1 mM ATP and 200 μM BzATP for 3 hr. The expression of cytokine and reference genes in mixed glia was examined by real-time qPCR. It shows that both ATP and BzATP increased the levels of IL-6, CCL2 and TNF-α mRNA. Data were normalized by control vehicle. Values represent the mean ± SEM of three independent experiment. Unpaird t-test, ***p<0.001, **p<0.01, *p<0.05 vs. vehicle. Volume of culture medium: 300 μL/well.
4.5.2 Elimination of microglia in cultured mouse primary mixed glia by clodronate-liposomes

To obtain purer astroglia from cultured primary mixed glia, cells were treated by clodronate-liposomes to eliminate the microglia. Mixed glia were trypsinized and replated into 24-well plates. Once the cells reached confluence, cells were treated 4 hr with 100-140 μg/ml clodronate-liposomes or vehicle (PBS-liposomes). After the treatment, cells were washed twice by warm PBS, and incubated 48 hr with DMEM containing 10% FCS at 37 °C. Afterwards, cells were lysed by GTC buffer and the total RNA was extracted. The expression of microglial marker CD11b and astroglial marker GFAP was investigated by PCR. Figure 34 shows that the expression of CD11b in clodronate-treated cells was almost completely vanished compared to that in vehicle-treated cells, while there is no obvious change in the expression of GFAP.

Figure 34: The expression of microglia marker CD11b and astroglial marker GFAP in the mixed glia treated with clodronate-liposomes or PBS-liposomes. To diminish the possible influence from co-cultured microglia, the mixed glia were treated 4 hr with clodronate-liposomes 100-140 μg/ml at 37°C. After the treatment, the cells were washed twice by DPBS. Forty-eight hours after the treatment, cells were utilized for further experiments. Number 1-4: mixed glial samples treated with
clodronate-liposomes. Number 5-8: mixed glial samples treated with PBS-liposomes. Number 9 in upper figure: cultured primary mouse microglia as positive control for CD11b expression. MM: mastermix for PCR reaction. CD11b: 219 bp, GFAP: 278 bp. Experiments were repeated three times.
4.5.3 Dose-dependent effects of ATP and BzATP on astroglial cytokine release and mRNA expression

The effects of ATP on astroglial cytokine release and mRNA expression were investigated. Purified primary astroglia were stimulated 24 hr with vehicle or different concentrations of ATP (500 μM and 1 mM). Culture supernatants were collected, and the levels of IL-6, IL-10, CCL2, IFN-γ, TNF-α and IL-12p70 were measured by CBA. On the other hand, cells were treated 3 hr with vehicle, 500 μM or 1 mM ATP, and the total RNA was extracted. The effects of ATP on the levels of cytokine mRNA were examined by real-time qPCR. We found that 1 mM ATP stimulated the most robust cytokine release and mRNA expression in primary astroglia (Figure 35A-B). Among the detected cytokines, only IL-6 and CCL2 production was significantly induced by 1 mM ATP in primary astroglia. Since ATP at 1 mM evoked the release of IL-6 and CCL2, we supposed that the receptor mediates IL-6 and CCL2 production may be the P2X7 receptors. To determine the role of the P2X7 receptors in astroglial cytokine regulation, we examined the effects of the potent P2X7 agonist BzATP on cytokine production. It shows that BzATP dose-dependently increased the levels of IL-6, CCL2 and TNF-α release in purified primary astroglia. BzATP at 200 μM significantly enhanced the mRNA expression of IL-6 and TNF-α (Figure 35C-D). However, compared with the effects of 200 μM BzATP on TNF-α secretion in microglia, the TNF-α increase induced by 200 μM BzATP was marginal.
Figure 35: Dose-dependent effects of ATP and BzATP on cytokine gene transcription and secretion in astroglia. A) The effects of ATP on astroglial cytokine release. After the clodronate-liposome treatment, purified astroglia were treated with 500 μM and 1 mM ATP for 24 hr. Culture supernatants were collected and the release of cytokine was detected by CBA. B) Cells were stimulated 3 hr with 500 μM and 1 mM ATP and the expression of cytokine and reference genes in purified astroglia was examined by real-time qPCR. It shows that ATP dose-dependently increased the levels of IL-6 and CCL2 release and mRNA. C) Effects of BzATP on astroglial cytokine release. Cells were stimulated 24 hr in the presence of vehicle or different concentrations of BzATP (50, 100 and 200 μM), and the supernatants were collected for CBA. BzATP dose-dependently increased the release of IL-6, CCL2 and TNF-α. D) Cells were stimulated 3 hr in the presence of vehicle or 200 μM BzATP, and the expression of cytokine and reference genes was measured. BzATP evoked a significant increase in IL-6 and TNF-α mRNA levels. Data were normalized by control vehicle. Values represent the mean ± SEM of three independent experiments. One-way ANOVA and Scheffe's post hoc test, ***p<0.001, **p<0.01, *p<0.05 vs. vehicle. Volume of culture medium: 300 μL/well.
4.5.4 Non-selective P$_2$ antagonists PPADs, suramin, and selective P$_2$X$_7$ antagonist BBG inhibited the cytokine release stimulated by 1 mM ATP in purified astroglia

To determine whether the 1 mM ATP-induced cytokine release is mediated via P$_2$ receptor activation, the effects of non-selective P$_2$ antagonists PPADs and suramin on BzATP-evoked cytokine induction were tested. Purified astroglia were pre-incubated 1 hr in the presence or absence of PPADs or suramin at 1 mM, and then stimulated 24 hr by 1 mM ATP. After the stimulation, culture supernatants were collected and the levels of cytokines were measured by CBA. It is found that 1mM PPADs significantly inhibited the release of IL-6 induced by ATP, while the production of IL-6 and CCL2 was significantly suppressed by PPADs and suramin at 1 mM (Figure 36A). The effects of the selective P$_2$X$_7$ antagonist BBG on the cytokine induction triggered by 1 mM ATP were also examined. Astroglia were pre-incubated in the presence or absence of 3 μM BBG for 1 hr, and stimulated 24 hr by 1 mM ATP. After the stimulation, culture supernatants were collected and the levels of cytokines were measured by CBA. Figure 36B shows that 3 μM BBG significantly inhibited the IL-6 and CCL2 secretion stimulated by 1 mM ATP.
Figure 36: Inhibitory effects of A) the non-selective P$_2$ antagonists PPAD, suramin, and B) the selective P$_2$X$_7$ antagonist BBG on ATP-induced cytokine release. One milimolar PPADs and suramin significantly blocked the CCL2 release induced by 1 mM ATP, while the release of IL-6 was repressed by 1 mM PPADs. BBG at 3 μM blocked the ATP-induced IL-6 and CCL2 production significantly. Values represent the mean ± SEM of three independent experiment. Two-way ANOVA, simple main effect, *** p<0.001, ** p<0.01, * p<0.05 vs. vehicle; ###,&&& p<0.001, # p<0.05 vs. 1 mM ATP. Volume of culture medium: 300 μL/well.
5. Discussion

Microglia

ATP is known as an important neuromodulator that regulates many aspects of CNS functions such as neurotransmission and cell-to-cell communication. Under physiological conditions, ATP is released from the cells at basal rates. ATP can be co-packaged with other neurotransmitters (e.g. acetylcholine, noradrenaline) and released from neurons (Sperlágh 1996). In non-neuron cells, ATP is mainly released from the membrane channels and the gap junction (Anselmi 2008, Bao 2004, Lohman 2012). ATP can elicit a series of actions by activating the P2 receptors distributed widely on CNS cells. The levels of extracellular ATP are normally maintained in low nanomolar range under physiological conditions. Once the cells are under pathological conditions such as hypoxia and injury, ATP can be powerfully released from damaged/dying cells, and it produces an extracellular milieu with high ATP levels (~ mM). The high extracellular ATP levels may stimulate the P2 receptors which have very low affinity for ATP. In the beginning of this study, we investigated the effects of different concentrations of extracellular ATP on the regulation of a panel of inflammatory cytokines (IL-6, IL-10, CCL2, IFN-γ, TNF-α, IL-12p70) in primary mouse microglia. It was found that in primary microglia high concentration of extracellular ATP (1 mM) potentially stimulated the release of pro-inflammatory cytokine TNF-α and chemokine CCL2. This increase in cytokine secretion resulted from an elevated cytokine gene transcription. The data obtained from ATP treatment suggest a role of the P2X7 receptors (EC50 = 300 - 400 μM for ATP) in cytokine synthesis and release in primary microglia. The P2X7 receptors are primarily expressed on the cells of haemopoietic origin, and ATP is the only known physiological ligand for the P2X7 receptors (Chakfe 2002). In the CNS, the functional P2X7 receptors are predominantly found on microglia. We demonstrated the
expression of the P<sub>2</sub>X<sub>7</sub> receptors on cultured primary mouse microglia. To examine the effects of the P<sub>2</sub>X<sub>7</sub> activation on cytokine production, BzATP, a 30-fold more potent agonist at the P<sub>2</sub>X<sub>7</sub> receptors (EC<sub>50</sub> = 18 μM) than ATP (Burnstock 2000, Donnelly-Roberts 2009, North 2002, Surprenant 1996) was used. We demonstrated that BzATP additionally evoked the production of IL-6, and had a more pronounced effect on TNF-α and CCL2 release in primary microglia. Three reasons may explain why BzATP induces more remarkable cytokine production than ATP: (1) the continual degradation of ATP to adenosine; (2) ATP may evoke the concomitant activation of other P<sub>2</sub> receptors (e.g. P<sub>2</sub>Y receptors), which exhibit inhibitory actions on P<sub>2</sub>X<sub>7</sub>-mediated cytokine production (Queiroz 2003); (3) because of the special amino acid residues in the P<sub>2</sub>X<sub>7</sub> receptors, P<sub>2</sub>X<sub>7</sub> receptors have differential sensitivity to ATP and BzATP. BzATP can elicit higher maximum currents than ATP (Young 2006). Among the cytokines provoked by BzATP, we found that BzATP stimulated a more robust induction of IL-6 and TNF-α release as well as mRNA transcription in comparison with CCL2 in primary microglia. Next, in order to further verify the role of P<sub>2</sub>X<sub>7</sub> receptors in microglial cytokine production, we examined the effects of the non-selective P<sub>2</sub> and the selective P<sub>2</sub>X<sub>7</sub> antagonists on BzATP-induced cytokine release. It was found that both PPADs (IC<sub>50</sub> ~ 1-50 mM, with the mouse receptor being the least sensitive) and suramin (IC<sub>50</sub> > 100 μM) (Burnstock 2012) at 1 mM completely suppressed the IL-6, CCL2 and TNF-α release evoked by BzATP, indicating that the BzATP effects are mediated by the P<sub>2</sub> receptors. Unexpectedly, the selective P<sub>2</sub>X<sub>7</sub> antagonists oxATP 300μM, BBG 50 μM (rat P<sub>2</sub>X<sub>7</sub> receptor IC<sub>50</sub> = 10 nM, human P<sub>2</sub>X<sub>7</sub> receptor IC<sub>50</sub> = 250 nM ) (Burnstock 2012), and A438079 10 μM (rat P<sub>2</sub>X<sub>7</sub> receptor IC<sub>50</sub> = 100 nM, human P<sub>2</sub>X<sub>7</sub> receptor IC<sub>50</sub> = 300 nM) (Xin 2011) did inhibit BzATP-induced secretion of IL-6, CCL2, but not TNF-α in primary microglia. To test whether we can see the inhibitory effects of the selective P<sub>2</sub>X<sub>7</sub>
antagonists on TNF-α release by increasing the dose of antagonists, we treated cells with A438079 at 50 μM instead of 10 μM. However, even increasing the concentrations of A438079 to 50 μM, its suppressive effects on BzATP-induced TNF-α secretion remain limited.

To directly address the role of the P₂X₇ receptors in cytokine release stimulated by ATP and BzATP, we utilized the primary microglia prepared from P₂X₇ receptor-deficient (P₂X₇⁻/⁻) mice. It was found that P₂X₇⁻/⁻ microglia responded to LPS 100 ng/mL and produced levels of IL-6, CCL2, and TNF-α comparable with those generated by wild-type cells. In contrast, in response to ATP or BzATP, the induction of IL-6, CCL2 and TNF-α release and gene transcription were abolished in P₂X₇⁻/⁻ microglia. These results show that P₂X₇-mediated signaling is necessary for ATP- and BzATP-induced cytokine production. Both pharmacological and knock-out studies clearly reveal that in primary mouse microglia the production of IL-6 and CCL2 induced by BzATP is mainly mediated by the P₂X₇ receptors. Because the selective P₂X₇ antagonists did not inhibit TNF-α secretion, the up-regulation of TNF-α levels may be modulated by more complex mechanisms which the P₂X₇ receptors are involved in. To explore more about BzATP-regulated TNF-α production, first we hypothesized that apart from stimulating the P₂X₇ receptors, BzATP might activate other P₂ receptors which induce the secretion of TNF-α. This hypothesis is supported by the evidence that BzATP can elicit its effects by binding to the P₂X₁ (EC₅₀ = 19 nM), P₂X₃ (EC₅₀ = 98 nM), and P₂Y₁₁ receptors (EC₅₀ = 8 μM) (Burnstock 2000, Tocris Bioscience, Biotrend, North 2002). To investigate this hypothesis, we treated the primary microglia with different agonists for the P₂ receptors. It was found that the potent P₂X agonist 2-MeSATP, potent P₂Y agonists UTP and UDP had no tendencies to enhance the production of cytokines after 24 hr stimulation. This result may suggest that activation of other P₂ receptors does not induce the release of
cytokines in microglia.

To further elucidate the action of BzATP on the P$_2$X$_7$ receptors, calcium microfluorometry is another approach undertaken in our study. The increase of intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_i$) is the most common signal associated with the activation of all P$_2$ receptors (Dubyak 1993). In regard to P$_2$Y receptors, the [Ca$^{2+}$]$_i$ increase relies on the PLC-dependent pathways which elicit the Ca$^{2+}$ release from intracellular stores. On the other hand, in P$_2$X receptors, Ca$^{2+}$ chiefly comes from the extracellular milieu, and enters the cells through ligand-gated ion channels according to the electrochemical gradient (Harden 1995, Nihei 2000, Vitiello 2012). On account of this rationale, calcium microfluorometry has been regarded as a useful tool for the functional and pharmacological studies of the P$_2$ receptors since the Ca$^{2+}$ response amplitude is correlated with the agonist potency and concentration.

Our findings show that in the presence of 1.3 mM extracellular Ca$^{2+}$, ATP at 1 mM elicited a biphasic Ca$^{2+}$ response in primary microglia. The Ca$^{2+}$ response consists of the first peak for Ca$^{2+}$ release from intracellular stores, followed by an elevated Ca$^{2+}$ influx from extracellular milieu. Like ATP, UTP at 100 μM also evoked a biphasic Ca$^{2+}$ response in microglia. This Ca$^{2+}$ response induced by UTP is composed of a sharp rise to a peak value which represents for Ca$^{2+}$ release from intracellular stores, followed by a rapid decline to an elevated plateau level which represents the influx of Ca$^{2+}$ from extracellular space. In Ca$^{2+}$-free condition, both ATP and UTP triggered only the peak representing for the Ca$^{2+}$ release from intracellular stores. Our data disclosed that the effects of ATP on the changes of [Ca$^{2+}$]$_i$ are mediated by both P$_2$X and P$_2$Y receptors. Although UTP is always utilized as an agonist to stimulate P$_2$Y receptors which activation leads to the Ca$^{2+}$ release from intracellular stores, our findings indicate that UTP stimulated not only Ca$^{2+}$ release from intracellular stores but also Ca$^{2+}$ influx from extracellular sources. This suggests that like ATP, UTP
acts on both P₂X and P₂Y receptors.

In contrast, BzATP evoked a sustained Ca\(^{2+}\) influx from extracellular sources in the presence of extracellular Ca\(^{2+}\), whereas its effects on the changes of [Ca\(^{2+}\)], were almost disrupted in Ca\(^{2+}\)-free condition. This result suggests that BzATP only exerts its effects on P₂X receptors. This suggestion is supported by the study of Witting and colleagues. They verified that in primary mouse microglia, ATP at 1 mM stimulated a biphasic calcium response by activating the P₂X and P₂Y receptors, while BzATP only stimulates the P₂X receptors to induce a sustained Ca\(^{2+}\) influx from extracellular space (Witting 2004).

It has been proven that in primary rat microglia BzATP elicits a sustained Ca\(^{2+}\) influx by activating the P₂X\(_7\) receptors (Parvathenani 2003). In our study we also examined the effects of the non-selective P₂ antagonist PPADs, the selective P₂X\(_7\) antagonists BBG and A438079 on ATP or BzATP-triggered Ca\(^{2+}\) response. We found that PPADs inhibited both intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) influx stimulated by 1mM ATP. The ATP-evoked Ca\(^{2+}\) influx from extracellular sources via P₂X receptor activation was suppressed by the selective P₂X\(_7\) antagonists. In addition, the BzATP-evoked Ca\(^{2+}\) influx was completely inhibited by PPADs. BBG and A438079 also significantly suppressed the Ca\(^{2+}\) response evoked by BzATP. It was observed that A438079, a selective, competitive P₂X\(_7\) antagonist that is devoid of activity at other P₂ receptors (IC\(_{50}\) >> 10 μM) (Donnelly-Roberts 2007), had a stronger inhibitory effect than the selective, non-competitive P₂X\(_7\) antagonist BBG on BzATP-induced intracellular Ca\(^{2+}\) changes. Here we clearly demonstrated that in primary mouse microglia, BzATP stimulated the Ca\(^{2+}\) response via activation of the P₂X\(_7\) receptors and furthermore, both BBG and A438079 are real antagonists on P₂X\(_7\) receptors. Therefore, the data obtained from calcium microfluorometry did not support our hypothesis that BzATP might stimulate other P₂ receptors which induce
the release of TNF-α.

Next, we hypothesized the possible involvement of receptor heteromerization in BzATP-induced cytokine release. The seven subtypes of P2X receptors have been identified that they can assemble as either homo- or heterotrimeric receptors (Barrera 2007, Lewis 1995), and both their functional and pharmacological properties can be altered by heteromerization. For instance, Compan and colleagues have indicated that the P2X2 and P2X5 receptors interact to form heteromeric receptors, which possess the P2X7 receptor-like properties (Compan 2012). Recent study by Guo and colleagues revealed that the P2X7 receptors can form functional heterometric subunits with another subtype of ionotropic P2X receptor, the P2X4 receptors (Guo 2007). Accordingly, it is reasonable to hypothesize that the P2X4/P2X7 heteromeric subunits might change the pharmacological properties of the P2X7 receptors, therefore the BzATP-induced TNF-α release cannot be inhibited by the selective P2X7 antagonists. The formation of functional P2X4/P2X7 heteromeric subunits could provide a critical mechanism for the modulation of P2X7 receptor signaling.

It has been reported that the P2X4 receptors are involved not only in P2X7-mediated cell death, but also multiple mechanisms of P2X7-mediated inflammation (Kawano 2012 (a) and (b)). In the stably P2X4-knockdowned RAW264.7 macrophages, the ATP- or BzATP-induced cell death and cytokine IL-1β production were repressed. These studies implicate that the P2X4 receptors may be associated with the P2X7-mediated signaling; co-expression of the P2X4 and P2X7 receptors may play an important role in regulating inflammatory responses associated with a variety of immunological and pathophysiological processes. According to these studies, we supposed that the P2X4 receptors might participate in the TNF-α release mediated by P2X7 receptor activation. To explicate the role of the P2X4 receptors in P2X7-mediated cytokine secretion, first we investigated whether P2X4 receptor
activation alone induces cytokine production in primary microglia. We stimulated the primary microglia with ATP at low concentration (50 μM, EC50 of ATP at P2X4 receptor: < 10 μM) (Townsend-Nicholson 1999), or incorporating with the selective P2X4 allosteric potentiator ivermectin at 3 μM (EC50 = 250 nM) (Faria 2012). It is reported that in patch clamp recording, ivermectin can enhance the P2X4 currents by stabilizing the agonist-induced open state and isolate the P2X4 currents from other P2 receptor currents (Bernier 2008). We found that neither ATP nor co-treatment of ATP with ivermectin stimulated IL-6, CCL2 and TNF-α production. It indicates that activation of the P2X4 receptors itself does not evoke the release of IL-6, CCL2 and TNF-α in primary microglia. Moreover, to investigate whether P2X4 receptor deficiency (P2X4−/−) changes P2X7-mediated cytokine induction, we examined the pharmacological action of BzATP, the selective P2X7 antagonists BBG and A438079 on cytokine production in P2X4−/− microglia. P2X4−/− microglia are verified to have no expression of functional P2X4 receptors, while the expression of functional P2X7 receptors is still present. It means that in the absence of P2X4 receptors, the potential changes in P2X7 pharmacological properties caused by the P2X4/P2X7 heteromeric subunits can be excluded, and the BzATP-induced TNF-α production should be blocked by the selective P2X7 antagonists in P2X4−/− microglia. Our data show that the effects of BzATP on inducing cytokines in P2X4−/− microglia were comparable to those in wild-type microglia. Moreover, in P2X4−/− microglia, like in wild-type cells, BBG and A438079 did not elicit significant suppressive effects on BzATP-evoked TNF-α secretion. These results suggest that the P2X4 receptors did not influence the effects of BzATP and the selective P2X7 antagonists on cytokine production in primary microglia.

Activation of the P2X7 receptors not only opens a typical ion channel which is selective for small cations such as Na+, K+, Ca2+, but also leads to the gradual opening
of a membrane pore permeable to molecules up to 900 Da. The pore-forming property of the P2X7 receptors is usually examined by the uptake of fluorescent dyes such as ethidium and Yo-Pro 1 (Nihei 2000, North 2002). Conventionally, it had been thought that the ion channel itself dilated to allow dye uptake. However, recent studies have concluded that pannexin-1 (panx-1), a protein that functions as a hemichannel, serves as the pore opened after the P2X7 activation and mediates the dye-uptake pathway. It is demonstrated that the increased K+ efflux induced by panx-1 activation through binding of ATP to the P2X7 receptors is a key component required for the processing of caspase-1 and release of mature IL-1β and IL-18. Inhibition of panx-1 abolished ATP-induced activation of caspase-1 and release of mature IL-1β and IL-18 (Pelegrin 2006, 2008 and 2009). In addition, panx-1 signaling is necessary for P2X7-mediated inflammasome activation (Silverman 2009, Zambetti 2012). Due to the essential role of panx-1 in the secretion of IL-1 cytokines, we hypothesized that the P2X7-mediated activation of panx-1 channel might be involved in the secretion of TNF-α induced by BzATP. To investigate this hypothesis, we stimulated the microglia with BzATP in the presence or absence of the panx-1 blocker, carbenoxolone (CBX). The data reveal that blockade of panx-1 channel did not change the levels of BzATP-induced cytokine release. This indicates that, unlike IL-1β and IL-18, panx-1 signaling may not involve in the secretion of IL-6, CCL2, and TNF-α elicited by BzATP-induced P2X7 receptor activation.

Taken together, the data collected from pharmacological, knock-out and calcium microfluorometry indicate that the P2X7 receptors are the primary receptor activated by BzATP in primary mouse micorglia. Additionally, we found that the P2X4 receptors and the panx-1 channel did not participate in P2X7-dependent IL-6, CCL2, and TNF-α secretion.

This is the first study that discloses the actions of the P2X7 receptors on the
synthesis and secretion of inflammatory cytokines in cultured primary mouse microglia. CBA is a flow cytometry application that allows users to quantify multiple proteins simultaneously. This method has higher sensitivity to detect cytokine concentrations than conventional ELISA. For instance, we have compared the levels of TNF-α in culture supernatants collected microglia treated with control vehicle, 500 μM BzATP and 100 ng/mL LPS by performing a spike recovery assay using ELISA following the CBA analysis. Surprisingly, we found that the significant increase of TNF-α secretion induced by BzATP was not observed in ELISA. In sample treated with LPS, the levels of TNF-α detected by ELISA were lower than those detected by CBA. This result reveals that there is a difference between ELISA and CBA in detection sensitivity. Because TNF-α secretion induced by ATP is not as remarkable as that induced by BzATP and is at low pg/mL levels (< 200 pg/mL, Figure 11), it may be more difficult to detect the ATP-induced TNF-α release by ELISA. In fact, there are inconsistent results of microglial TNF-α production measured by ELISA after ATP treatment. Su et al. have reported that extracellular ATP increased the release of TNF-α in rat microglia (Su 2010). The study of Hide et al. have also proven that stimulation of ATP at 1 mM for 3 hr enhanced the levels of TNF-α secretion (Hide 2002). In contrast, McIlvain and colleagues indicated that they did not detect the TNF-α increase induced by 1 mM ATP in rat microglia after 3 hr stimulation (McIlvain 2010). Based on these studies and our findings, we suggest that the detection limit of the methods chosen for cytokine measurement after stimulation may influence the final outcome of study.

Neuroinflammation has been suggested to be involved in the pathogenesis of many neurological disorders. Activation of microglia is generally observed in these pathological events. Once activated, microglia undergo dramatic changes in their morphology, initiating the phagocytosis, and secrete a variety of inflammatory
cytokines such as IL-1β, IL-6, and TNF-α. This increase in cytokines is thought to contribute to the secondary brain injury by inducing neuronal death and amplifying the disease state (Kerschensteiner 2009). Thus, controlling the production of microglial cytokines during neuroinflammation may prevent the aggravation of the diseases. We demonstrated that activation of the P₂X₇ receptors triggers the production of pro-inflammatory cytokines IL-6, TNF-α, and chemokine CCL2 from primary mouse microglia. These cytokines can be up-regulated in CNS injury and neurodegenerative diseases and individually exert differential, but essential actions. Under pathological conditions in which cytokine levels increase (e.g. cerebral ischemia, AD), the levels of extracellular ATP and the P₂X₇ expression on microglia also rise. Immediately after the cerebral ischemia, there is a dramatic decrease in the cerebral blood flow in the infarct core. This decrease in the cerebral blood flow leads to imbalance in cellular oxygen consumption and ionic environment (Bai 2013). These events can cause the death of neurons and non-neuron cells, provoking a strong release of ATP into the extracellular space, and eventually lead to activation of the P₂X₇ receptors. It has been shown that inhibition of the P₂X₇ receptors reduced the cytokine levels and brain damage after the ischemia (Chu 2012). As for the AD, the study of Sanz et al. has demonstrated that the Aβ plaques stimulated the release of ATP from microglia; ATP released from microglia induced an increase in [Ca²⁺]ₙ and production of IL-1β by activating the P₂X₇ receptors in microglia (Sanz 2009). They also found that intra-hippocampal Aβ administration results in a large accumulation of IL-1β in wild-type mice, but not in P2X7⁻ mice. In this study, Aβ activates an autocrine/paracrine stimulatory loop which the P₂X₇ receptors are obligate. Furthermore, the IL-1β secreted in response to inflammation was reported to enhance the expression of APP and Aβ (Goldgaber 1989, Dash 1995). This implicates that the P₂X₇-mediated cytokine production may contribute to the augmentation of disease
conditions. The evidence listed above suggests that at least by potentiating the production of microglial cytokines, the P₂X₇ activation plays a pivotal role in neurological disorders; antagonism or deficiency of the P₂X₇ receptors can reduce cytokine levels and ameliorate the disease conditions. In neuropsychiatric disorder MDD, the role of the P₂X₇ receptors remains not understood. It is found that the P₂X₇ genotype can influence the susceptibility of the MDD. In addition, a sustained elevation in blood levels of cytokines is observed in MDD patients. According to our findings, as well as the actions of the P₂X₇ activation in neurological diseases, we supposed that the P₂X₇ receptors are involved in the MDD as a result of its role in regulating cytokine production.

Among the cytokines secreted from microglia, TNF-α is considered as a master regulator of the immune responses. It serves as a central initiator of immune-mediated inflammation. In the CNS, microglia are the sole source of TNF-α in response to inflammatory insults (Figiel 2007, Welser 2012). In addition to enhance the expression of cell adhesion molecules in endothelial cells and astrocytes, TNF-α can drive the infiltration of leukocytes into the brain and elicits destructive effects on injured sites (Altmann 2008, Merrill 1996). The detrimental actions of TNF-α over-production in neuroinflammation has also been verified by the evidence that blockade of TNF-α can improve the conditions of CNS injury and neurodegenerative diseases (Brambilla 2011, Shohami 1997). These findings make microglial TNF-α an attractive target for the treatment of neurological disorders (McCoy 2008, Tweedie 2007).

Additionally, enhanced TNF-α levels are also noted in the patients with MDD. TNF-α appears to be unique in its role in the CNS, as other cytokines do not similarly regulate the excitatory synaptic scaling (Steinmetz 2010, Stellwagen 2006). The evidence has revealed that synaptic plasticity mechanisms contribute to the MDD
(Marsden 2012) and the efficacy of antidepressants (Baudry 2011, Djordjevic 2012). Thus, TNF-α dysregulation may cause synaptic dysfunction and lead to the MDD. Kaster and colleagues have demonstrated that the intracerebroventricular administration of TNF-α resulted in depressive behavior (Kaster 2012). Conversely, chronic administration of TNF-α inhibitor Infliximab ameliorated depression and anxiety-like behavior generated in chronic mild stress (Karson 2012). Lastly, it is reported that reduced neurogenesis in hippocampal dentate gyrus may be involved in MDD (Kempermann 2002). TNF-α is known to reduce hippocampal neurogenesis (Iosif 2006). These suggest that maintaining moderate TNF-α levels may help alleviate MDD. Overall, since activation of the P2X7 receptors is demonstrated to stimulate microglial TNF-α production, inhibition of the P2X7 activity may prevent the over-production of TNF-α and display neuroprotective effects in neurological and neuropsychiatric disorders.

However, it is surprising for us to find that the selective P2X7 antagonists oxATP, BBG and A438079 blocked the P2X7-dependent production of IL-6, CCL2, but not TNF-α in microglia. Although it has been reported that these three antagonists can significantly block the P2X7-mediated TNF-α release in microglia (Chu 2012, Murasaki 2013, Suzuki 2004), we did not see their inhibitory effects on TNF-α secretion in our experiments. Among these antagonists, oxATP was found to induce the secretion of TNF-α via the P2X7-independent pathways. Curiously, it still exhibited inhibitory actions on P2X7-mediated IL-6 and CCL2 secretion. Although it has been demonstrated that pre-incubation of oxATP in mouse macrophages caused a complete and irreversible antagonism of the P2X7 receptors (Murgia 1993), the converse study indicated that oxATP can attenuate pro-inflammatory signaling by mechanisms independent of the P2X7 receptors (Beigi 2003, Sikora 1999). Hide and colleagues have also reported that oxATP alone induced the TNF-α release in rat
microglia (Hide 2000). They assumed that it might be due to the degranulation of oxATP. These results made it difficult to investigate the effects of oxATP on P2X7-mediated TNF-α release. Therefore, we did not examine the actions of oxATP on P2X7-mediated Ca\(^{2+}\) influx in calcium microfluorometry. Nevertheless, it has been proven that BBG and A438079 indeed blocked the P2X7-mediated Ca\(^{2+}\) influx. On the basis of these findings, it remains difficult for us to explain the mechanisms underlying the unexpected effects of the selective P2X7 antagonists on P2X7-dependent TNF-α release.

Although TNF-α is generally considered to be cytotoxic and involved in the pathogenesis of CNS diseases, it is suggested that TNF-α can exert neuroprotective actions as well. It is possible that the dual effects of TNF-α relies on the time course and expression levels after the injury. It is reported that TNF-α, at acutely high levels, possesses the capacity to enhance injury associated with neurological diseases, whereas the effect of TNF-α at low levels is nutritive over time (Kraig 2010). Chertoff and colleagues have demonstrated that in the 6-OHDA mouse model of PD, constitutive, chronic low levels of TNF-α reduced neurodegeneration of the dopaminergic neurons in substantia nigra, as well as exhibited protective effects on the striatal dopaminergic terminals. Conversely, chronic high expression of TNF-α in substantia nigra caused progressive neurodegeneration in nigrostriatal dopaminergic system. (Chertoff 2011). Notably, compared with the strong TNF-α induction caused by LPS stimulation (roughly > 10 ng/ml), we found that the extent of TNF-α release stimulated by ATP is fairly small (TNF-α ~ 2.6 fold to unstimulated cells) in primary microglia. The neuroprotective effects of ATP-induced microglial TNF-α was once suggested and demonstrated by Suzuki et al. They indicated that P2X7-provoked microglia protect neurons from glutamate toxicity by releasing TNF-α (Suzuki 2004). The presence of two distinct TNF-α receptors may also explain the neurotoxic and
neuroprotective actions of TNF-α. Recent study from Lambertsen and colleagues have identified a neuroprotective role of microglia-derived TNF-α by using TNF-α and two TNF-α receptors, TNFR1 and TNFR2 deficient (KO) mice. They reported that in ischemic injury, there was a significant increase of TNF-α synthesis in the ischemic hemisphere. They also indicated that the deficiency in TNF-α, or TNFR1 exacerbated ischemic injury. It suggests that microglia-derived TNF-α is neuroprotective in conjunction with the action of TNFR1 (Lambertsen 2009). Similar results were also found by Devin et al. (Devin 1998). In addition, the transcription factor NFκB is implicated as a key molecule to decide death or survival of the cells in response to TNF-α (van Antwerp 1998).

Several lines of evidence in our study support that the production of microglial IL-6 after BzATP stimulation relies on the P2X7 signaling, but the data from Shigemoto-Mogami and colleagues revealed a P2X7-independent pathway for IL-6 production after ATP treatment in microglia (Shigemoto-Mogami 2001). They found that ATP at 1 mM enhanced the levels of IL-6 in MG-5 microglia, but BBG did not inhibit the ATP-induced IL-6 release. The P2X7 agonist BzATP did not elicit the production of IL-6. However, the ATP-induced IL-6 production was inhibited by a Ca2+-dependent protein kinase C (PKC) inhibitor. Therefore, they suggested a potential role of pertussis toxin (PTX)-insensitive, PLC-linked P2Y receptors in ATP-mediated IL-6 production in MG-5 microglia. Although MG-5 is a microglial cell line which is reported to preserve the morphological, biological, and physiological properties of primary microglia (Ohsawa 1997), they might not exactly exhibit the same receptor expression pattern and functional profile as primary microglia.

In fixed, unstimulated microglia, endogenous IL-6, CCL2, but not TNF-α can be immunostained and clearly observed in intracellular space. We found that in the absence of exogenous stimuli, microglia constitutively secreted basal levels of CCL2
and TNF-α into culture supernatants (CCL2 ~ 500-1000 pg/ml, TNF-α ~ 30-50 pg/ml), whereas the basal release of IL-6 was not detectable. In CNS, in addition to serve as a chemokine that exerts its chemotactic functions, the constitutive release of CCL2 may play a leading role in the early development of the ventral midbrain. CCL2 has been demonstrated to direct the differentiation of neural precursors towards the dopaminergic phenotype, and promote the neuritogenesis of dopaminergic neurons (Edman 2008). CCL2 is also one of the crucial factors which maintain the permeability of BBB by regulating the tight junction proteins in endothelial cells (Stamatovic 2005). As mentioned earlier, studies have suggested that the continual, low levels of TNF-α release from glial cells is essential in the regulation of synaptic scaling and the maintenance of synaptic strength.

We demonstrated that ATP and BzATP per se can act as full stimuli to elicit gene transcription and the subsequent release of IL-6, CCL2 and TNF-α from primary mouse microglia by activating the P2X7 receptors. Several studies have also documented that ATP or BzATP alone stimulates the release of pro-inflammatory cytokines TNF-α or chemokines CCL3, CXCL2 in primary rat microglia, as well as in immortalized murine microglial cell lines (Hide 2000, Kataoka 2009, Shiratori 2010). These findings consolidate the concept that the elevated extracellular ATP can serve as an essential signal to initiate the immune reactions such as cytokine release in the presence of tissue damage, and the P2X7 receptors can independently serve as a specific detector of large increase in extracellular ATP due to the requirement of high concentration ATP for its activation (Trautmann 2009, Vitiello 2012).

Several findings have indicated that ATP and BzATP exhibit their effects on cytokine release only in LPS-primed microglia (Friedle 2011, Lambert 2010). In this study, we also examined the effects of ATP and BzATP in LPS-primed primary microglia in order to make a comparison with the studies which primed their cells
with LPS before stimulating with ATP or BzATP. We found that 1 mM ATP significantly attenuated the TNF-α secretion evoked by LPS, whereas the release of IL-6 and CCL2 was not altered. Because the inhibitory effects of adenosine on LPS-induced TNF-α release have been previously demonstrated (Prabhakar 1995, Wagner 1998), the suppressive action of ATP on LPS-induced TNF-α release may be mediated through the adenosine degraded from ATP. However, non-hydrolysable BzATP also had tendencies to decrease the levels of LPS-induced TNF-α, but not IL-6 and CCL2. These implicate that the P2X7 stimulation reduced LPS-evoked TNF-α release in microglia. It is interesting to find that activation of the P2X7 receptors elicited diverse actions on the production of IL-6, CCL2 and TNF-α in LPS-primed microglia. Lambert et al. also indicated that activation of the P2X7 receptors down-regulated the secretion of TNF-α in human microglia primed by LPS (Lambert 2010). Nevertheless, the contradictory results reported by Friedle et. al. showed that activation of the P2X7 receptors raised the TNF-α mRNA transcription induced by LPS in microglial cell line N9 cells (Friedle 2011). Because of these controversial data, the effects of P2X7 receptor activation on LPS-evoked TNF-α production in microglia needs further elucidation.

The P2X7 receptors are also demonstrated to be expressed on the inflammatory cells such as mast cells, monocytes and macrophages (Ward 2010, Wareham 2009, Wewers 2009). In mast cells, the activation of the P2X7 receptors by ATP is verified to elicit the gene transcription of inflammatory cytokines IL-6, TNF-α, oncostatin M (OSM), and chemokines CCL2, CCL7, CXCL2 (Kurashima 2012). In monocytes and macrophages, it is difficult to interpret the role of P2X7 receptor activation in cytokine regulation because there are few reports in the literature indicating the effects of P2X7 receptor activation on inflammatory cytokine production in cells that have not been previously primed with LPS (Fujimoto 2012, Le Feuvre 2001, Perregaux 2000).
In this study, we did not determine the downstream signal transduction pathways and transcription factors responsible for the cytokine production induced by P$_2$X$_7$ receptor activation, but there is a wealth of data available suggesting the potential candidates. ATP- or BzATP-evoked P$_2$X$_7$ receptor activation has been previously been demonstrated to be dependent on extracellular Ca$^{2+}$, and induce the regulated secretion of cytokines by activation of mitogen-activated protein (MAP) kinases i.e. extracellular signal receptor-activated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Chu 2010, Hide 2000, Kataoka 2009, Panenka 2001, Suzuki 2004). Therefore, it can be supposed here that ATP- and BzTP-induced IL-6, CCL2 and TNF-α via P$_2$X$_7$ receptor activation in primary mouse microglia may be also mediated by ERK, JNK, and p38 activation. In addition, several transcription factors such as nuclear factor κB (NFκB) (O’Neill 1997), activating protein-1 (AP-1) (Herdegen 2001), signal transducers and activator of transcription (STAT) (Campbell 2005), peroxisome proliferator-activated receptor γ (PPARγ) (Kielian 2003), CCAAT/enhancer-binding protein β (C/EBPβ) (Ejarque-Ortiz 2007) have been demonstrated to regulate the production of cytokines in microglia. It is reported that P$_2$X$_7$ receptor activation can activate the nuclear factor of activated T cells (NFAT) inducing the production of chemokines CCL3 and CXCL2 in microglial cells (Ferrari 1999 (a), Kataoka 2009, Shiratori 2010). Therefore, the role of NFAT in P$_2$X$_7$-mediated IL-6, CCL2 and TNF-α production is worthy of being further investigated.

The ATP-dependent molecular environment is more complex than we imagine. In pathological situations that lead to large increases in extracellular ATP also lead to the formation of adenosine from ATP degradation (Fredholm 2007). Several studies have shown that adenosine can serve as an immunosuppressor that limits tissue damage during inflammation (Boison 2007, Cunha 2005, Heijne 2000, Lubitz 1994,
Additionally, adenosine can also regulate the cytokine releasing activity by activating the adenosine P₁ receptors. For instance, the suppressive action of A₂₅ receptor on TNF-α production has been verified (Alam 2009, Buenestado 2010, Fotheringham 2004, Sun 2008, Wagner 1998). In primary astroglia, activation of the A₃ receptors induced the release of CCL2 (Wittendorp 2004), whereas A₂B receptor activation evoked the synthesis and secretion of LIF (Moidunny 2012). According to these studies, we supposed that adenosine and the P₁ receptors may participate in the regulation of cytokines in primary mouse microglia. Unstimulated primary mouse microglia are demonstrated to express A₁, A₂B and A₃ receptor mRNA. Once activated, the expression of A₂₅ receptor increases (Wittendorp 2004). To examine the potential effects of endogenous adenosine on P₂X₇-mediated microglial cytokine release, one hour before P₂X₇ stimulation we preincubated the cells with vehicle or 1 U/mL adenosine deaminase (ADA), an enzyme which deaminates adenosine to its metabolite inosine (Figure 1). ADA can eliminate the adenosine present in cultured medium, and thus we can exclude the influence of endogenous adenosine on cytokine production. Figure 28A-B show that ADA pretreatment alone increased the levels of TNF-α release (~2.4 fold to vehicle) and mRNA expression (~3.7 fold to vehicle). The 1 mM ATP-induced TNF-α release and gene transcription were also significantly enhanced by ADA pretreatment (releasae: ~2.1 fold and mRNA expression: ~2.9 fold to ATP-stimulated cells). In contrast to TNF-α, the production of CCL2 were not altered by ADA preincubation. To determine the effects of the P₂X₇ receptors on cytokine release, we stimulated the microglia with BzATP in stead of ATP. It shows that ADA enhanced the TNF-α induction evoked by BzATP, while the production of CCL2 was not influenced. These results implicate that endogenous adenosine can activate the P₁ receptors and inhibit the TNF-α release triggered by P₂X₇ receptor activation. One thing that attracts our attention is finding
that ADA per se can enhance TNF-α synthesis in the absence of ATP or BzATP. This suggests that ADA itself can modulate the TNF-α production by regulating the levels of adenosine. ADA is detectable in normal tissues, and its expression is increased in inflammed tissues (Beyazit 2012, Calis 2005, Ozturk 2008). Due to the anti-inflammatory properties of adenosine, it is implicated that blockade of ADA activity may ameliorate the inflammation through the increase of adenosine levels. Several studies have also demonstrated that repressing the activity of ADA attenuates the inflammation and the production of cytotoxic mediators (Antonioli 2007 and 2010, Uzar 2006). Thus, the efficient control of ADA activity may provide a potential target for the treatment of inflammatory disorders (Antonioli 2012, Honma 2001).

Next, to determine the role of the P₁ receptors in the regulation of TNF-α, we treated the cells with non-hydrolysable, non-selective P₁ receptor agonist 5′-N-ethylcarboxamido-adenosine (NECA). To avoid the binding of endogenous adenosine and the adenosine degraded from exogenous ATP to P₁ receptors, we pretreated the microglia 1 hr with 1U/ml ADA for adenosine clearance. Thereafter, the cells were stimulated with 1 μM NECA in the presence or absence of 1 mM ATP and 500 μM BzATP for 24 hr. It is found that co-stimulation of ATP and NECA slightly (~ 20%), but significantly attenuated the release of CCL2 induced by ATP. Co-stimulation of ATP and NECA also had tendencies to suppress the release of TNF-α (~ 20%). In this experiment, we also used BzATP to determine the effects of the P₂X₇ receptors. It is shown that NECA significantly inhibited the TNF-α production stimulated by BzATP(~ 25%), while CCL2 was not affected by NECA treatment. This experiment reveals that activation of the P₁ receptors can inhibit the TNF-α secretion induced by activation of the P₂X₇ receptors, suggesting the opposing effects of adenosine and ATP in inflammatory conditions. Further pharmacological and knock-out studies targeting individual P₁ receptor subtype can be designed to find
which P₁ receptor subtype is involved in the suppression of TNF-α production. The A₂A receptors are most likely the P₁ receptor subtype which involves in the suppression of TNF-α. It is reported that activation of the A₂A receptors by the selective agonist 2-[p-(2-carboxyethyl)phenylethylamino]-50 ethylcarboxamidoadenosine (CGS 21680) reduces the severity of inflammatory conditions such as rheumatoid arthritis (RA) and chronic granulomatous disease (CGD)-induced hyper-inflammation (Chehata 2011, Mazzon 2011, Varani 2011). The production of pro-inflammatory cytokines was also down-regulated by CGS 21680 in these conditions. However, role of the A₂A receptors in regulating microglial TNF-α production needs be elucidated.

Overall, we found that not only the P₂X₇ receptors but also the P₁ receptor subtypes contribute to the regulation of TNF-α production in primary microglia. In contrast, adenosine and the P₁ receptors do not influence the production of IL-6 and CCL2 in the presence or absence of P₂X₇ ligands. These findings increase the complexity for understanding the mechanisms underlying TNF-α regulation.

**BV-2 and N9 cells**

The effects of ATP and BzATP on murine microglial cell line BV-2 and N9 cells were also investigated in our study. Expression of the P₂X₇ receptors was observed on our BV-2 and N9 cells. Studies have also reported that the P₂X₇ receptors are expressed on both BV-2 and N9 cells (Brautigam 2005, Friedle 2011). We stimulated BV-2 and N9 cells with ATP and BzATP, finding that ATP at different concentrations and BzATP did not increase the transcription of cytokine mRNA in both BV-2 and N9 cells. Interestingly, Friedle and colleagues have shown that P₂X₇ receptor activation by BzATP promotes IL-6 gene transcription in N9 cells (Friedle 2011), but we did not see the effects of BzATP on inducing cytokines in the N9 cells we used.

We also compared the Ca²⁺ responses evoked by 1 mM ATP between primary
microglia and N9 cells. It is found that the effects of ATP on the P$_2$X receptor-induced Ca$^{2+}$ influx were fairly weak (Figure 32A). In addition, we cannot observe the suppressive effects of the selective P$_2$X$_7$ antagonists on the inhibition of ATP effects (Figure 32D). We supposed that the weak effects of ATP on inducing Ca$^{2+}$ responses may be due to the low expression of the P$_2$X$_7$ receptors on N9 cells (Figure 30).

**Mixed glia and purified astroglia**

Astroglia play an important role in the maintenance of CNS homeostasis both in normal conditions and after injury. Like activated microglia, reactive astrogliosis is observed in all forms of CNS insults. Astroglia can respond to a number of important cytokines which influence the cellular state of the surrounding cells. In addition, reactive astroglia themselves release various inflammatory mediators and neurotrophic factors. Because of the importance of P$_2$X$_7$ activation in evoking cytokine release in pathological events, we also investigated the effects of P$_2$X$_7$ receptor activation on the induction of inflammatory cytokines in astroglia. We have demonstrated that the P$_2$X$_7$ receptors are expressed on primary mouse astroglia.

First, in order to compare with the effects of ATP and BzATP in pure microglia and purified astroglia, we examined the effects of ATP and BzATP in primary mixed glia. In mixed glia, both ATP and BzATP increased the gene transcription of IL-6, CCL2 and TNF-α. It is found that ATP at 1mM stimulated much more remarkable IL-6 and CCL2 mRNA expression than 200 μM BzATP, whereas the effects of BzATP on the induction of TNF-α mRNA were more pronounced than those of ATP (Figure 33).

To obtain purified astroglia from mixed glia, the cells were pre-treated with clodronate-liposomes. The bisphosphonate clodronate is known to deplete cells of monocyte lineage (Frith 1997), and it markedly reduces the microglia in hippocampal
organotypic cultures (Kohl 2003). Kumamaru and colleagues have indicated that three days treatment of clodronate-liposomes at 100 μg/mL could eliminate the microglia from primary glial cultures (Kumamaru 2012). In our study, we observed that 4 hr of 100-140 μg/mL clodronate-liposomes treatment almost eliminated the expression of microglial marker CD11b (Figure 34). This reveals that treatment of clodronate-liposomes at 100-140 μg/mL for 4 hr may be sufficient to achieve depletion of microglia.

In purified, unstimulated astroglia, IL-6 and CCL2 were constantly released into the culture medium. Extracellular ATP was demonstrated to stimulate the release and mRNA expression of IL-6 and CCL2 in astrocytes. We found that the IL-6 and CCL2 levels induced by 500 μM ATP were similar to those induced by 1 mM ATP (Figure 35A-B). BzATP increased the production of IL-6, CCL2 and TNF-α. It shows that the extent of IL-6, CCL2 or TNF-α mRNA transcription increased by 200 μM BzATP was similar, but the CCL2 release stimulated by BzATP was more robust than IL-6 and TNF-α (Figure 35C-D). It is notable here that the effects of 200 μM BzATP on TNF-α release in purified astroglia were marginal (Figure 34C) while comparing with those in pure microglia (Figure 13). We would ask whether astroglia indeed produced TNF-α after BzATP stimulation. Some studies have indicated that astroglia secrete TNF-α after treating several stimuli (Chung 1990, Kucher 2005), however, the most recent evidence from Welser-Alves and Milner demonstrated that cultured primary astroglia did not produce TNF-α in response to the stimuli like LPS and IFN-γ. To determine the cellular source of TNF-α, they employed a modified method in which microglia-free astroglia are prepared from neural stem cells (Wang 2012, Welser 2012). They compared TNF-α production after stimulation in mixed glial cultures, pure microglia and pure astroglia, finding that microglia are the sole source of TNF-α. Therefore, we considered that microglia but not astroglia, are the only source of
TNF-α after P₂X₇ stimulation. In further experiments, the immunofluorescent double staining of glial markers and cytokines can help us obtain more determinant evidence for the cellular source of released cytokines.

We also investigated the effects of the non-selective P₂ antagonists PPADs, suramin, and the selective P₂X₇ antagonists BBG on ATP-induced cytokine production in purified astroglia. It is demonstrated that the production of IL-6 and CCL2 induced by 1 mM ATP was significantly suppressed by PPADs, suramin, and BBG. Since we have demonstrated formerly that the P₂X₇ receptors are the primary receptor activated by BzATP, these results implicate that effects of ATP and BzATP on IL-6 and CCL2 release in purified astroglia are mainly mediated by the P₂X₇ receptors. It was found that both microglia and astroglia produced IL-6 and CCL2 after the P₂X₇ stimulation.

IL-6 is a pleiotropic cytokine which exhibits both pro-inflammatory and anti-inflammatory features. Under physiological conditions, IL-6 is expressed in low levels and it contributes in the normal brain function. During CNS development, the IL-6 promotes the vasculogenesis (Fee 2000). IL-6 can also induce the differentiation of astroglia (Taga 2005, Yanagisawa 2001). In CNS injury and inflammation, IL-6 levels become elevated. Several reports suggest the beneficial effects of IL-6 on improving the disease states of CNS injury (Swartz 2001, Yamashita 2005). A complete lack of IL-6 may decrease the proliferation of neuronal progenitors and be harmful to neurogenesis in adult CNS (Bowen 2011). Conversely, it is reported that chronic exposure to astroglial IL-6 reduces adult neurogenesis (Vallières 2002). Elevated IL-6 levels suppress the neuronal differentiation (Monje 2003, Nakanishi 2007).

CCL2 is a chemokine which serves as a potent chemotactic factor for monocytes. After the brain injury, CCL2 is up-regulated and it participates in the infiltration of
CCR2+ monocytes to the lesion site. In a study of intrahippocampal injections of various chemokines, CCL2 was found to be the most potent stimulus of monocyte recruitment to the site of injection (Bell 1996). Many studies have revealed a devastating role of CCL2 in neurological disorders. For instance, CCL2 was found to facilitate Aβ oligomer formation and accelerate the memory dysfuction in AD (Kiyota 2009). In addition, CCL2−/− mice with TBI had decreased monocytes accumulation and improved neurological function (Semple 2009). The neuroprotective actions of CCL2 deficiency were also observed in mouse model of ischemia (Hughes 2002). However, converse evidence indicates that CCR2-dependent monocyte accumulation can delay the disease progression of AD by phagocytosing Aβ deposits. Astroglia are predominant source of IL-6 and CCL2 in response to CNS insults (Gabryel 2001, Klegeris 2006, Lau 2001, Lehmann 2006, van der Voorn 1999). Role of the ATP-induced P2X7 activation in astroglial CCL2 production has been previously proven by Panenka et al. They indicated that activation of the P2X7 receptors induced CCL2 production in rat astroglia, and the P2X7-evoked CCL2 is dependent on the activation of the MAPKs ERK1/2 and p38. They also performed in vivo experiments, finding that antagonism of the P2X7 receptors blocked the CCL2 increase after cortical trauma (Panenka 2001). Therefore, they suggested that the P2X7 receptors may serve an integral component for astroglial CCL2 production in response to CNS insults. In this study, the actions of P2X7 stimulation on CCL2 production were verified again in mouse astroglia, and the role of the P2X7 receptors in astroglial IL-6 regulation was revealed. Both IL-6 and CCL2 are important regulators of the inflammatory responses, and their up-regulation is associated with the pathogenesis of neurological disorders. Since the P2X7 activation can provoke IL-6 and CCL2 release from both microglia and astroglia in response to CNS insults, targeting the P2X7 activity may provide therapeutic approach for CNS diseases.
Several *in vitro* studies have proven that the P$_2$X$_7$ receptors are expressed on cultured primary astroglia (Duan 2003, Gao 2011, Panenka 2001); however, *in vivo* evidence shows that in the CNS, the P$_2$X$_7$ receptors are restricted to be expressed on microglia, but not on neurons and astroglia (Chu 2010, He 2012, Melani 2006). In addition, we did not find any studies in literature demonstrating that activation of the P$_2$X$_7$ receptors induces cytokine production from astroglia *in vivo*. Therefore, contributions of the P$_2$X$_7$ receptors to astroglial cytokine regulation in the CNS may not fit in with the data we have presented.
6. Conclusions

A large body of evidence has displayed the importance of the P$_2$X$_7$ receptors in CNS diseases by triggering cytokine production in glial cells. Here we clearly proved that stimulation of the P$_2$X$_7$ receptors evoked the production of IL-6 and CCL2 in mouse microglia and astroglia, while TNF-$\alpha$ secretion was induced after the P$_2$X$_7$ activation in mouse microglia. Among these cytokines secreted by microglia, TNF-$\alpha$ is a central cytokine that regulates many aspects of inflammatory reactions. In CNS, TNF-$\alpha$ is exclusively produced by microglia. The noxious actions of microglial TNF-$\alpha$ over-production in neurological and neuropsychiatric disorders have been demonstrated. Since activation of the P$_2$X$_7$ receptors potentiates TNF-$\alpha$ release from microglia in pathological conditions, antagonizing the P$_2$X$_7$ activity may prevent TNF-$\alpha$ over-expression and exhibit protective effects. Surprisingly, we found that the selective P$_2$X$_7$ antagonists are not able to block the P$_2$X$_7$-dependent TNF-$\alpha$ release in microglia, whereas they still exert inhibitory effects on IL-6 and CCL2. This finding is contradictory to several studies which declared that the selective P$_2$X$_7$ antagonists significantly block the P$_2$X$_7$-mediated TNF-$\alpha$ secretion. Because of the potential anti-inflammatory actions of the selective P$_2$X$_7$ antagonists, people have suggested their therapeutic effects for the CNS diseases. Recently, more novel selective P$_2$X$_7$ antagonists are developed, and some of them have been applied in early clinical trials of the inflammatory disease rheumatoid arthritis (Arulkumaran 2011, Friedle 2010, Nagy 2012). However, our findings suggest that the inhibitory effects of novel selective P$_2$X$_7$ antagonists on P$_2$X$_7$-induced cytokine production should be carefully investigated both in vitro and in vivo.

In addition to find the unanticipated effects of selective P$_2$X$_7$ antagonists on P$_2$X$_7$-induced TNF-$\alpha$ secretion, we demonstrated that activation of the P$_2$X$_7$ receptors reduced the LPS-induced TNF-$\alpha$, but not IL-6 and CCL2 release in microglia.
Moreover, we noticed that in the absence of exogenous stimuli, ADA, an enzyme which ubiquitously presents in tissues and metabolizes adenosine to inosine, can per se regulate the synthesis and release of microglial TNF-α by modulating the levels of adenosine. In contrast, the production of IL-6 and CCL2 are not affected by ADA. We also revealed that activation of the P₂X₇ receptors and the P₁ receptors exert opposing actions on TNF-α production. In summary, these data indicate that compared with IL-6 and CCL2, TNF-α may be differentially regulated by more complicated mechanisms in microglia. Since TNF-α plays an important role in both healthy and pathological brain, elucidating the mechanisms underlying its regulation may provide therapeutic strategies for the CNS diseases.
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