Role of microglia and astrocytes in inflammatory processes involving neurological diseases, chronic pain, and psychiatric disorders, with emphasis on the purinergic P2X7 receptor

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ABSTRACT

Under pathological conditions microglia (resident central nervous system (CNS) immune cells) become activated, and produce reactive oxygen and nitrogen species and pro-inflammatory cytokines: molecules that can contribute to disorders including stroke, traumatic brain injury, progressive neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, multiple sclerosis, and several retinal diseases. Given that ATP is frequently released from CNS neurons during tissue damage and inflammation, its actions on microglia-mediated toxicity are especially pertinent. For example, the ATP-gated P2X7 purinergic receptor (P2X7R) cation channel is up-regulated around amyloid β-peptide plaques in transgenic mouse models of Alzheimer's disease and co-localizes to microglia and astrocytes. Upregulation of P2X7R on microglia occurs also following spinal cord injury and after brain ischemia. ATP, via activation of P2X7R, is one of the most powerful stimuli for secretion of the key pro-inflammatory cytokine interleukin-1β (IL-1β) in its mature form. This project investigates the pharmacological and biochemical behaviors of P2X7R on microglia and astrocytes cultured from rat cerebral cortex, spinal cord and cerebellum, and the relationship between these two glial cell types.

ATP is an efficient stimulus for IL-1β secretion only after the cells have undergone a short 'priming' with endotoxin (lipopolysaccharide (LPS)). Indeed LPS, but not ATP caused release of IL-1β from cortical microglia. However, it is known that the greater part of the IL-1β thus released is the precursor (biologically inactive) form. Purified (>99%) cortical microglia and enriched (≥95%) astrocytes were primed for 2 hours with LPS, followed by addition of ATP for 1 hour. Culture medium was then collected and the content of IL-1β quantified by ELISA. The effects of LPS and ATP were concentration-dependent; although LPS alone (but not ATP) modestly stimulated IL-1β release, levels of cytokine release were much higher from primed cells incubated with ATP. The ATP-dependent component was fully blocked by selective P2X7R antagonists, and followed their
known rank order of target potency. The P2X7R priming response was also seen with spinal cord and cerebellar microglia, a finding not described in the literature until now.

To rule out a contribution by the minor population of microglia in our astrocyte cultures, the latter were treated with the lysosomotropic agent L-leucyl-L-leucine methyl ester (L-LME) which selectively eliminates cells with cytotoxic potential (e.g. macrophages, microglia). Immunocytochemical and molecular biological evaluation showed L-LME-treated cortical and spinal cord astrocytes to be fully depleted of microglia. These purified astrocytes failed to respond to LPS, and did not show the ATP priming behavior. Responsiveness was recovered upon addition of microglia to the L-LME-treated astrocytes and, moreover, a far more robust release of IL-1β occurred than that achieved with the same numbers of microglia alone. This astrocyte-microglia interaction was also observed for LPS-stimulated release of nitric oxide and IL-6, and was not mediated by astrocyte-derived soluble factors.

Lastly, the LPS/ATP priming behavior was studied by examining the ability of other agents, linked to neuropathology, to replace either LPS or ATP. Neither ethanol (ethanol intoxication; in place of LPS) nor amyloid β-peptides (Alzheimer disease; in place of ATP) were able to provoke IL-1β release from microglia. However, both zymosan and poly(I:C), agonists of Toll-like receptors -2 and -3, respectively, were capable of substituting LPS (a Toll-like receptor 4 agonist) in the P2X7R priming response. Release of IL-1β in all these cases was antagonized by inhibitors of p38 mitogen-activated protein kinase (a stress response kinase).

TLRs contribute to CNS immunocompetent cell activation and the resulting pro-inflammatory cascade producing pathological pain. TLR4 recognizes not only LPS, but also ligands called damage associated molecular patterns, released by the injured tissue. The involvement of extracellular TLR4 and TLR2, as well as TLR3 in preclinical pain models has been demonstrated. The findings described here further support the notion of astrocyte/microglia interaction, which may improve our understanding in how these cells respond to CNS injury or inflammation, in particular where TLRs are involved.
RIASSUNTO

Le ricerche svolte hanno riguardato lo studio dei comportamenti della microglia e degli astrociti derivati da corteccia, midollo spinale e cervelletto di ratto in presenza di uno stimolo infiammatorio, in particolare quello associato alla famiglia dei recettori toll-like (TLR) e il recettore purinergico P2X\textsubscript{7} (P2X7R). E’ ormai riconosciuto il coinvolgimento della glia nello sviluppo delle malattie neurodegenerative e nel dolore neuropatico, sia nel midollo spinale che nelle aree corticali. In particolare, il rilascio di ATP da cellule danneggiate o morenti (cellule gliali, neuroni e cellule endoteliali) può agire come un segnale di ‘pericolo’ attraverso un sottotipo di recettori purinergici come il P2X\textsubscript{7}. I recettori P2X\textsubscript{7} possono influenzare la morte cellulare attraverso la capacità di regolare il processo e il rilascio di interluchina-1\textbeta (IL-1\textbeta), un mediatore chiave nella neurodegenerazione, infiammazione e dolore cronico. L’IL-1\textbeta è rilasciata dai macrofagi e dalla microglia esposta a endotossina batterica (lipopolisaccaride, LPS). Scopo della ricerca svolta è stato quindi la caratterizzazione di queste popolazioni di cellule gliali con metodi immunologici, molecolari, biochimici e farmacologici in presenza o assenza di stimolo infiammatorio (LPS) e l’identificazione del meccanismo del ‘priming’ (le cellule di microglia trattate per breve tempo con LPS diventano più sensibili all’azione dell’ATP) in termine di rilascio di IL-1\textbeta. Fino ad ora la maggior parte degli studi presenti in letteratura hanno esaminato il ruolo della microglia ma non degli astrociti nella neuroinfiammazione.

Il lavoro svolto nel primo anno è consistito principalmente nella caratterizzazione del comportamento della microglia e degli astrociti ottenuti da corteccia, midollo spinale e cervelletto, in termini di risposta ad ATP, in presenza o assenza di uno stimolo infiammatorio come LPS. Per questo scopo è stato utilizzato un modello in vitro di microglia preparata da corteccia cerebrale di ratto di 1-2 giorni d’eta’ o da midollo spinale. Dopo 1-2 settimane la popolazione di cellule di microglia vengono separate dagli astrociti e raccolte. Queste colture secondarie di microglia purificate (>99%) e astrociti arricchiti (>95%) (come determinato mediante colorazione immunocitochimica e l'espressione genica (RT-PCR), sono state trattate e il terreno di coltura è
stato utilizzato per l’analisi (ELISA) delle citochine (IL-1β, TNF-α, IL-6), rilasciate. Inoltre, nel terreno di coltura si è provveduto a valutare la quantità di ossido d’azoto (NO). La microglia e gli astrociti di controllo e trattati con ATP (fino a 5 mM) non producono una quantità misurabile di IL-1β. Il LPS, alla dose di 100 ng/ml, produce invece un considerevole aumento di NO e IL-1β in funzione del tempo in tutte e due i tipi cellulari.

Successivamente abbiamo analizzato la risposta della microglia e degli astrociti esposti per tempi brevi prima a LPS e poi ad ATP per stimolare il rilascio di una quantità maggiore di IL-1β matura (‘priming’). Sia la microglia che gli astrociti, stimolati con LPS e ATP, rilasciano IL-1β. La parte della risposta data dall’ATP e non dall’LPS viene bloccata dagli antagonisti del P2X7R. L’SB-202190, un inibitore selettivo della miogeno-activated-protein (MAP) chinasi p38, riduce il rilascio di IL-1β indotto da LPS nel ‘priming’, ma lo aumenta (da 6 a 10 volte) se incubato con LPS in presenza di siero per 24 ore. L’SB-202190 inoltre, inibisce il rilascio di NO in cellule trattate con LPS per 24 ore. L’SP600125, un inibitore del c-Jun N-terminale chinasi (JNK), aumenta il rilascio di IL-1β indotto da LPS (24 ore) in microglia. In contrasto, nel priming l’SP600125 riduce il rilascio di IL-1β. I due meccanismi di rilascio di IL-1β sono quindi presumibilmente diversi, ma legati alla cascata del segnale della MAP chinasi. La risposta 'priming' è stata anche dimostrata in microglia ottenuta da cervelletto di ratto. Questi risultati rappresentano la prima descrizione della risposta 'priming' nelle cellule gliali del midollo spinale e del cervelletto collegato al P2X7R.

Il lavoro svolto nel secondo anno è consistito principalmente nel caratterizzare dal punto di vista molecolare e farmacologico il rapporto tra microglia e astrociti in queste culture. Siccome le colture arricchite in astrociti contengono una piccola percentuale di microglia (<5%), abbiamo voluto verificare se il rilascio di citochine in seguito a uno stimolo infiammatorio dipende da una azione diretta sugli astrociti o una azione indiretta tramite la presenza della microglia. Per eliminare la microglia residua le colture arricchite in astrociti (corticali, spinali o cerebellari), sono state trattate per un ora con l’agente lisosomotropico L-leucine methyl ester (L-LME) (50 mM), che è tossico per i macrofagi. Dopo un ora di incubazione la soluzione di L-LME è stata sostituita da terreno di
coltura per 24 ore, al termine delle quali sono stati iniziati i trattamenti con lo stimolo infiammatorio. Da ora in poi definiamo tali colture come ‘astrociti purificati’. L’RNA messaggero (mRNA) per l’ Iba1, proteina espressa solo nella microglia, non è stato rilevato tramite la tecnica RT-PCR negli astrociti purificati, mentre il segnale è presente nelle cellule prima del trattamento con L-LME. Il trattamento con L-LME elimina anche la risposta LPS/ATP (priming) negli astrociti corticali, spinali e cerebellari purificati. L’aggiunta di una quantità nota di microglia agli astrociti purificati, ripristina la risposta degli astrociti al priming. Tuttavia, lo stesso numero di microglia, da sola, senza la presenza degli astrociti, è insufficiente a produrre il segnale. Lo studio dell’interazione tra microglia e astrociti purificati, è stato esteso anche alla produzione dell’NO e mRNA per iNOS (NO sintasi inducibile). È stato dimostrato che l’aumento della produzione di NO in seguito a stimolazione con LPS, è stata abolita negli astrociti purificati. L’aggiunta di una quantità nota di microglia alle cellule di astrociti purificati, ripristina la risposta degli astrociti al LPS e di conseguenza un aumento di iNOS e NO. Inoltre, il terreno di coltura dagli astrociti trattati con LPS non è grado a stimolare il rilascio di NO da microglia. Un comportamento simile è stato osservato per IL-6 mRNA e proteine. Questi dati mostrano chiaramente che la microglia, ma non gli astrociti rispondono al LPS, e che il comportamento degli astrociti dipende dalla presenza di microglia.

L’obiettivo ultimo era quello di esaminare, oltre al LPS o ATP, la capacità di altri agenti potenzialmente patologici, ad esempio il peptide beta-amiloide (Aβ), l’etanolo, e agonisti del recettore TLR2 e TLR3 nella risposta 'priming' della microglia. Aβ, il principale costituente delle placche che si trovano nel cervello dei pazienti affetti dalla malattia Alzheimer (AD), può attivare la microglia. È stato pubblicato che la proteina Aβ (25-35) aumenta la secrezione di IL-1β nella microglia esposta a LPS. Siccome nel cervello dei pazienti affetti da AD si accumula la forma (1-42) della Aβ e non la (25-35), tutte e due le forme sono state testate per vedere se sono in grado di sostituire l’ATP nella microglia pretrattata con LPS. Nessuno dei due peptidi però si sono dimostrati efficaci.
L’intossicazione da etanolo cronica e acuta promuove i processi infiammatori nel cervello e nelle cellule gliali agendo sul TLR4. L’etanolo, dunque, potrebbe attivare la microglia e sostituire LPS nella risposta 'priming' per l'ATP. Per dimostrare questa teoria, la microglia corticale e da midollo spinale è stata incubata con 100 mM etanolo o 1 µg/ml LPS per due ore, seguita da incubazione con 5 mM ATP per un'ora. Successivamente è stato misurato il rilascio di IL-β nel mezzo di coltura che ha dimostrato l’effetto dell’ATP, ma non dell’etanolo, sulle cellule pretrattate con LPS.

La microglia oltre al TLR4 esprime anche TLR2 e TLR3 funzionali che, quando attivati, partecipano al dolore neuropatico causato da lesioni nervose. Finora non sono stati fatti studi che dimostrano il coinvolgimento degli agonisti TLR2 e TLR3 sulla microglia ‘primed’ e il rilascio di citochina IL-1β. L’agonista TLR2 (Zymosan) e TLR3 (Poly(I:C)), sono stati dunque testati nella microglia al posto del LPS e si sono dimostrati capaci di attivare la microglia, corticale, spinale e cerebellare. In presenza di ATP sono stati in grado, come il LPS, di essere efficaci come ‘priming’ in termini di rilascio di IL-1β. Un inibitore selettivo della MAP chinasi p38 (SB-202190) riduce il rilascio di IL-1β indotto da Zymosan o Poly(I:C) nel 'priming', ma lo aumenta (circa 4 volte) se incubato per 24 ore, in presenza di siero.

Questo studio costituisce un contributo originale alla ricerca nel campo della neuroinfiammazione a livello cellulare e in particolare il rapporto tra astroцитi e microglia. Dato che gli astrocitii sono molto più numerosi della microglia nel sistema nervoso centrale, questi dati suggeriscono che una simile interazione tra astrocitii e microglia in vivo può essere un elemento importante per l'evoluzione di una patologia infiammatoria. Inoltre, questo lavoro dimostra per la prima volta che l'attivazione del P2X7R si verifica in microglia da midollo spinale e da cervelletto, che può verificarsi con agonisti TL2 e TLR3 oltre che con TLR4. Questo amplia notevolmente le possibilità di partecipazione del recettore P2X7 nella neuroinfiammazione.
Acknowledgements

I would like to start by expressing my thanks to Prof. Pietro Giusti, my supervisor during these three years, for his constant support, guidance and encouragement. Thanks also go to those members of our laboratory for their collaborative input to this project, especially Dr. Massimo Barbierato for his expert molecular biological skills in performing the gene expression experiments, and Dr. Carla Argentini and again Dr. Barbierato for their important contributions to the preparation and characterization of the spinal cord glia cultures. A note of thanks is also due Massimo Rizza and our animal support facility for the timely provision of animals for this project, as well as our departmental support staff. Last, but not least, I would like to express my appreciation to Dr. Stephen Skaper for critically reading of this thesis and provision of numerous suggestions for improvement.

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## Abbreviations

Aβ = Amyloid β-peptide  
AD = Alzheimer disease  
ADP = Adenosine diphosphate  
AMP = Adenosine monophosphate  
AP-1 = Activator protein-1  
ATP = Adenosine triphosphate  
ATP-P = Periodate-oxidized ATP  
BzATP = 3’-O-(4-benzoyl)benzoyl-ATP  
CNS = Central nervous system  
COX-2 = Cyclooxygenase-2  
CRE = Cyclic AMP response element  
CREB = CRE binding protein  
DMEM = Dulbecco’s modified Eagle medium  
ELISA = Enzyme-linked immunosorbent assay  
E-NPPs = Ectonucleotide pyrophosphatase and/or phosphodiesterases  
ERK = Extracellular signal-regulated kinase  
FBS = Fetal bovine serum  
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase  
GFAP = Glial fibrillary acidic protein  
HD = Huntington disease  
IL-6 = Interleukin-6  
IL-1β = Interleukin-1β  
IFN-γ = Interferon γ  
iNOS = Inducible nitric oxide synthase  
LDH = Lactate dehydrogenase  
L-LME = L-Leucyl-L-leucine methyl ester  
LPS = Lipopolysaccharide  
MAPK = Mitogen-activated protein kinase  
MEK = MAPK kinase  
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide  
NFAT = Nuclear factor of activated T cells  
NF-κB = Nuclear factor κB
NMDA = N-methyl-D-aspartic acid
NO = Nitric oxide
P2X7R = P2X7 receptor
PanX1 = Pannexin 1
PBS = Phosphate buffered saline
PD = Parkinson disease
Poly(I:C) = Poly inosinic:polycytidylic acid
RT-PCR = Real-time polymerase chain reaction
SEM = Standard error of the mean
SNP = Single nucleotide polymorphism
TLR = Toll-like receptor
TM = Transmembrane
TNF-α = Tumor necrosis factor α
1. INTRODUCTION

1.1. PURINERGIC NEUROTRANSMISSION AND SIGNALING - A HISTORICAL PERSPECTIVE

It has been estimated that adenosine triphosphate (ATP) participates in more chemical reactions than any other molecule, except water. This is due, in no small measure to the evolutionarily progressive development of both complementary binding sites for ATP on cellular proteins, and various receptors for extracellular ATP on the plasma membrane of almost all cell phenotypes – in addition to the ancestral function of ATP as the main source of energy. Moreover, purine derivatives have been viewed as primordial precursors in the evolution of neurochemical transmission (Trams, 1981) – extracellular actions of ATP have been reported in very primitive organisms, including bacteria, diatoms, algae and slime moulds (Burnstock, 1996; Burnstock and Verkhratsky, 2009). Therefore, when examining the ontogeny and phylogeny of ATP functions and ATP receptors, one must keep in mind both short-term signalling, as typified by neurotransmission and secretion, and long-term signalling which involve not only cell proliferation, differentiation and death, but also plasticity of expression during pathological states.

The concept of purinergic neurotransmission was introduced in 1972 (Burnstock, 1972) after it was shown that ATP was a transmitter in non-adrenergic, noncholinergic inhibitory nerves in the guinea-pig taenia coli. Subsequently, ATP was identified as a co-transmitter in sympathetic and parasympathetic nerves (Burnstock, 1976), and it is now recognized that ATP acts as either sole transmitter or a cotransmitter in most nerves in both the peripheral nervous system and central nervous system (CNS) (Burnstock, 2007) (Table 1).

Implicit in the concept of purinergic neurotransmission was the existence of postjunctional purinergic receptors, and the potent actions of extracellular ATP on many different cell types also implicated membrane receptors. Purinergic receptors were first defined in 1976 (Burnstock, 1976). Two years later a basis for distinguishing two types of purinoceptors, identified as P1 and P2 (for
adenosine and ATP/ADP, respectively), was proposed (Burnstock, 1978). At about the same time, two subtypes of the P1 (adenosine) receptor were recognised (Londos et al., 1980; Van Calker et al., 1979), but it was not until 1985 that a proposal suggesting a pharmacological basis for distinguishing two types of P2 receptors (P2X and P2Y) was made (Burnstock and Kennedy, 1985). In 1993, the first G protein-coupled P2 receptors were cloned (Lustig et al., 1993; Webb et al., 1993), and a year later two ion-gated receptors were cloned (Brake et al., 1994; Valera et al., 1994). In 1994 Abbracchio and Burnstock (1994), on the basis of molecular structure and transduction mechanisms, proposed that purinoceptors should belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. This nomenclature has been widely adopted and currently seven P2X subtypes and eight P2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as purines (Abbracchio et al., 2006; North, 2002; Ralevic and Burnstock, 1998) (Table 2).

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**Table 1. ATP as a ubiquitous co-transmitter in peripheral and central nervous systems**

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<tr>
<td>Sympathetic nerves</td>
<td>ATP + NA + NPY</td>
</tr>
<tr>
<td>Parasympathetic nerves</td>
<td>ATP + ACh + VIP</td>
</tr>
<tr>
<td>Sensory-motor</td>
<td>ATP + CGRP + SP</td>
</tr>
<tr>
<td>NANC enteric nerves</td>
<td>ATP + NO + VIP</td>
</tr>
<tr>
<td>Motor nerves (in early development)</td>
<td>ATP + Ach</td>
</tr>
<tr>
<td><strong>Central nervous system</strong></td>
<td></td>
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<tr>
<td>Cortex, caudate nucleus</td>
<td>ATP + ACh</td>
</tr>
<tr>
<td>Hypothalamus, locus coeruleus</td>
<td>ATP + NA</td>
</tr>
<tr>
<td>Hypothalamus, dorsal horn, retina</td>
<td>ATP + GABA</td>
</tr>
<tr>
<td>Mesolimbic system</td>
<td>ATP + DA</td>
</tr>
<tr>
<td>Hippocampus, dorsal horn, cortex</td>
<td>ATP + glutamate</td>
</tr>
</tbody>
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ACh, acetylcholine; ATP, adenosine 5'-triphosphate; CGRP, calcitonin gene-related peptide; DA, dopamine; GABA, γ-aminobutyric acid; NA, noradrenaline; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal polypeptide.

(Compiled from Burnstock (2007)).
1.1.1. Storage, Release and Breakdown of ATP and Related Nucleotides

The cytoplasm of most neurons contains ~2-5 mM ATP, and higher concentrations of ATP (up to 100 mM) are stored in synaptic vesicles. Synaptic vesicles also contain other nucleotides such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), diadenosine tetra- and pentaphosphate, and guanosine triphosphate, but at lower concentrations (Novak, 2003; Sperlágh and Vizi 1996). Accumulation of ATP into vesicles (Figure 1) can be mediated by a Cl⁻-dependent vesicular nucleotide transporter, which belongs to the SLC17 anion transporter family, which includes also vesicular glutamate transporters (Sawada et al., 2008). This transporter is highly expressed in brain localized to chromaffin granules and subpopulations of astrocytes. ATP is probably present in every synaptic and/or secretory vesicle, although at different concentrations, and can be co-stored and co-released with other neurotransmitters (e.g. γ-aminobutyric acid, noradrenaline or glutamate; Table 1).

At present, it is unclear whether various physiological nucleotide-receptor agonists (e.g. ATP, ADP, UTP, UDP, UDP sugars and NAD⁺) are released by common mechanisms. There is strong evidence for exocytotic neuronal vesicular release of ATP (Pankratov et al., 2007) (Figure 1); there is also support for vesicular release of ATP from astrocytes (Bowser and Khakh, 2007), which may involve lysosomes (Zhang et al., 2007). ATP released from nerves, or by autocrine and paracrine mechanisms from nonneuronal cells, is involved in a wide spectrum of physiological and pathophysiological activities, including synaptic transmission and modulation, pain and touch perception.

After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which is functionally important because ATP metabolites act as physiological ligands for various purinergic receptors (Figure 1). The ectonucleotidases not only control the lifetime of nucleotide ligands but, by degrading or interconverting the originally released ligands,
they also produce ligands for additional P2 receptors and nucleosides (Figure 1; Table 2) (Zimmermann, 2006). All ectonucleotidase families hitherto identified are expressed in the brain. These include the ectonucleoside triphosphate diphosphohydrolases, E-NPPs (ectonucleotide

Table 2

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
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<tbody>
<tr>
<td>1929</td>
<td>Purines hypothesized as extracellular signaling molecules</td>
</tr>
<tr>
<td>1930’s &amp; 1940’s</td>
<td>Effects of ATP and other nucleotides on tissues explored</td>
</tr>
<tr>
<td>1950’s</td>
<td>ATP release from sensory nerves</td>
</tr>
<tr>
<td>1972</td>
<td>ATP proposed as a neurotransmitter</td>
</tr>
<tr>
<td>1975</td>
<td>α,β- and β,γ-MeATP first used on gastrointestinal tissues</td>
</tr>
<tr>
<td>1978</td>
<td>&quot;P2 purinoceptor&quot; coined</td>
</tr>
<tr>
<td>1979</td>
<td>Reactive blue-2 first used as a P2 antagonist</td>
</tr>
<tr>
<td>1985</td>
<td>P2 receptors subclassified as P2X and P2Y</td>
</tr>
<tr>
<td>1988</td>
<td>Suramin identified as a P2 antagonist</td>
</tr>
<tr>
<td>1989</td>
<td>BzATP activates P2Z (P2X7)</td>
</tr>
<tr>
<td>1992</td>
<td>PPADS identified as a P2X antagonist</td>
</tr>
<tr>
<td>1993</td>
<td>Oxidized ATP blocks P2Z (P2X7)</td>
</tr>
<tr>
<td>1994/1996</td>
<td>All seven P2X subtypes cloned</td>
</tr>
<tr>
<td>1998</td>
<td>TNP-ATP identified as selective antagonist of P2X1, 3, 2/3</td>
</tr>
<tr>
<td>2002</td>
<td>Selective small-molecule antagonists of P2X1,3, 2/3, 7 first identified</td>
</tr>
<tr>
<td>present</td>
<td>Drug-like antagonists emerging; first clinical trials in man underway</td>
</tr>
</tbody>
</table>
pyrophosphatase and/or phosphodiesterases), alkaline phosphatases and ecto-50-nucleotidase (Zimmermann, 2006). Individual enzymes differ in substrate specificity and product formation. Ectonucleoside triphosphate diphosphohydrolases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-50-nucleotidase. Alkaline phosphatases equally hydrolyse nucleoside tri-, di- and mono-phosphates. Dinucleoside polyphosphates, NAD$^+$ and UDP sugars are substrates solely for E-NPPs. Besides the catabolic pathways, nucleotide interconverting enzymes exist for nucleotide rephosphorylation and extracellular synthesis of ATP (e.g. ectonucleoside diphosphate kinase and adenylate kinase). Although usually adenosine is produced by ectoenzymatic breakdown of ATP, there might be subpopulations of neurons and/or astrocytes that release adenosine directly (Wall and Dale, 2007).

**Figure 1.** Mechanisms of ATP release, degradation and reception

Abbreviations: Alk Phos, alkaline phosphatase; Myok, myokinase (adenylate kinase); NDK, nucleoside diphosphate kinase; NPPs, nucleotide pyrophosphatase and/or phosphodiesterases; 50-Nuc, 50-nucleotidase; VNUT, vesicular nucleotide transporter. (Taken from Abbracchio et al., Trends Neurosci. 2009).
1.2. RECEPTORS FOR PURINES AND PYRIMIDINES

1.2.1. P1 Purinergic Receptors

The four adenosine receptors A1, A2A, A2B and A3 are G-protein coupled (Figure 1). Typically, A1 and A3 couple to the G\textsubscript{i/o} family of G proteins inhibiting cyclic AMP (cAMP) production, whereas A2A and A2B stimulate cAMP production via G\textsubscript{s}. Other G-protein combinations have been revealed as a function of cell type; however, all adenosine receptors activate at least one subfamily of mitogen-activated protein kinases (MAPKs) (Schulte and Fredholm, 2003). Accordingly, in the CNS, adenosine exerts a multitude of functions, including modulation of neural and glial functions, of neuron–glia signalling or of neural development (Daré et al., 2007; Fellin et al., 2006). Furthermore, adenosine plays an important part in the control of the innate and adaptive immune systems, and dysregulation of the adenosine system is involved in pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions (Boisen, 2008).

1.2.2. P2Y Receptors

The first G protein-coupled P2Y receptors were cloned in 1993 (Lustig et al., 1993; Webb et al., 1993), and there are currently eight accepted P2Y receptors: P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, P2Y\textsubscript{6}, P2Y\textsubscript{11}, P2Y\textsubscript{12}, P2Y\textsubscript{13}, and P2Y\textsubscript{14}. The missing numbers represent either non-mammalian orthologs or receptors that have some sequence homology to P2Y receptors but for which there is no functional evidence of responsiveness to nucleotides (Abbracchio et al., 2006). P2Y receptors are 308-377 amino acid proteins with a mass of 41-53 kDa after glycosylation. The seven transmembrane (TM) domain tertiary structure of P2Y receptors is common to other G protein-coupled receptors (Figure 1). P2Y are activated by purine or pyrimidine nucleotides or sugar-nucleotides with subtype-dependent heterotrimeric G-proteins (Abbracchio et al., 2006). Site-directed mutagenesis studies have shown that some positively charged residues in TM3, 6 and 7 are crucial for receptor
activation by nucleotides (Jiang et al., 1997). Each P2Y receptor binds to a single heterotrimeric G protein (Gαq/11 for P2Y1, 2, 4, 6), although P2Y11 can couple to both Gαq/G11, and Gαs, whereas P2Y12 and P2Y13 couple to Gαi and P2Y14 couples to Gαi/G0 (Abbracchio et al., 2006). In response to nucleotide activation, recombinant P2Y receptors either activate the phospholipase C-inositol trisphosphate pathway through Gαq/G11, which in turn releases intracellular calcium and activates protein kinase C, or affect adenylyl cyclase and alter cAMP levels through Gαi/G0 (Abbracchio et al., 2006). Furthermore recent studies have shown that several P2Y receptors (P2Y2, P2Y6 and P2Y12) also coupled to Gα12/13, and nucleotide stimulation also activates Rho (Liao et al., 2007; Nishida et al., 2008) (Figure 2).

P2Y receptors are expressed very early in the embryonic CNS and are broadly distributed on both neurons and glia. In contrast to P2X receptors (discussed below), P2Y receptors are activated not only by ATP but also by its metabolite ADP, uracil nucleotides (UTP, UDP), sugar nucleotides, or both adenine and uracil nucleotides (i.e. P2Y2) (Abbracchio et al., 2006). For P2Y1 receptors, ADP is a more potent agonist than ATP (their 2-methylthio derivatives are even more potent). The most effective antagonists to display selectivity for the P2Y1 receptor are MRS2179 (Boyer et al., 1998), MRS2279 (Boyer et al., 2002) and MRS2500 (Kim et al., 2003). P2Y2 receptors are fully activated by equivalent concentrations of ATP and UTP, whereas ADP and UDP are much less effective agonists. P2Y6 receptors are selective for UDP (Abbracchio et al., 2006). A 1,4-di-(phenylthioureido) butane derivative (MRS2578) (Mamedova et al., 2004) has been shown to selectively inhibit UDP-induced phospholipase C activity through P2Y6 receptors. P2Y12 receptors are also activated selectively by ADP and blocked by the 5′-triphosphate derivative AR-C69931MX (Ingall et al., 1999) (although this antagonist also blocks P2Y11 and P2Y13 receptors (Abbracchio et al., 2006; Communi et al., 1999)). The active metabolite of the antiplatelet drug clopidogrel is a potent and selective antagonist of P2Y12 receptors (Savi and Herbert, 2005).
P2Y receptors can be divided into two principal subtypes (a and b) as a function of their G-protein coupling. DAG, diacylglycerol; IP₃, inositol trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C. PLC, phospholipase C.

P2Y receptors might generate homodimers or heterodimers with other P2Y receptors (Ecke et al., 2008) or with other transmitter receptors (e.g. A₁ adenosine receptors (Fischer and Krügel, 2007). Interactive molecular pathways include: nucleotide-induced transactivation of tyrosine kinase receptors (such as epidermal growth factor receptor, platelet-derived growth factor receptor and vascular endothelial growth factor receptor-2); activation of soluble tyrosine kinases (such as Src, or of ectometalloproteases (Camden et al., 2005)); and interaction with integrins or the nerve growth factor receptor and their signalling pathways (Arthur et al., 2005; Erb et al., 2006; Milenkovic et al., 2003; Neary and Zhu, 1994).
1.2.3. P2X Receptors

P2X receptors are classical cationic ligand-operated channels that upon ATP binding open the pore permeable to Na\(^+\), K\(^-\), and Ca\(^{2+}\) (Figure 1). The P2X receptors are trimers (Nicke et al., 1998) formed from individual subunits encoded by seven distinct genes (designated P2X\(_1\) to P2X\(_7\) according to historical order of cloning (North, 2002; Roberts et al., 2006)). Diversity of the P2X receptor phenotypes is determined by assembly of individual subunits; hitherto six homomeric (P2X\(_1\)-P2X\(_5\) and P2X\(_7\); P2X\(_6\) subunits apparently do not oligomerize (Nicke et al., 1998)) and six heteromeric (P2X\(_{1/2}\), P2X\(_{1/4}\), PX\(_{1/5}\), P2X\(_{2/3}\), P2X\(_{2/6}\), and P2X\(_{4/6}\)) channels have been identified (Roberts et al., 2006). Homomeric P2X\(_7\) receptors (P2X7R) are activated at 100-1000 \(\mu\)M ATP concentrations and the rest of receptors have an EC\(_{50}\) of \(~1\) to \(~10\) \(\mu\)M. All P2X subunits are expressed in neural cells; this expression is heterogeneous in different brain regions and cell types. In many central neurons, expression is mosaic (and far from being fully characterised), which determines exceptional variability of ATP-induced current responses (Pankratov et al., 2003). Peripheral neurons (both sensory and autonomic) predominantly express P2X\(_3\) and P2X\(_{2/3}\) receptors, which are implicated in pain and temperature perception. The homomeric P2X\(_3\) channels have a unique temperature sensitivity (Khmyz et al., 2008); their rate of desensitisation is temperature insensitive, whereas the recovery from desensitization shows exceptional temperature dependence (temperature coefficient, Q\(_{10}\) \(~10\)).

All P2X receptors are permeable to Ca\(^{2+}\); the ratio of Ca\(^{2+}\) to monovalent cations permeability varies between 1 and \(>5\)-10 for various P2X subunit combinations (Egan et al., 2006; Pankratov et al., 2009). Native ATP-induced currents in several types of central neurons, such as neurons from medial habenula and somatosensory cortex, have an exceptionally high Ca\(^{2+}\) permeability (\(P_{Ca}:P_{\text{monovalent}} = \sim10\)-12 (Edwards et al., 1997; Pankratov et al., 2002)), which is comparable to the Ca\(^{2+}\) permeability of N-methyl-D-aspartate (NMDA) receptors. As a result, P2X receptors might provide the most important route for Ca\(^{2+}\) influx at the postsynaptic density at resting membrane potentials when NMDA receptors are unavailable owing to Mg\(^{2+}\) block (Pankratov et al., 2003).
Indeed, stimulation of P2X receptors triggers cytosolic Ca²⁺ signals in many CNS neurons (Pankratov et al., 2009), and P2X-mediated presynaptic Ca²⁺ signals can regulate neurotransmitter release (Sperlágh et al., 2007). The involvement of the P2X7R pore in apoptosis induction via sustained entry of Ca²⁺ into cells is discussed later.

Postsynaptic P2X receptors interact with several ionotropic receptors including nicotinic acetylcholine receptors, GABA_A receptors and NMDA receptors; for all these a reciprocal inhibition was demonstrated. The mechanisms for these interactions might be mediated by intracellular Ca²⁺, by Ca²⁺-activated kinases phosphorylating the receptors, or by direct interactions between receptors molecules (Khakh et al., 2000; Pankratov et al., 2008).

1.2.4. P2X7 receptors

Keller in 1966 was the first to discover that DNA synthesis and cytotoxicity in human and rat lymphocytes and mast cells required high concentrations (100 µM) of extracellular ATP, and was furthermore a receptor-mediated process (Keller, 1966). In 1979, Cockcroft and Gomperts recognized that some of the biological effects induced by extracellular ATP in rat mast cells, above all permeability to metabolites and nucleotides, degranulation and histamine release, also required very high concentrations of ATP, selectively binding to a receptor in the form of ATP₄⁻ (Cockcroft and Gomperts, 1979). These pore-forming, high extracellular ATP receptors were also found in transformed rat fibroblasts (Heppel et al., 1985; Rozengurt and Heppel, 1979). Gordon (1986) later classified all these activities as mediated by a receptor defined as P2Z. Pore-forming P2Z receptors were subsequently demonstrated to play an important role in ATP-mediated plasma membrane permeability and cytotoxicity of mouse lymphocytes (Di Virgilio et al., 1989), and in the modulation of macrophage functions (el-Moatassim and Dubyak, 1992).

Surprenant et al. (1996) subsequently published the cDNA sequence of the rat P2X7R gene, identified as the cytolytic P2Z receptor for extracellular ATP. The following year, Rassendren et al.
(1997) cloned the human gene for a receptor, called $P2X7R$ that was structurally related to the P2X family and exhibited most of the properties of a P2Z receptor. They screened a human monocyte cDNA library with the rat $P2X7R$ gene as a probe, and recovered a cDNA encoding a predicted 595-amino acid protein that was 80% identical to the rat P2X7R protein. $P2X7R$ is expressed as a 6-kb mRNA in many tissues. Buell et al. (1998) determined that the $P2X7R$ gene contains 13 exons, is localized close to the tip of the long arm of human chromosome 12(q24.31) and is <24 kb pairs apart from the $P2X4R$ gene and presumably reflects gene duplication, given that P2X4R and P2X7R subunits are among the most closely related P2X receptor pairs in amino acid sequences (with 48.6% pairwise amino acid identity). **Box 1** presents an ‘identity card’ for the P2X7R.

### 1.2.4.1. Biologically significant coding sequence variants

Genetic variability is important for biodiversity and underlies the differential resistance of organisms to diseases and their sensitivity to toxic agents and conditions, or drugs. Without variability, mediated for instance by homologous recombination or genetic mutations, a population, a gene, or a protein adapts poorly to environmental changes and becomes more prone to extinction. ATP appears to have played a crucial and active role since early evolution, and the progressive generation of a wide range of ATP receptors mediating its extracellular actions has certainly contributed to expand and consolidate ATP functions, while at the same time increasing the potential genetic variability of ATP receptors. **Box 2** summarizes the taxonomic relationships of P2X7R expression.

Regarding P2X7R subtypes, in some healthy subjects the receptor appears to be non-functional in, for example, lymphocytes and monocytes. To study a possible genetic basis for this, in a population study Gu et al. (2001) sequenced the DNA coding for the carboxyl-terminal tail of the P2X7R protein. Unresponsive subjects were characterized by a 1513A-C substitution, which resulted in a glu496-to-ala (E496A) mutation. Monocytes and lymphocytes from E496A homozygote subjects expressed non-functional receptors, whereas heterozygotes showed P2X7R
**BOX 1**

**P2X7R ID chart**

**Chromosomal location:** 12(q24.31) in human, chromosome 12 in chimpanzee, 26 in dog, 17 in cow, 14(q14) in pig, 5(62.5cM) in mouse, 12(q16) in rat, 15 in chicken, 8 in zebrafish.

**Gene structure:** 13 exons (Buell et al., 1998) and multiple splice variants (Cheewatrakoolpong et al., 2005).

**Protein properties:**
- **molecular mass:** 85, 65, and 18 KDa. The 65-KDa P2X7R, the most common isoform, is a mature receptor, whereas the 85-KDa protein is a naive receptor, and the 18-kDa protein is the degraded form. Receptor maturation likely involves glycosylation events, with substantial differences in glycosylation between human and other species (Feng et al., 2005).
- **average isoelectric point:** 8.008, according to DTASelect, EMBOSS, Grimsley, Lehninger, Rodwell, Sillero, Solomon, ProMoST scales; 8.085, as determined by net charge values available from Lehninger's scale.

**Post-translational modifications:**
- phosphorylation: phosphotyrosine at residue 343 results in receptor inactivation (Kim et al., 2001).
- ADP-ribosylation: ADP-ribosylarginine at Arg-125, but not Arg-133, is necessary and sufficient to activate P2X7R and gate the channel (Hong et al., 2009; Seman et al., 2003).
- N-linked glycosylation, at residue 187, 202, 213, 241, 284: N-linked acetyl-glucosamine sites are important for receptor function and essential for trafficking to the cell surface (Lenertz et al., 2010).

**Interactants:**
- ABL1 (Abelson tyrosine-protein kinase 1): non-receptor tyrosine-protein kinase playing a role in cytoskeleton remodeling in response to extracellular stimuli, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response and apoptosis (Wu et al., 2007).
- GRB2 (growth factor receptor-bound protein 2): adapter protein providing a link between cell surface growth factor receptors and Ras signaling pathway (Wu et al., 2007).
- NCK1 (non-catalytic region of tyrosine kinase 1): signalling and transforming cytoplasm protein containing Src homology 2 and 3 domains and involved in transducing signals from receptor tyrosine kinases to downstream recipients such as Ras (Wu et al., 2007).
- EMP3 (hematopoietic neural membrane protein): involved in cell proliferation, cell-cell interactions (Wu et al., 2007).
- PANX1 (pannexin 1): structural component of gap junctions and hemichannels, playing a role as a Ca\(^{2+}\)-leak channel to regulate endoplasmic reticulum Ca\(^{2+}\) homeostasis (Iglesias et al., 2008).
- LAMA3 (extracellular matrix protein laminin alpha3), ITGB2 (membrane-spanning protein integrin subunit beta-2), ACTB (cytoskeletal beta-actin), ACTN4 (cytoskeletal alpha-actinin 4), SVIL (cytoskeletal supervillin), MAGuK (scaffolding protein membrane-associated guanylate kinase P55), heat shock proteins Hsp90, Hsc71 and Hsp70, PIK3C3 (phosphatidylinositol 4-kinase 230) and PTPRβ (membrane-spanning receptor protein tyrosine phosphatase beta) (Kim et al., 2001).

**Cellular components:** bleb, cytoplasm, integral to plasma membrane (Morelli et al., 2003), nuclear envelope (Atkinson et al., 2002).

(Modified from Volontè et al. CNS Neurol Disord Drug Targets 2012)
BOX 2

P2X7R taxonomic groups

- VERTEBRATES
  - MAMMALS
    - Primates Hominidae:
      - Homo: *Homo sapiens* (human) 595 aa
      - Pan: *Pan troglodytes* (chimpanzee) 595 aa
    - Rodents
      - Muridae
        - Mus: *Mus musculus* (mouse) 595 aa
        - Rattus: *Rattus norvegicus* (rat) 595 aa
        - Meriones: *Meriones unguiculatus* (mongolian gerbil) 126 aa
      - Caviidae: *Cavia porcellus* (guinea pig) 594 aa
    - Carnivores: *Canis lupus familiaris* (dog) 595 aa
  - Even-toed ungulates
    - Suidae: *Sus scrofa* (pig) 318 aa
    - Bovidae: *Bos taurus* (cattle) 168 aa
  - Rabbits & hares: *Oryctolagus cuniculus* (rabbit) 595 aa
  - AMPHIBIANS: *Xenopus Laevis* (frog) 553 aa
  - BONY FISHES
    - Cypriniformes: *Danio rerio* (zebrafish) 596 aa
    - Perciformes: *Sparus aurata* (silver seabream) 576 aa
  - AVES: *Gallus gallus* (chicken) 560 aa

Cloned and conserved in: human (Rassendren et al., 1997), chimpanzee, dog (Roman et al., 2009), cow (Zimin et al., 2009), mouse (Chessell et al., 1998b), rat (Surprenant et al., 1996), chicken and zebrafish (Kucenas et al., 2003). Also cloned in Xenopus laevis (only 50% homology to the mammalian receptor) (Paukert et al., 2002), guinea pig (70% homology to the rat receptor) (Fonfria et al., 2008), gerbil, pig, rabbit, and silver seabream (unpublished). There are no reports of homologous sequences from invertebrate species, although there is considerable functional evidence that extracellular ATP and other nucleotides can directly gate ion channels in invertebrates.

(Modified from Volontè et al. CNS Neurol Disord Drug Targets 2012)

function that was half that of germline P2X7R. P2X7R-mediated apoptosis of lymphocytes was impaired in homozygous mutant P2X7R protein compared with germline, while surface expression of P2X7R on lymphocytes was not affected by this E496A polymorphism. Single nucleotide splice site polymorphism at position +1 in intron 1, SNP 151+1g>t, led to a null allele of the P2X7R gene in 1-2% of the Caucasian population (Skarratt et al., 2005). As further examples, Adriouch et al. (2002) demonstrated that splenic T cells from C57BL/6 mice were less sensitive to extracellular ATP-induced calcium uptake and apoptosis than were those from BALB/c mice. The authors identified a single nucleotide allelic mutation, a T-to-C transition at nucleotide 1352, leading to a
pro451-to-leu (P451L) substitution in the long cytoplasmic COOH tail of the P2X7R, which harbors a putative tumor necrosis factor receptor-related death domain. By transfecting Xenopus oocytes with either wild-type or site-directed mutants of P2X7R protein, Worthington et al. (2002) highlighted the importance of various residues in ATP binding to human P2X7R, finding that point mutation of residues K193 and K311 confers loss-of-function in terms of channel/pore activity. The variant lacking the entire C-terminal cytoplasmic tail is generally highly expressed in human tissues and does not effect ion movement; rather, it severely affects the ability to form a large pore and to induce activation for instance of caspases (Cheewatrakoolpong et al., 2005). The variant lacking the first transmembrane domain is also highly expressed in various human tissues and results in impaired channel activity (Cheewatrakoolpong et al., 2005). Conversely, the P2X7R splice variant with an alternative intracellular N terminus and first transmembrane domain encoded by a novel exon 1 in the rodent P2X7R gene, has increased agonist sensitivity and higher propensity to form N-methyl-D-glucamine permeable pores (Nicke et al., 2009). The tissue and cellular distribution of P2X7R in human, rat and mouse is given in Box 3.

1.2.4.2. Ion permeability and current properties

Highly selective for the ATP$^+$ species, stimulation of P2X7R with high concentrations of ATP triggers massive trans-membrane, poorly selective, ion fluxes (particularly influx of Ca$^{2+}$ and Na$^+$, and efflux of K$^+$) and, at lower concentrations, slowly inactivating cation currents, thus exhibiting complex gating kinetics. The ability of P2X7R to act as a direct conduit for Ca$^{2+}$-influx, and indirect activator of voltage-gated Ca$^{2+}$-channel underlies its multiple role in Ca$^{2+}$-based signalling responses. In addition, P2X7R channel opening gives rise to Ca$^{2+}$-independent anion currents, while addition of excess Mg$^{2+}$ closes the receptor channel (Wiley et al., 1996). Extracellular ATP through P2X7R also induces equal efflux and influx of Rb$^+$ (in isotonic KCl medium) and of Na$^+$ (in isotonic NaCl medium) (Sluyter et al., 2004). P2X7R also elicits chloride conductance I (ATP$^+$Cl)$^2$
BOX 3

P2X7R tissue and cell distribution

*Human:*
- high in heart, liver, skeletal muscle, pancreas, thymus, tonsils, monocytes, macrophages, osteoclasts (Buell et al., 1998; Gartland et al., 2003; Rassendren et al., 1997);
- medium in brain, lung, prostate, leukocytes, fibroblasts, dendritic cells, osteoblasts, B lymphocytes, T lymphocytes, keratinocytes, erythrocytes, microglia (Amadio et al., 2010, 2011; Berchtold et al., 1999; Buell et al., 1998; Gartland et al., 2003; Greig et al., 2003; Rassendren et al., 1997; Sluyter et al., 2001, 2004; Solini et al., 1999; Yiangou et al., 2006; Yip et al., 2009);
- low in bladder, astrocytes (Narcisse et al., 2005; O'Reilly et al., 2001).

*Rat:*
- high in newborn and adult brain, bone marrow, retina, salivary glands, parotid gland, lacrimal glands, lung, spleen, pancreas, liver, testis, ependyma, neurons from olfactory nucleus, cerebral cortex, striatum, piriform cortex, lateral septal nucleus, hippocampal pyramidal cells, oligodendrocytes, microglia, macrophages, osteoclasts (Brändle et al., 1998; Cavaliere et al., 2004; Collo et al., 1997; Coutinho-Silva et al., 2003; Emmett et al., 2008; Franke et al., 2004; Frizzo et al., 2010; Hodges et al., 2009; Melani et al., 2006; Naemesch et al., 2001; Sugiyama et al., 2010; Tenneti et al., 1998; Yu et al., 2008).

*Mouse:*
- high in bone marrow, submandibular glands, lung, liver, kidney, macrophages, granulocytes, B lymphocytes, mast cells, microglia, Schwann cells, osteoclasts, osteoblasts (Chessell et al., 1998b; Collo et al., 1997; Colomar et al., 2001; D’Ambrosi et al., 2009; Emmett et al., 2008; Ferrari et al., 1997a; Grol et al., 2009; Hillman et al., 2002; Ke et al., 2003; Sim et al., 2004).

(Modified from Volontè et al. CNS Neurol Disord Drug Targets 2012)

when a Ca$^{2+}$-independent Cl$^-$ channel is gated in the P2X7R directly by external ATP (Reyes et al., 2008). In Na$^+$-free solutions, chloride conductance I (ATPCl) of P2X7R has an apparent anion permeability sequence of SCN$^-$ > I$^-$ > NO3$^-$ > Br$^-$ > Cl$^-$ > acetate (Arreola and Melvin, 2003). The P2X7R is activated and deactivated monophasically at low and biphasecally at higher agonist concentrations. The binding of orthosteric agonists at the ectodomain moreover induces a conformational change in the receptor complex that favours a gating transition from closed, to open, to dilated states. The slow secondary growth of current in the biphasic response coincides temporally with pore dilation. Once a steady level of the secondary current is reached, responses at high agonist concentrations are no longer biphasic but monophasic. Repetitive stimulation with the same agonist concentration causes receptor sensitization, which manifests as a progressive increase
in current amplitude, accompanied by slower deactivation rate. Sensitization of the receptor is independent of Na\(^+\) and Ca\(^{2+}\) influx and about 30 minutes of washout is needed to re-establish the initial gating properties. Thus, the complex pattern of gating exhibited by P2X7R channels includes negative cooperativity of agonist binding to unsensitized receptors (caused by occupancy of one or two binding sites), opening of the channel pore to a low conductance state (when two sites are bound), and sensitization with pore dilation to a high conductance state (when three sites are occupied) (Yan et al., 2010). Figure 3 illustrates the structure and functioning of the P2X7R. Table 1 summarizes the pharmacology of P2X7R.

1.2.4.3. Topology and structure

Understanding receptor structure-function, in this case P2X7R, depends not only on the exact "shape" of the receptor itself, but rather on the way the receptor is "assembled". In essence, a problem of continuity (topology) and connectivity (biology), in other words the properties that are preserved under continuous deformations of objects, and namely the stretching of a receptor on a differently fluid plasma membrane, its conformational twisting after ligand binding, and the layout pattern of interconnections with various elements: the basic characteristics of P2X7R.

In 1997, Hansen et al. (1997) studied the topology of P2X7R on the plasma membrane, establishing that amino acids N-1-25 reside on the cytoplasmic side; amino acids 26-46 constitute the highly hydrophobic transmembrane TM1 region; amino acids 47-334 are responsible for the extracellular loop; amino acids 335-355 form the transmembrane TM2 region; amino acids 356-595-C are present on the cytoplasmic side. The N terminus has residues related to selectivity and activity of the ion channel and interaction with mitogen-activated protein kinases (Amstrup and Novak, 2003). Only one \(\alpha\)-helix is predicted in the TM1 segment, and a major propensity for \(\beta\)-sheet conformation is expected in the TM2 region (Teixeira et al., 2009). Some residues of the
(a) Each functional P2X7R is a trimer, with the three protein subunits arranged around a cation-permeable channel pore. The subunits all share a common topology, possessing two plasma membrane spanning domains (TM1 and TM2), a large extracellular loop with the ATP binding site, and containing 10 similarly spaced cysteines and glycosylation sites, and intracellular carboxyl and amino termini. (b) Brief ATP activation (<10 seconds) of the P2X7R results in rapid and reversible channel opening that is permeable to Na⁺, K⁺, and Ca²⁺. Acute receptor activation also triggers a series of cellular responses, such as depolarization, degranulation, and membrane blebbing, along with signaling cascades (see Figure 3). (c) Continued stimulation results in the formation of a larger plasma membrane pore, which facilitates the uptake of cationic molecules up to 900 Da. ATP-induced IL-1β release is mediated mainly through the activation of IL-1β converting enzyme (also known as caspase-1). Activation of P2X7R triggers the efflux of K⁺ from cells which in turn activates IL-1 converting enzyme, leading to cleavage of pro-IL-1β to mature IL-1β and release from the cell.

(Taken from Skaper et al. FASEB J. 2010)
## Table 1. P2X7R Pharmacology

<table>
<thead>
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<th>Compound</th>
<th>Pharmacological Characteristics</th>
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<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BzATP (3'-O-(4-benzoyle)benzoyl-ATP)</td>
<td>EC50 = 35-617 µM</td>
<td></td>
<td>Donnelly-Roberts et al. (2004)</td>
</tr>
<tr>
<td><strong>Antagonists (updated to June 2012 from <a href="http://www.clinicaltrial.gov">www.clinicaltrial.gov</a>)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSK1482160</td>
<td>(Phase I) for inflammatory pain</td>
<td></td>
<td>Arulkumaran et al. (2011); Ali et al. (2012)</td>
</tr>
<tr>
<td>AZD9056</td>
<td>(Phase II) for rheumatoid arthritis</td>
<td></td>
<td>Arulkumaran et al. (2011); Keystone et al. (2011)</td>
</tr>
<tr>
<td>CE-224,535</td>
<td>(Phase III) for rheumatoid arthritis</td>
<td></td>
<td>Arulkumaran et al. (2011); Stock et al. (2012)</td>
</tr>
<tr>
<td><strong>Antagonists (preclinical phase)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A438079</td>
<td>Competitive antagonist (pIC50 = 6.9 for inhibiting Ca²⁺ influx in human recombinant P2X7R cells)</td>
<td>Antinociceptive role in neuropathic pain <em>in vivo</em> models</td>
<td>Donnelly-Roberts et al. (2007); McGaraughty et al. (2007)</td>
</tr>
<tr>
<td>A740003</td>
<td>Potent, selective and competitive antagonist (IC50 values of 18 and 40 nM for rat and human receptor, respectively)</td>
<td>Antinociceptive in neuropathic pain and inflammatory animal models</td>
<td>Donnelly-Roberts et al. (2009)</td>
</tr>
<tr>
<td>A839977</td>
<td>Potent antagonist of BzATP-evoked calcium influx at recombinant human, rat, mouse receptors (IC50 = 20, 42, 150 nM, respectively)</td>
<td>Antinociceptive in inflammatory pain, rat, mouse models, CNS permeant</td>
<td>Honore et al. (2009); Friedle et al. (2010)</td>
</tr>
<tr>
<td>AZ-1060620</td>
<td>Potent antagonist and negative allosteric modulator (KD = 1.4 and 19 nM at human and rat receptor, respectively). It cooperatively binds to sites distinct but coupled to the ATP binding sites</td>
<td></td>
<td>Michel et al. (2008)</td>
</tr>
<tr>
<td>AZ-11645373</td>
<td>Potent selective antagonist (KB = 5-7 and &gt; 10,000 nM at human and rat, respectively). It inhibits BzATP-calcium influx, ATP- IL-1β release <em>in vitro</em> (KB =15 and 92 nM at human and rat, respectively)</td>
<td></td>
<td>Michel et al. (2009)</td>
</tr>
<tr>
<td>Oxidized ATP</td>
<td>Irreversible inhibitor, covalent modification of receptor (IC50 = 30 µM)</td>
<td></td>
<td>Murgia et al. (1993)</td>
</tr>
<tr>
<td>KN-62</td>
<td>Non-competitive antagonist (IC50=15 nM). Selective, cell-permeable inhibitor of CaM kinase II (IC50 = 0.9 µM)</td>
<td></td>
<td>Chessell et al. (1998a)</td>
</tr>
<tr>
<td>Modulators</td>
<td></td>
<td></td>
<td>Reference</td>
</tr>
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<tr>
<td><strong>Brilliant Blue G</strong></td>
<td>Non-selective antagonist (IC50 = 10 nM and 267 nM at rat and human receptor, respectively)</td>
<td>Protects cortical neurons from BzATP-activated microglia</td>
<td>Friedle et al. (2010); Skaper et al. (2006); Gunosewoyo et al. (2007); Takenouchi et al. (2010)</td>
</tr>
<tr>
<td><strong>Modulators</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Calcium</strong></td>
<td>At physiological concentrations acts as negative allosteric modulator by decreasing the affinity for orthosteric agonists</td>
<td></td>
<td>Yan et al. (2011)</td>
</tr>
<tr>
<td><strong>Zinc, Copper</strong></td>
<td>Potent inhibitors at submicromolar ranges, due to interaction with ectodomain His(62) and Asp(197)</td>
<td></td>
<td>Liu et al. (2008)</td>
</tr>
<tr>
<td><strong>Extracellular acidification</strong></td>
<td>Functional inhibition by acidic pH is potently affected by the extracellular His(85), Lys(110), Lys(137), Asp(197), His(219) residues, with Asp(197) residue being the most critical one</td>
<td></td>
<td>Liu et al. (2009)</td>
</tr>
<tr>
<td><strong>Extracellular anions</strong></td>
<td>Chloride, iodide, nitrate, sulfate inhibit ATP-induced human P2X7R-mediated currents, allosterically affecting channel opening in fully ATP^4- liganded P2X7R, through extracellular anion binding site</td>
<td></td>
<td>Kubick et al. (2011)</td>
</tr>
<tr>
<td><strong>Compound-17:</strong></td>
<td>(N-[2-[(2-hydroxyethyl)amino]ethyl]amino)-5-quinolinyl]-2-tricyclo[3.3.1.1(3,7)]dec-1-ylacetamide</td>
<td>Negative allosteric modulator of human and rat P2X7R</td>
<td>Michel et al. (2008)</td>
</tr>
<tr>
<td><strong>GW791343:</strong></td>
<td>(N(2)-(3,4-difluorophenyl)-N(1)-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamidedihydrochloride)</td>
<td>Negative allosteric modulator of human P2X7R (pIC50=6.9-7.2), but positive allosteric modulator at rat P2X7R</td>
<td>Michel et al. (2008)</td>
</tr>
<tr>
<td><strong>PIP2</strong></td>
<td>Activator of ATP-gated currents, mutations of charged residues in C-terminus of P2X7R reduces the apparent affinity for PIP(2) and ATP-mediated cell death</td>
<td></td>
<td>Zhao et al. (2007)</td>
</tr>
<tr>
<td><strong>Decavanadate</strong></td>
<td>Reversible, competitive antagonist (against pyridoxal 5-phosphate, oxidised ATP, but not KN62)</td>
<td></td>
<td>Michel et al. (2006)</td>
</tr>
<tr>
<td><strong>Propofol</strong></td>
<td>Intravenous anesthetic, increases P2X7R current amplitudes and inactivation times</td>
<td></td>
<td>Liu et al. (2012)</td>
</tr>
<tr>
<td><strong>Clemastine (H1 antihistaminic)</strong></td>
<td>Positive allosteric modulator</td>
<td></td>
<td>Nörenberg et al. (2011)</td>
</tr>
</tbody>
</table>

(Adapted from Volontè et al. CNS Neurol Disord Drug Targets 2012)
extracellular loop displaying three different binding sites for ATP are glycosylated in the ATP-interacting sequence. The intracellular COOH terminus (239 amino acids) is much longer than in all other P2XR subtypes, and is involved in the majority of functions related to P2X7R and contains an additional hydrophobic domain (residues 510–530) sufficiently long to traverse the plasma membrane (Costa-Junior et al., 2011).

Substantial evidence supports a trimeric structure for P2X7R, although scattered evidence suggests that it might aggregate to form examers (Kim et al., 2001b). It has been suggested that it forms heteromers with P2X4R (Casas-Pruneda et al., 2009; Dubyak, 2007; Guo et al., 2007). However, in another study using subtype-specific antibodies in combination with blue native polyacrylamide gel electrophoresis to directly visualize P2X receptor complexes solubilized from membrane extracts of a wide variety of tissues, homotrimeric complexes were the dominant assembly state of P2X7R complexes (Nicke, 2008). No complexes corresponding to more than three subunits or heterotrimeric P2X4R/P2X7R were detected, suggesting that either higher heteromerization between P2X4R and P2X7R subunits results in unstable heteromeric complexes, or that such P2X4R/P2X7R heteromers do not represent a dominant subtype in the tissues investigated (Nicke, 2008). However, a variety of proteins interacting with the P2X7R have been identified by immunoprecipitation of P2X7R over-expressed in HEK cells (Kim et al., 2001a) and, moreover, in a yeast two-hybrid screen (Wilson et al., 2002). Transient interaction via one of these proteins could also account for these copurification results (Guo et al., 2007). The potential contribution of recently identified splice variants of the human P2X7R (Cheewatrakoolpong et al., 2005) to subunit assembly represents another element for consideration. Further studies will be needed to resolve this issue.

In contrast to other P2X receptors, but in certain aspects similar to the activation profile found in some cells expressing P2X2R and P2X4R, stimulation of the P2X7R subtype with high concentrations of ATP is associated with two different membrane permeability states: a small non-selective monovalent and divalent cation conductance which opens within milliseconds after brief
agonist stimulation, leading to depolarization of the plasma membrane (North, 2002), followed by increased non-selective membrane permeability to larger cations such as N-methyl-D-glucamine (molecular weight 195) after prolonged and repetitive agonist stimulation (Rassendren et al., 1997). Membrane permeability increases with time, allowing cellular uptake of higher molecular weight fluorescent dyes such as ethidium bromide (molecular weight 394) or Yo-Pro-1 (molecular weight 629). This phenomenon has been attributed to the formation of large cytolytic pores in the plasma membrane, leading to cell death (Pelegrin and Surprenant, 2009; Virginio et al., 1999). This time-dependent increase in permeability has been ascribed to two contrasting mechanisms. The first model allows the coexistence of two functions (channel and large pore) within a single structure (Teixeira et al., 2009) and predicts that a conformational change initially forms a channel permeable to small cations, then leading to dilation of the integral P2X7R pore. When ATP at high concentration is linked to P2X7R, eliciting cation influx and intracellular signalling cascades, the β-sheet structure in the TM2 region then assumes a configuration allowing the passage of molecules up to 1 KDa, with the channel transiently acting as a large pore. However, P2X7R-mediated pore formation was reported to result from a coordinated signalling cascade involving both the p38 mitogen-activated protein kinase and caspase pathways that is distinct from other cytolytic pore-forming mechanisms. A selective p38 (mitogen-activated protein kinase) MAPK inhibitor indeed potently inhibits receptor agonist BzATP-induced pore formation, without altering P2X7R-mediated calcium influx or interleukin-1β (IL-1β) release. In contrast, caspase inhibitors attenuate both BzATP-induced pore formation and IL-1β release. Taken together, these results support the hypothesis that downstream signalling mechanisms, rather than channel dilation, mediate cytolytic pore formation after prolonged agonist activation (Donnelly-Roberts et al., 2004). Moreover, pore opening does not occur in all cell types and may be dependent upon receptor density (North, 2002). P2X7R-mediated changes in calcium influx and pore-opening are species-specific, showing different pharmacological properties between recombinant mouse, rat and human P2X7R (Donnelly-Roberts and Jarvis, 2007).
An alternative mechanism to pore-opening postulates the activation of a distinct channel protein permeable to higher molecular weight cations (North, 2002). In this scenario, P2X7R is responsible only for permeability to small cations and interacts instead (directly or through second messengers) with this distinct channel protein, thereby allowing permeability to larger cations. At present, there is no widely accepted hypothesis to explain this phenomenon, and evidence for and against these two models has been forthcoming. A study by Marques-da-Silva et al. (2011) shows that colchicine, independently from disruption of cytoskeletal microtubules, inhibits P2X7R-dependent dye uptake without affecting receptor channel ionic currents, thus supporting the hypothesis of a distinct permeation pathway for high MW dyes. However, the molecular nature of this permeation pathway remains unknown. One of the P2X7R activated pore pathways has been attributed to the opening of pannexin-1 (Panx1) hemichannels, allowing the passage of ions and small molecules such as ATP between the intracellular and the extracellular space (Pelegrin and Surprenant, 2006). This permits further P2X7R activation and induces physiological responses such as spreading of cytoplasmic calcium waves. In particular, the release of ATP through the interaction between Panx1 and P2X7R leads to the release of IL-1β involved in early stages of innate immunity (Iglesias et al., 2008; Pelegrin and Surprenant, 2006). However, transient P2X7R activation and Ca$^{2+}$ overload can act as a death trigger for native mouse macrophages independently from Panx1 recruitment (Hanley et al., 2012). Gulbransen et al. (2012) using in vivo models of experimental colitis, reported that inflammation causes enteric neuron death by activating a neuronal signaling complex composed of P2X7R, Panx1 channels, the Asc adaptor protein and caspases. Inhibition of P2X7R, Panx1, Asc or caspase activity prevented inflammation-induced neuronal death. Preservation of enteric neurons by inhibiting Panx1 in vivo prevented the onset of inflammation-induced colonic motor dysfunction. The authors concluded that activation of neuronal Panx1 underlies neuron death.

Yan and colleagues (2010) have formulated a mathematical model to harmonize the above studies. Using P2X7R transfected cells, responses to single applications of increasing concentrations of BzATP were analyzed utilizing the Markov state mathematical model. This model
reproduced the complex pattern of P2X7R gating, including the initial rise in current \(I_1\) and decrease in \(I_2\) amplitude with elevation in agonist concentration, or the transition from biphasic to monophasic signalling during repetitive agonist application. All receptor responses to the P2X7R-specific antagonist AZ10606120 were also reproduced by the model. Based on evidence that the P2X7R intrinsic pore can dilate over time in physiological conditions, Yan et al. (2010) modelled the channel in terms of mutable symmetry within the trimeric receptor. When no ATP is bound, P2X7R is symmetrical and closed. When one ATP is bound, the receptor is distorted in such a way as to reduce the affinity of the remaining binding sites and remains closed. Binding of the second ATP molecule thus requires a higher concentration but leads to further distortion of the receptor (additionally decreasing the affinity of the third site), and to the opening of a low-conductance pore permeable to small cations. A five-fold difference in the potency of agonist for \(I_1\) and \(I_2\) rise supports the concept of asymmetry, and small amplitude monophasic currents observed at low agonist concentrations support the presence of a low-conductance state. When the third ATP binds, the symmetry is restored, putatively relieving the mechanical stress, and the receptor is fully activated with the pore dilated to the high conductance state. The restoration of symmetry may thus facilitate an unknown persistent change of state that is required for dilation (Yan et al., 2010). This model would explain the facilitation observed for activation of the P2X7R (Roger et al., 2010), the two components of the P2X7R-associated current and the opposite effects of P2X7R on cellular functions, such as cell growth and differentiation (occupancy of two ATP binding sites) versus cell death (occupancy of 3 ATP binding sites accompanied by pore dilation).

1.2.4.4. P2X7R signaling

In macrophages/monocytes, P2X7R stimulation rapidly activates c-Jun N-terminal kinases 1 and 2 (Humphreys et al., 2000), extracellular signal-regulated kinase (ERK-1/2), and p38 MAPK (Aga et al., 2002). The P2X7R agonist BzATP activates the same pathways in mouse N9 microglia (Potucek et al., 2006), and increases the nuclear translocation of NF-κB in mouse BV-2 microglia.
Dephosphorylation of NFAT (nuclear factor of activated T cells) by calcineurin exposes a nuclear localization sequence, permitting nuclear translocation and transcriptional activation (Rao et al., 1997). In N9 cells, ATP activates NFAT via the P2X7R in a calcineurin-dependent fashion (Ferrari et al., 1999). The cyclic AMP response element- (CRE-) binding protein (CREB), a member of the activating transcription factor 1 family of transcription factors, is involved in cytokine gene regulation (Mayr and Montminy, 2001). P2X7R-dependent induction of CREB and activating transcription factor 1 phosphorylation occurs in BV-2 cells via an MAPK kinase (MEK)/ERK-dependent pathway (Potucek et al., 2006). Activation of activator protein-1 (AP-1) is another transcription factor associated with regulation of inflammatory genes (Foletta et al., 1998). Multiple members of the c-Fos and c-Jun families dimerize to form AP-1. In serum-starved Jurkat T-cells, activation of P2X7Rs induced AP-1 DNA binding activity as a result of increased c-Jun and c-Fos expression (Budagian et al., 2003). ATP treatment also increased the phosphorylation of ERK-1/2 and c-Jun N-terminal kinases 1 and 2, but not p38 MAPK, providing a potential mechanism for these effects (Figure 4).

Stimulation of P2X7Rs increases protein tyrosine phosphorylation (Adinolfi et al., 2003; Watters et al., 2001) ultimately leading to MAPK pathway activation. Many events downstream of P2X7R activation are dependent upon extracellular calcium influx (Ferrari et al., 1999; North, 2002), and activation of MAPK pathways by P2X7Rs may involve calcium signaling. In RAW 264.7 macrophages, the calcium-dependent kinase Pyk2, which facilitates Ras activation, is tyrosine phosphorylated in response to treatment with BzATP (Aga et al., 2004; Watters et al., 2001), potentially linking calcium fluxes, Ras activation, and MAPK pathways with P2X7Rs. P2X7Rs also induce the activation of other small molecular weight G-proteins. For example, the Rho/p38 pathway may be involved in the shedding of IL-1β-containing vesicles (Pfeiffer et al., 2004) through actin filament reorganization and membrane blebbing (MacKenzie et al., 2004; Pfeiffer et al., 2004), conceivably providing a mechanism whereby MAPKs can mediate increased microglial pro-inflammatory cytokine release.
Figure 4. Schematic depiction of the signal transduction events occurring in microglia following P2X7R activation

Extracellular calcium influx triggered by activation of ionotropic P2X7Rs leads to activation of calcineurin and dephosphorylation/activation of NFAT (nuclear factor of activated T cells). P2X7R activation also results in activation of phospholipases A2 and D (PLA2, PLD), as well as tyrosine phosphorylation (P-Tyr) and activation of mitogen-activated protein kinase (MAPK) pathway proteins (MAPK kinase, MEK; extracellular signal-regulated kinase, ERK). The latter can then influence the activity of transcription factors like NF-κB (nuclear factor-κB), CREB (cyclic AMP response element (CRE)-binding protein), and AP-1 (activator protein-1) which upregulate expression of pro-inflammatory genes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Activation of P2X7Rs also leads to p38 MAPK activation with consequent phosphorylation/activation of CREB. Broken lines indicate multistep pathways.

(Taken from Skaper and Giusti, Cardiovasc. Psychiatry Neurol. 2009)
The P2X7R engages several disparate functions in many distinct cell populations, or even in the same cell type, comprising a complex role in the nervous system and neuroinflammation (Duan and Neary, 2006; Le Feuvre et al., 2002, Skaper et al., 2010; Weisman et al., 2012). From basic extracellular ATP binding, protein oligomerization and pore complex assembly, ATP-gated ion channel transport and conductance, membrane depolarization, and calcium signalling, the functions of P2X7R extend to lipopolysaccharide (LPS) binding, regulation of MAPK kinase cascades, caspases and phospholipases, production of free radicals, and IL-1β secretion. While LPS is an efficient stimulus for IL-1β release, rodent microglial cells secrete predominantly the inactive 33-kDa precursor of IL-1β in response to LPS (Chauvet et al., 2001). Indeed, activation of P2X7R is one of the most powerful stimuli for secretion of IL-1β in its mature form (Ferrari et al., 2006; Perragaux and Gabel, 1994). However, ATP is an efficient stimulus for IL-1β secretion only after the cells have undergone a short 'priming' with LPS (Ferrari et al., 1997b, 2006; Sanz and Di Virgilio, 2000). The priming response may be relevant for neuroinflammation, as tissue injury can activate Toll-like receptors (TLRs), including TLR4, on glia (Casula et al., 2011).

Specifically within different tissues, P2X7R can then regulate cell cycle, apoptosis, lysis of antigen-presenting cells and killing of foreign cells, T cell maturation, activation of innate immune responses, epithelial secretion, regulation of bone resorption and mineralization, fast synaptic transmission, sensory perception of pain, as well as playing a role in neurological diseases. Since its discovery in lymphocytes and mast cells (Cockcroft and Gomperts, 1979; Keller, 1996), the P2X7R has been viewed as a key mediator of inflammation and immunity (Arulkumaran et al., 2011; Di Virgilio, 1995; Surprenant et al., 1996). Rassendren et al. (1997) indeed found that treatment of cloned P2X7R-transfected human embryonic kidney cells and human macrophages with ATP or BzATP elicited cation-selective currents, while longer agonist application caused noxious permeabilization of the cells, thus establishing a toxic role for the receptor. In the nervous system,
mRNA encoding P2X7R was detected by situ hybridization in excitatory synaptic terminals in CA1 and CA3 regions of rat hippocampus targeting dendrites of pyramidal cells and parvalbumin-labelled structures, and regulating the release of glutamate and γ-aminobutyric acid. P2X7R mRNA has also been detected in hippocampal inhibitory neurons (Sperlágh et al., 2002). Sugiyama et al. (2002) provided evidence that P2X7R activation in rat retinal microvessels triggers apoptosis with lethal consequences. Lemaire et al. (2006), using rat lung alveolar macrophages expressing native P2X7R and human embryonic kidney cells ectopically expressing full-length rat P2X7R or a C-terminally truncated P2X7R mutant, showed that the P2X7R is involved in the fusion process leading to multinucleated giant cells. This process contributes to many important biological mechanisms in mammals during both normal processes and disease such as the development of multinucleated osteoclasts during bone resorption, or the fusion of macrophages during granulomatous inflammation.

Apart from the detrimental functions ascribed to P2X7R, trophic properties apparently coexist. Depending on its level of activation, P2X7R may induce cell proliferation or apoptosis. For example, P2X7R activation has been correlated with disease severity in B-cell chronic lymphocytic leukaemia due to cell cycle stimulation, together with increased intracellular calcium fluxes, plasma membrane depolarization, formation of a nonselective membrane pore and proliferation in neuroblastoma cell lines (Raffaghello et al., 2006). In microglia, using a P2X7R mutant (P2X7R-G345Y) with intact channel function but ablated pore-forming capacity, Monif et al. (2009) provided evidence that the trophic effects of P2X7R expression are exclusively mediated by pore conductance and not by the cation channel. Moreover, the authors reported that P2X7R overexpression, in the absence of pathological insults, was sufficient to drive activation and proliferation of microglia in rat primary hippocampal cultures; culture treatment with the antagonist oxidized ATP significantly decreased the number and activation of microglia. Ortega et al. (2011) demonstrated that stimulation of cerebellar granule neurons with BzATP, similarly to brain-derived neurotrophic factor, increased ERK1/2 phosphorylation and protected from excitotoxic
concentrations of glutamate, indicating that P2X7R shares survival pathways with trophic factors. Using a selective P2X7R antagonist and small interfering RNA knockdown of P2X7R, Thompson et al. (2012) reported a pro-survival role in mouse embryonic stem cells in the presence of leukemia inhibitor factor. However, chronic exposure to exogenous ATP still led to rapid P2X7R-dependent necrotic cell death. Further, these data demonstrate the dual role for P2X7R as a pro-survival or pro-death signal, depending on its mode of activation. Finally, when the C-terminal truncated P2X7R splice variant (also called isoform B or ΔC) that is widely distributed especially in human immune and nervous systems, was over-expressed in HEK293 cells, it mediated ATP-stimulated channel activity but not plasma membrane permeabilization, raised endoplasmic reticulum Ca\(^{2+}\) content, increased the cellular ATP pool and especially stimulated growth. Consistently, P2X7R expression increased after mitogenic stimulation of peripheral blood lymphocytes (Adinolfi et al., 2010).

1.2.4.6. P2X7R and pathological states

1.2.4.6.1. Polymorphisms in human diseases

The P2X7R gene is highly polymorphic with many single nucleotide polymorphisms (SNPs) affecting receptor function and activity having been documented (www.ncbi.nlm.nih.gov/SNP). For example, a role for P2X7R has been demonstrated in susceptibility to infections with intracellular pathogens such as tuberculosis. Saunders et al. (2003) found that neither apoptosis nor killing of mycobacteria occurred after brief exposure to ATP in macrophages from individuals homozygous for inheritance of the 1513A>C loss-of-function polymorphic variant of P2X7R, resulting in the glu496-to-ala (E496A) substitution. E496A mutation moreover confers increased lifetime risk of extra-pulmonary tuberculosis (Fernando et al., 2007; Niño-Moreno et al., 2007). A recent study on papillary thyroid carcinoma revealed strong association between E496A polymorphism and follicular variant of this carcinoma (Dardano et al., 2009). Cabrini et al. (2005) noted that in patients affected by chronic lymphocytic leukaemia, several other polymorphisms in addition to E496A cause receptor loss of function. Moreover, these authors established that the 489C-T polymorphism,
which causes a His155-to-Tyr (H155Y) change in the extracellular portion of the receptor, corresponds instead to a gain-of-function polymorphism as assessed both by $[\text{Ca}^{2+}]_i$, influx and ethidium bromide uptake. The P2X7R plays a role also in bone homeostasis and disease, and recent studies have suggested its function as a mechano-transducer in osteocytes. Inheritance of loss-of-function $P2X7R$ variants E496A and I568N (Ile568-to-Asn) is associated with increased bone fracture risk in post-menopausal females (Ohlendorff et al., 2007; Nissen et al., 2009). Allelic, genotypic or family-based case-control association studies using synonymous and non-synonymous SNPs revealed $P2X7R$ as a susceptibility gene in the nervous system for mood disorders. Three studies with a total of 2,500 patients with bipolar or major depressive disorders found disease association with a non-synonymous SNP (rs2230912) within the coding region of the $P2X7R$ gene. This SNP codes for Gln460-to-Arg (Q460R) substitution in the carboxyl terminus of the receptor that is conserved between humans and rodents and is essential for receptor function (Barden et al., 2006; Lucae et al., 2006; McQuillin et al., 2009). Finally, a recent study found that $P2X7R$ gene variants resulting in receptor gain-of-function display increased frequency in multiple sclerosis (Oyanguren-Desez et al., 2011).

1.2.4.6.2. Animal models

Studies on animal models lacking the P2X7R derive from two different strains of P2X7R knockout (KO) mice generated by Solle et al. (2001) and Chessell et al. (2005), respectively. Solle and colleagues generated their mice by inserting a neomycin cassette into exon 13, replacing a region that encodes Cys-506–Pro-532 of the intracellular C terminus of the receptor. In the mouse line generated by Chessell and coworkers, the $P2X7R$ gene was knocked out by insertion of a lacZ transgene into exon 1. The former model demonstrated P2X7R involvement in bone formation, inflammation, and mood disorders, while the study by Chessell et al. (2005) established a role for the receptor in inflammatory and neuropathic pain. While the P2X7R was knocked out in both
mouse strains, at least two functional receptor splice variants seem to have escaped deletion in these KO mice. The P2X7R splice variant with an alternative intracellular N-terminus and first transmembrane domains (expressing increased agonist sensitivity and higher propensity to form permeable pores) escapes inactivation (Nicke et al., 2009). On the other hand, the KO mice utilized by Solle et al. (2001) are not completely null for P2X7R expression but express C-terminal truncated (ΔC) variants of the receptor with reduced function (Masin et al., 2012).

Macrophages from the mutant mice of Solle et al. (2001) failed to respond to extracellular ATP, as measured by fluorescent dye accumulation. In addition, after ATP or LPS stimulation, macrophages from these P2X7R-deficient mice produced levels of cyclooxygenase-2 and accumulated 35-kD pro-IL-1β in amounts comparable to wild-type mice, but did not secrete the mature 17-kD IL-1β form, due to impaired post-translational processing. Likewise, mutant mice primed with LPS and challenged with ATP failed to generate significant levels of IL-1β, but not of IL-6, by impairment of cytokine signalling cascades (Solle et al., 2001). Absence of the P2X7R gene moreover alters leukocyte function and attenuates inflammatory responses (Labasi et al., 2002). P2X7R KO mice also demonstrate a unique skeletal phenotype that involves deficient periosteal bone formation together with excessive trabecular bone resorption (Ke et al., 2003). Lack of P2X7R prevents ATP-evoked γ-aminobutyric acid and glutamate release in the hippocampus (Papp et al., 2004) and microglia activation by amyloid β-peptide (Sanz et al., 2009), but does not change survival rate or depletion of striatal endogenous dopamine content after in vivo dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) or in vitro rotenone treatment (Hracskó et al., 2011). P2X7R−/− mice are resistant to contact hypersensitivity, and injection of IL-1β restores the capacity to develop contact hypersensitivity. P2X7R−/− dendritic cells also fail to release IL-1β in response to LPS and ATP, suggesting that the P2X7R is a crucial receptor for extracellular release of ATP and IL-1β in skin in response to contact allergens (Weber et al., 2010). Absence of P2X7R gene moreover has protective effects in different experimental
models of lung inflammation (Lucattelli et al., 2011; Monção-Ribeiro et al., 2011; Muller et al., 2011; Riteau et al., 2010), affects fluid secretion in pancreas, salivary glands and tear glands (Novak et al., 2010), exhibits an antidepressant-like profile (Basso et al., 2009) and shows a mood-stabilizing phenotype alleviating stress-induced responses in several behavioural models (Csölle et al., 2012).

Chessell et al. (2005) showed that chronic inflammatory (in an adjuvant-induced model) and neuropathic (in a partial nerve ligation model) hypersensitivity was completely absent to both mechanical and thermal stimuli, while normal nociceptive processing was preserved in P2X7R−/− mice. Although KO mice were still able to produce pro-IL-1β mRNA, release of IL-1β and IL-10 was impaired, with systemic reductions in adjuvant-induced increases in IL-6 and monocyte chemotactic protein-1 (Chessell et al., 2005). Conversely, Hansen et al. (2011) found that BALB/cJ P2X7R-deficient mice were susceptible to bone cancer pain and, moreover, had an earlier onset of pain-related behaviours compared to cancer-bearing wild-type mice. These findings support the notion that bone cancer pain is a separate pain state compared with neuropathic and inflammatory pain. Finally, lack of P2X7R gene attenuated renal injury in experimental glomerulonephritis (Taylor et al., 2009). Physiopathological correlations of altered P2X7R gene or protein expression are summarized in Table 2.

1.2.4.6.3. Neurodegenerative movement disorders

Parkinson’s disease (PD) and Huntington’s disease (HD) are the two most common chronic progressive neurodegenerative movement disorders. PD is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of protein inclusions called Lewy bodies. HD, on the other hand, is associated with atrophy of the striatum and cerebral cortex, which leads to a loss of motor control, deterioration in cognitive function, and dementia (Burnstock, 2008).
Mitochondrial dysfunction and neuroinflammation have been implicated in PD pathophysiology. Midbrain astrocytes have been described to express the P2X7R, and exposure to rotenone (a mitochondrial poison and PD model) increased receptor current density and inhibited the secretion of TNF-α (Gao et al., 2011). In a study by Hracskó et al. (2011), in vivo 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment (a dopaminergic toxin and model of PD) increased the mRNA expression of P2X7R in the striatum and substantia nigra of wild-type mice. Genetic deletion or pharmacological inhibition of P2X7R, however, did not change survival rate or depletion of striatal endogenous dopamine content after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment.

There is little information on a potential role for the P2X7R in HD, although in one study increased P2X7R levels and altered receptor-mediated calcium permeability in somata and terminals of neurons from HD mutant mice was observed (Díaz-Hernández et al., 2009). Furthermore, cultured neurons expressing mutant huntingtin showed increased susceptibility to apoptosis triggered by P2X7R stimulation. In vivo administration of the P2X7R-antagonist Brilliant Blue-G to HD mice prevented neuronal apoptosis and attenuated body weight loss and motor-coordination deficits, suggesting that altered P2X7R levels and function contribute to HD pathogenesis.

1.2.4.6.4. Epilepsy and neuropsychiatric disorders

PanX1, a vertebrate homologue of the invertebrate innexin gap junction proteins, acts as a channel and can be opened at the resting membrane potential by ATP via the P2X7R, as previously described. Besides causing cell death, Panx1 opening induces aberrant bursting in vitro (Thompson et al., 2008). It has been suggested that the P2X7R-Panx1 complex may play an important role as a negative modulator of muscarinic acetylcholine M1 receptor-mediated seizure activity in vivo, as P2X7R KO mice showed greater susceptibility to seizures induced by pilocarpine, an M1 receptor
<table>
<thead>
<tr>
<th>Variation</th>
<th>Tissue/Cell</th>
<th>Physiopathological Action(s)</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td><strong>Gene deletion</strong></td>
<td>macrophages, microglia</td>
<td>defective IL-1β release,</td>
<td>Solle et al. (2001); Sanz et al. (2009); Fairbairn et al. (2001)</td>
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<tr>
<td></td>
<td></td>
<td>defective intraphagosomal killing</td>
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<td></td>
<td>joint tissue</td>
<td>reduced experimental arthritis</td>
<td>Labasi et al. (2002)</td>
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<td></td>
<td>long bones</td>
<td>defective bone homeostasis</td>
<td>Ke et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>cornea</td>
<td>epithelial migration and stromal organization</td>
<td>Mayo et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>reduced smoke-induced lung inflammation</td>
<td>Lucattelli et al. (2011)</td>
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<tr>
<td></td>
<td>liver</td>
<td>reduced autoimmune hepatitis</td>
<td>Kawamura et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td>reduced inflammation and fibrosis following ureteral obstruction,</td>
<td>Gonçalves et al. (2006); Taylor et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>defective experimental encephalomyelitis, anti-depressant-like behaviour, spatial memory impairment, mood-stabilizing phenotype</td>
<td>Sharp et al. (2008); Basso et al. (2009); Labrousse et al. (2009); Csöllő et al (2012)</td>
</tr>
<tr>
<td></td>
<td>nervous system</td>
<td>reduced neuropathic pain</td>
<td>Chessell et al. (2005)</td>
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<tr>
<td><strong>RNA interference</strong></td>
<td>mouse microglia</td>
<td>decreased proliferation</td>
<td>Bianco et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>rat spinal cord</td>
<td>reduced long term potentiation and allodynia</td>
<td>Chu et al. (2010)</td>
</tr>
<tr>
<td><strong>Upregulation</strong></td>
<td>human neuroblastoma cells</td>
<td>Carcinomas</td>
<td>Raffaghello et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>human spinal cord and cerebral cortex</td>
<td>amyotrophic lateral sclerosis,</td>
<td>Yangou et al. (2006); D’Ambrosi et al. (2006); Amadio et al. (2010, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>multiple sclerosis</td>
<td></td>
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<td></td>
<td>human B lymphocytes</td>
<td>chronic lymphocytic leukaemia</td>
<td>Adinolfi et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>human kidney</td>
<td>autosomal recessive polycystic kidney disease</td>
<td>Hillman et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>rat hippocampus, striatum and frontoparietal cortex</td>
<td>hypoxic/hypoglycaemic damage and perinatal rat asphyxia</td>
<td>Cavaliere et al. (2004); Melani et al. (2006); Frizzo et al. (2010)</td>
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<tr>
<td></td>
<td>mouse retina</td>
<td>retinal degeneration</td>
<td>Franke et al. (2005)</td>
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agonist, than their wild-type littermates (Kim and Tang, 2011). Further, administration of P2X7R antagonists and gene silencing of P2X7R or Panx1 in wild-type mice increased pilocarpine-induced seizure susceptibility (Burnstock, 2007; but see Engel et al., 2012).

The case for P2X7R gene association with neuropsychiatric diseases is controversial. Several linkage and association studies have claimed an association with bipolar- and unipolar affective disorders (McQuillin et al., 2009; Soronen et al., 2011) and depression (Burnstock et al., 2011; Hejjas et al., 2009; Lucae et al., 2006). In addition, P2X7R KO mice are reported to display alteration in mood-related behaviour (Csölle et al., 2012) an anti-depressant-like profile, but no significant differences between genotypes were observed in models of anxiety (Basso et al., 2009). In contrast, other studies have failed to find association between P2X7R gene polymorphisms and major affective disorders (Grigoroiu-Serbanescu et al., 2009; Viikki et al., 2011).

1.2.4.6.5. Amyotrophic lateral sclerosis and multiple sclerosis

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive degeneration of motor neurons in the spinal cord, brainstem and motor cortex, leading to respiratory failure and death of affected patients within a few years from diagnosis (Cozzolino et al., 2012). The discovery of mutations in the gene encoding the antioxidant enzyme Cu/Zn superoxide dismutase-1 (SOD1) in a subset of patients with familial ALS has led to the development of transgenic animal models expressing different SOD1 mutations (Gurney, 1997). Accumulating evidence indicates that non-neuronal cells contribute to motor neuron dysfunction and death in ALS, by the maintenance of a chronic inflammatory response (Boillée et al., 2006). Extracellular ATP levels markedly increase in the nervous system in response to ischemia, trauma and inflammatory insults (Burnstock, 2008), although the situation in ALS has not been studied. In ALS patients as well as SOD1G93A animals, increased immunoreactivity for P2X7R has been found in spinal cord microglia (Yiangou et al., 2006). Furthermore, SOD1G93A microglia in culture display an increased sensitivity to ATP, and P2X7R activation drives a pro-inflammatory activation that leads to decreased survival of neuronal
cell lines (D'Ambrosi et al., 2009). Moreover, P2X7R activation in spinal cord astrocytes has been described to initiate a neurotoxic phenotype that leads to motor neuron death (Gandelman et al., 2010). The neurotoxic phenotype of SOD1\textsuperscript{G93A} astrocytes depended upon basal activation of the P2X7R.

Multiple sclerosis (MS) is a chronic, degenerative disease of the CNS, which is characterized by focal lesions with inflammation, demyelination, oligodendrogial death and axonal degeneration (Nylander and Hafler, 2012). Although the etiology of the disease is still unknown, both genetic and environmental factors contribute to MS susceptibility. In addition, blockade of P2X7R prevents ATP excitotoxicity to oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis (Matute et al., 2007) also in a P2X7R KO model (Sharp et al., 2008). Increased P2X7R immunoreactivity has been observed in post-mortem spinal cord microglia of MS patients (Matute et al., 2007; Yiangou et al., 2006) (Figure 5). From a therapeutic perspective, a new study reports that glatiramer acetate modulates monocyte P2X7R expression in MS (Caragnano et al., 2012).

1.2.4.6.6. Neuroblastoma

Neuroblastoma is the most common extracranial tumor of childhood, derived from the sympathetic nervous system, often characterized by low response to conventional treatments and poor prognosis. P2X7R is expressed in neuroblastoma primary tumors and cell lines (Raffaghello et al., 2006; Sun, 2010), and functional activation by ATP is coupled to massive increases in cytosolic calcium, membrane depolarization, and uptake of larger hydrophilic molecules. Further, P2X7R stimulation by ATP induces early morphologic changes without signs of apoptosis and late increase of cell proliferation mediated by substance P secretion (Raffaghello et al., 2006), suggesting that neuroblastoma cells have shaped P2X7R function to their advantage.
Figure 5. P2X7R levels are increased in multiple sclerosis

A, Hoechst (top) and immunofluorescence staining of oligodendrocytes (APC+ cells) with antibodies to P2X7Rs in the human optic nerve from controls and normally appearing multiple sclerosis (MS) samples. Merge images show that P2X7Rs are located in oligodendrocytes in both control (C) and multiple sclerosis samples. In turn, cells of the microglial lineage (CD68+) also show P2X7R immunolabeling. Scale bar, 20 μm. B, Western blot of optic nerve homogenates from sex- and age-matched controls (C) and multiple sclerosis cases. Sample loading was normalized to the intensity of bands corresponding to actin. From Matute et al. (2007), Fig. 5.

1.2.4.6.7. Alzheimer’s disease

Alzheimer’s disease (AD), the most common cause of dementia, is characterized histopathologically by the appearance of senile plaques composed of the amyloid β-peptide (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein. The cause for most AD cases is still essentially unknown (except for 1% to 5% of cases where genetic differences have been identified), although inflammation is believed to be an important component of the etiopathology. Increased expression of P2X7R mRNA has been described in AD-derived microglia compared to non-demented brain, along with prominent P2X7R protein immunoreactivity in
association with Aβ plaques and localized to HLA-DR-immunoreactive microglia (McLarnon et al., 2006). Intrahippocampal injection of Aβ in rats resulted in strong P2X7R colocalization with microglia (McLarnon et al., 2006) and accumulation of IL-1β in wild-type, but not in P2X7R-deficient mice (Sanz et al., 2009). In a genetic mouse model of AD, the P2X7R was predominantly expressed in CD11b-immunopositive microglia from 3 months of age before Aβ plaque formation (Lee et al., 2011). A catalytic subunit of NADPH oxidase, gp91phox, was detected in P2X7R-positive microglial cells of 6-month-old animals, suggesting the potential for P2X7R-positive microglia to generate reactive oxygen species. Damaged postsynaptic density 95-positive dendrites (‘synaptotoxicity’) were found in regions positive for P2X7R in the cerebral cortex of 6-month-old mice (Lee et al., 2011).

1.2.4.6.8. Brain ischemia

ATP outflow increases after an ischemic insult in the brain, which could activate P2X receptors. Several studies have reported increased P2X7R expression, by immunohistochemistry and Western blot, in the peri-infarct region after middle cerebral artery occlusion in rats (Franke et al., 2004; Melani et al., 2006; Yanagisawa et al., 2008). P2X7R antagonists such as Brilliant Blue G reduced the extent of brain damage (Arbeloa et al., 2012; Melani et al., 2006) and improved sensorimotor deficit in ischemic animals (Melani et al., 2006). However, Le Feuvre et al. (2003) found that cell death induced by temporary cerebral ischemia was not altered in P2X7R KO mice, but was reduced by treatment with IL-1 receptor antagonist. Treatment of mice with P2X7R antagonists did not affect ischemic or excitotoxic cell death, suggesting that P2X7R is not a primary mediator of experimentally induced neuronal death. In a more recent study, Yanagisawa el al. (2008) reported that intracerebroventricular injection with the P2X7R agonist BzATP improved behavioural dysfunction and ischemic neural injury induced by middle cerebral artery occlusion, while the P2X7R antagonist adenosine 5′-triphosphate-2′,3′-dialdehyde exacerbated ischemic brain damage. Collectively, these results leave open the question of P2X7R as 'friend or foe' in brain ischemia.
Activation of P2X receptors in the spinal cord was shown to elicit allodynia (Fukuhara et al., 2000) and in a seminal publication in 2003, P2X4R on spinal cord microglia was shown to be upgraded in neuropathic pain, which was reduced after P2X4R antagonism (Tsuda et al., 2003). An explosion of work then followed and P2X7R and P2Y12R on microglia were also shown to be involved in neuropathic pain (Jarvis, 2010; Smith, 2010; Tsuda et al., 2010). However, the underlying mechanism whereby antagonists to P2X7R, P2Y12R, as well as P2X4R all reduce neuropathic pain is still unclear. The selective antagonists to P2X7R, A-438079 and A-740003 produced dose-dependent antinociceptive effects in models of neuropathic (Honore et al., 2006, McGarraughty et al., 2007; Nelson et al., 2006) (Figure 6) and inflammatory (Honore et al., 2006) pain, as did Brilliant Blue G (He et al., 2012), cyanoguanidine (Perez-Medrano et al., 2009) and A-839977 (Honore et al., 2009). It has also been suggested that P2X7R plays a role in neuron-glial interactions associated with ongoing pain (Donnelly-Roberts et al., 2007). Chronic inflammatory and neuropathic pain, and also release of the inflammatory cytokine IL-1β, was abolished in P2X7R KO mice (Chessell et al., 2005) (Figure 7). The authors suggested that the P2X7R, via regulation of mature IL-1β production played an upstream transductional role in the development of neuropathic and inflammatory pain (Clark et al., 2010). In recent studies, P2X7R was shown to be associated with TNF-α production in microglia through the p38-mitogen-activated protein kinase system and treatment with inhibitors of either TNF-α or p38 resulted in reduction of allodynia (Leung and Cahill, 2010; Teixeira et al., 2010). P2X7R expressed by immune cells plays a pivotal role in changing pain thresholds (Chessell et al., 2005). Sorge et al. (2012) recently showed that variation
Figure 6. Antinociceptive effects of A-740003 in the spinal nerve ligation model of neuropathic pain

Two weeks following L5-L6 spinal nerve injury, A-740003 was injected i.p. 30 min before testing. A-740003 demonstrated significant antiallodynic effects in neuropathic pain \( [F(5,152) = 109.42, p < 0.0001] \). Data represent mean ± S.E.M. **, p < 0.01 as compared with vehicle-treated animals (n = 6–12 per group). From Honore et al. (2006), Fig. 8.

Figure 7. Responses to noxious thermal and mechanical stimuli in P2X7R\(^{+/+}\) and P2X7R\(^{-/-}\) mice following partial nerve ligation

(a) Following ligation, thermal hypersensitivity developed after 3 days in P2X7R\(^{+/+}\) animals, and was significantly different from pre-surgery values for at least 21 days. In P2X7R\(^{-/-}\) animals, hypersensitivity completely failed to develop. (b) P2X7R\(^{+/+}\) mice developed significant mechanical hypersensitivity 3–7 days following ligation, which was maintained until day 21. P2X7R\(^{-/-}\) mice completely failed to display any significant hypersensitivity at any time point studied. From Chessell et al. (2005), Fig. 2.
within the coding sequence of the *P2X7R* gene affects chronic pain sensitivity in both mice and humans. Using genome-wide linkage analyses, they discovered an association between nerve injury–induced pain behavior (mechanical allodynia) and the P451L mutation of the mouse *P2X7R* gene. Mice with this mutation impairing calcium influx and pore formation also showed less allodynia than mice with the pore-forming *P2X7R* allele. Administration of a peptide corresponding to the P2X7R C-terminal domain, which blocked pore formation but not cation channel activity, selectively reduced nerve injury and inflammatory allodynia only in mice with the pore-forming *P2X7R* allele (Sorge et al., 2012). While this variant has been reported to be non-functional in this model, the P451L mutation found in C57BL/6 mice is instead fully functional and pharmacologically indistinguishable from wild-type P2X7R when expressed in human astrocytoma 1321N1 cells (Donnelly-Roberts et al., 2009). This of course becomes relevant in terms of human therapeutics. Moreover, in two independent human chronic pain cohorts a genetic association was observed between lower pain intensity and hypofunctional His270 (rs7958311) allele of *P2RX7*. Selectively targeting P2X7R pore formation may be a new strategy for the treatment of chronic pain (Sorge et al., 2012).

1.2.4.6.10. Cardiovascular disorders

Apoptotic cell death occurs in a number of vascular diseases, including atherosclerosis and hypertension (Mallat and Tedgui, 2000). Shear stress that occurs during changes in blood flow causes a substantial release of ATP from vascular endothelial cells (Burnstock, 1999). ATP may also be released from cardiomyocytes in ischemic or hypoxic conditions (Dutta et al., 2004). P2X7R-associated production of proinflammatory cytokines like tumor necrosis factor-α could promote endothelial cell apoptosis (Alesci et al., 2005), and play a role in vascular remodeling in hypertension (Gibbon, 1993). P2X receptor channels are involved in transducing aldosteronemediated signaling in the distal renal tubule and are potential candidate genes for blood pressure regulation (Zhang et al., 2007). On an intriguing note, there is evidence to suggest that
elevated night-time diastolic blood pressure is associated with single nucleotide polymorphisms of the P2X7R gene (Palomino-Doza et al., 2008). P2X7Rs are expressed in human saphenous vein myocytes (Cario-Toumaniantz et al., 1998), and venous diseases may favor conditions allowing P2X7R activation and lysis of venous myocytes. ATP released after hypoxia, stress and inflammation, or membrane damage, conditions found in the vessel wall of varicose veins, may lead to P2X7R-induced pore formation, the disorganization and loss of contractile myocytes in the muscle layers of the media of varicose veins, and venous disease.

Fibroblasts are a key structural element of the arterial wall known to play a major role in atherosclerosis and diabetic angiopathy (Duner et al., 1997). Fibroblasts from type-2 diabetes patients are characterized by a hyperactive purinergic loop (Solini et al., 2004).

While depression and cardiovascular co-morbidity have been recognized for some time (Glassman, 2007), a pro-inflammatory link has only recently been investigated (Stewart et al., 2009). Extensive prospective studies are required to confirm this idea, and to investigate whether a link exists between illnesses with a pro-inflammatory component (e.g., inflammatory and chronic neuropathic pain) and cardiovascular disease, for example, hypertension, and whether patients treated with anti-inflammatory drugs have a lower incidence of cardiovascular complications.
THESIS AIMS

Neuroinflammation in nervous system disorders was viewed at one time as an epiphenomenon following neuronal cell damage. Emerging evidence now challenges this earlier "neuron-oriented" perspective and points to a more active role of neuroinflammation in pathophysiology onset and progression, with glia having key roles in conditions from chronic pain and epilepsy to neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and amyotrophic lateral sclerosis – and may even contribute to schizophrenia, depression, and other psychiatric disorders. The initiation and propagation of neuroinflammation appears to rely very much on the interaction between glia, immune cells. Glial cells, in particular microglia, show increased activity in multiple pain processing pathways in response to peripheral injury, and systemic inflammation gives rise to signals that communicate with the brain and lead to changes in metabolism and behavior. The healthy brain often responds to stress and insults by transiently up-regulating inflammatory processes which are kept in check by endogenous protective elements. Upsetting this homeostatic balance can result in disease or exacerbation of initiating factors that result in disease.

This research project was organized around 3 main objectives:

1. To establish cultures of microglia and astrocytes from neonatal rat cortex, spinal cord and cerebellum, and to characterize these cells utilizing immunocytochemical, molecular biological, and biochemical techniques, their responses to a well accepted inflammatory stimulus (LPS) and ATP, including the functionality of P2X7R ('priming response');

2. To understand the interaction between astrocytes and microglia in response to the selected inflammatory stimulus (LPS), as well as in the action of P2X7R-mediated IL-1β release ('priming response');

3. To study the generality of the LPS/ATP priming behavior by examining the ability of other agents linked to neuropathology, to replace either LPS or ATP. These will include ethanol, amyloid β-peptides, and agonists of TLR2 and TLR3.
2. MATERIALS AND METHODS

2.1. Cell culture

2.1.1. Equipment and labware

1. Stereo dissecting microscope (backlighting of stage is preferred) with fiber optic light source
2. Horizontal flow cabinet for dissections
3. Laminar flow biological safety cabinet (CL2)
4. Humidified, water-jacketed culture incubator at 37°C and 5% CO₂/95% air
5. Water bath set at 37°C
6. Dissecting tools
7. Bench centrifuge to accommodate 15 mL and 50 mL tubes
8. Orbital shaker with enclosed, temperature controlled chamber
9. 10 cm Ø sterile tissue culture dishes (any supplier)
10. 15 mL and 50 mL polypropylene plastic centrifuge tubes (sterile)
11. Sterilin 10 cm Ø sterile petri plastic dishes (Bibby-Sarstedt)
12. Tissue culture flasks (T-75; 75cm²) with canted neck and 0.2 µm vented cap (Corning) or without vented cap (BD Falcon).
13. Cell scrapers, 25 cm handle, 1.8 cm blade (BD Falcon)
14. 0.22 µm filters (Millipore Corporation)

2.1.2. Reagents

1. Phosphate-buffered saline (PBS) (pH 7.4, sterile) (Invitrogen)
2. Dulbecco’s modified Eagle’s medium (DMEM) (+4.5 g/L glucose, L-glutamine, pyruvate) (Invitrogen)
3. Trypsin inhibitor (Sigma)
4. DNAse (Type I) (Sigma)
5. L-15 medium (+L-glutamine, L-amino acids) (Invitrogen)
6. Trypsin inhibitor, type I from soybean (Sigma)
7. Papain (Worthington (Lorne))
8. Bovine serum albumin, Cohen fraction V (Sigma)
9. Penicillin/streptomycin (100X stock), sterile, for cell culture (Invitrogen)
10. Gentamicin (50 mg/mL stock), sterile, for cell culture (Sigma)
11. Fetal bovine serum (FBS) (Invitrogen)
12. Poly-L-lysine (MW 70,000-150,000), sterile, for cell culture (Sigma P-6282)
13. Poly-D-lysine (MW 30,000-70,000), sterile, for cell culture (Sigma P-7280)
14. Collagen, Type I from rat tail (Sigma C-3867)
15. L-Glutamine (200 mM stock), sterile, for cell culture (Invitrogen)
16. Sodium pyruvate (100 mM stock), sterile, for cell culture (Sigma)
17. HEPES (1 M), sterile, for cell culture (Sigma)
18. LPS (E. coli 026:B6) (Sigma)
19. Griess Reagent (Sigma)
20. Adenosine 5’-triphosphate (ATP) (Sigma)
21. L-Leucine methyl ester HCl (L-LME) (Sigma)

2.1.3. Culture media and other solutions

1. **DNase:** Dissolve 40 mg of DNase in 10 mL of L-15 medium (100X stock, 4 mg/mL), filter-sterilize, aliquot and store at -20°C.
2. **L-cysteine:** Dissolve 24 mg of L-cysteine in 1 mL of L-15 medium (100X stock). Prepare fresh each time.
3. **Trypsin inhibitor:** Dissolve 100 mg of trypsin inhibitor in 1 mL (100X stock) of L-15 medium. Filter-sterilize, aliquot and store at -20°C.
4. **Bovine serum albumin:** Dissolve 5 mg of bovine serum albumin in 1 mL (100X stock) of L-15 medium. Filter-sterilize, aliquot and store at -20°C.
5. **0.15 M Borate buffer pH 8.4**: Dissolve 28.6 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$) in 500 mL water (pH will be ~9.2). Adjust pH to 8.4 with 5N HCl. Filter-sterilize and store at 4°C (up to 6 months).

6. **Plating medium/growth (maintenance) medium**: To a 500 mL bottle of DMEM add: 50 mL FBS (10% final), 5 mL stock penicillin/streptomycin (100 U/mL penicillin + 100 µg/mL streptomycin final), and 0.5 mL of gentamicin stock (50 µg/mL final).

### 2.1.4. Preparation of substratum

1. Dissolve a 5 mL bottle of poly-L-lysine (MW 70,000-150,000) in 5 mL 0.15 M borate buffer (pH 8.4). This 1 mg/mL stock solution can be stored at +4°C for up to 2 months.

2. Dilute the 1 mg/mL stock solution of poly-L-lysine 1:100 (1 mL in 99 mL sterile water) and add 10 mL to each of the T-75 culture flasks. Rock the flask to cover the entire culture surface and place flask in the 5% CO₂ incubator (37°C) overnight.

3. The following day aspirate the poly-L-lysine coating solution and leave the flask(s) under the CL2 cabinet uncapped to dry. This will usually take several hours. Once dried the poly-L-lysine-coated flasks can be stored at +4°C for one month.

### 2.1.5. Dissection of rat cortical tissue

1. Rat cultures are routinely performed using animals at postnatal day 1-2. Following appropriate national and institutional guidelines for animal sacrifice, pregnant rats (CD strain, Sprague Dawley) are euthanized following approved national and institutional guidelines.

2. Dissect out cerebral hemispheres following standard techniques and anatomical landmarks. The procedure is applicable to either rat or mouse pups.

3. Make an incision under the skin along the midline of the dorsal surface and peel the skin back.
4. Make a second incision close to the midline along the exposed dorsal surface (the cut should be along the complete anteroposterior axis of the lateral ventricles).

5. Splay open the hemisphere along the cut surface and continue the cut along the dorsal surface of the hemisphere.

6. Remove the cerebral hemispheres (minus cerebellum and brain stem) and peel off meninges. The latter step is best accomplished by using two fine-tipped #5 Dumont forceps, using one pair to anchor the hemisphere while peeling off the meninges with the second pair.

7. Place the dissected hemisphere into a separate 10 cm $\odot$ dish of cold L-15 medium.

8. Repeat this procedure for the other hemisphere and remaining pups.

9. The hippocampal area can be dissected away and retained or discarded, depending on need.

2.1.6. Enzymatic digestion of cortical tissue and preparation of cell suspension for plating

1. Grip a straight edge razor blade with a haemostat, dip in 70% ethanol, and pass through a flame to sterilize.

2. Mince the collected cortical tissue with the razor blade.

3. Collect the minced tissue with L-15 and transfer to a 15 mL centrifuge tube.

4. Centrifuge collected cortical tissue for 30 seconds (200 x g) to compact the minced tissue.

5. While the tissue is centrifuging, prepare the following: 3 mL of L-15 medium containing 140 $\mu$L stock papain solution (as purchased), 30 $\mu$L of 100X stock L-cysteine solution, and 30 $\mu$L of 100X DNase stock solution. Filter-sterilize before use.

6. Remove L-15 medium from the tube with cortical tissue pellet, and add the L-15 solution containing papain, DNase, and L-cysteine.

7. Incubate tissue for 60 minutes in a 37°C water bath with occasional swirling.

8. During this time, prepare the ovomucoid solution: to 3 mL of L-15 medium add 30 $\mu$L of 100x stock DNase solution, 30 $\mu$L of 100X stock bovine serum albumin solution, and 30 $\mu$L of 100X stock trypsin inhibitor solution.
9. Upon completion of the enzyme incubation step remove the supernatant and replace with 1.5 mL ovomucoid solution.

10. Incubate for 2 minutes in a 37°C water bath.

11. Centrifuge at 250xg for 3 minutes.

12. Remove the supernatant and add 1.5 mL fresh ovomucoid solution.

13. Triturate 20-25 times with a long (9-inch) cotton plugged Pasteur pipette.

14. Add 4 mL plating medium and centrifuge at 200xg for 5 minutes.

15. Using a 5 mL tissue culture pipette, re-suspend the cell pellet in 3 mL plating medium. Add an additional amount of plating medium such that the final volume is equivalent to 2 mL x number of T-75 flasks to be used (e.g. 20 mL final for 10 flasks).

16. Add 18 mL plating medium to each flask, followed by 2 mL of cell suspension. Normally, we seed cultures at a ratio of 1.5 brains per flask.

17. The following day, replace the culture medium with 15 mL fresh plating (growth) medium.

18. Twice per week, replace one-half the volume of culture medium with an equal volume of fresh growth medium.

19. The mixed glial cell cultures are incubated for 7-10 days, after which time confluence will have been reached and can be taken for harvesting different cell populations.

2.1.7. Isolation of Microglia

1. Remove 3 mL medium from each T-75 flask; this should leave about 12 mL of medium.

2. As the culture medium is bicarbonate buffered, to prevent pH excursions close the flask cap (or cover with parafilm if the cap is of the filter type).

3. Place the flasks on an orbital shaker fitted with a temperature controlled chamber. Flasks can be fastened to the shaker platform using double-sided adhesive tape, or taped directly to the platform using high strength industrial ducting tape.

4. Shake the flasks for 1 hour at 200 cycles per minute (37°C).
5. Remove the culture medium (containing mainly microglia) from the flasks and transfer to 10 cm Ø Sterilin petri plastic dishes. A volume of 20-25 mL per dish is best.

6. Place the Sterilin dishes in the 5% CO₂ incubator (37°C) and leave for 45-60 minutes.

7. Aspirate the medium on the Sterilin plates and add 6 mL fresh maintenance medium.

8. Use a cell scraper to remove the attached microglia from the Sterilin dishes. Transfer the medium with cells to a 50 mL centrifuge tube. Rinse the dishes with 5 mL maintenance medium and transfer to the collection tube.

9. Re-suspend the microglia cell pellet in maintenance medium and plate into poly-L-lysine-coated multiwall plates as dictated by the experimental design. Typically, we plate 10^5 cells in a 96 well plate (equivalent to 3.5x10^5 cells per cm²). Microglia obtained are ≥99% pure. Cultures will maintain their viability for approximately 10 days.

2.1.8. Isolation of an enriched population of astrocytes

1. The T-75 flasks remaining after shaking to recover microglia are used as a source of highly enriched astrocytes.

2. Aspirate the medium from the flask and rinse the cell monolayer with 10 mL sterile phosphate-buffered saline.

3. Add to the flask 3 mL of 0.25% trypsin/EDTA solution, rock the flask to spread the trypsin solution over the entire monolayer, and then remove by aspiration. This will leave a thin film of trypsin solution over the cells.

4. Incubate the flask(s) at 37°C for 10 minutes.

5. Tap the flask against the palm of the hand to dislodge cells and add 5 mL maintenance medium per flask; the FBS in the medium inactivates the trypsin.

6. Rinse the flask with the medium added, collect cells and transfer to a 15 mL or 50 mL centrifuge tube, as needed.
7. Pellet cells by centrifugation at 200xg for 5 minutes, re-suspend pellet in maintenance medium and count. The typical cell yield is 4-5 x 10^6 cells per T-75 flask. On this basis, dilute the cell suspension to 1-1.5 x 10^6 cells per mL for ease of counting. Astrocytes thus obtained are ≥95% pure.

8. If needed, dilute further the cell suspension for plating into poly-L-lysine-coated culture vessels are dictated by the experimental design. Typically, we plate 5 x 10^4 cells in a 96 well plate (equivalent to 1.75x10^5 cells per cm^2).

2.1.9. Preparation of spinal cord glia

1. The procedure is essentially as that described for cortical tissue, except for the dissection.

2. Sacrifice pups by decapitation.

3. Place the body face down on a paper towel. Spray 70% ethanol over the back.

4. Remove the back skin with surgical scissors to expose the vertebral column.

5. Entering from the anterior side of the body, make a cut through the midline as far as the tail.

6. Using a fine pair of surgical scissors trim back, at a 45° angle, the tissue on either side of the spinal canal (being careful not to disrupt the spinal cord itself).

7. Carefully strip off the spinal cord with a fine pair of forceps and transfer to a 10 cm Ø dish of cold L-15 medium.

8. Repeat this procedure for the remaining pups.

9. Peel off the meninges using fine tipped forceps, and transfer the tissue pieces to a 15 mL centrifuge tube. It is not necessary to mince the tissue as for cortex; the spinal cord is much smaller, and will already have been fragmented to some extent in the course of removing the meninges.

10. Centrifuge the collected tissue for 30 seconds (200xg) to compact.

12. Incubate tissue for 60 minutes in a 37°C water bath with occasional swirling.

13. Proceed with tissue dissociation as for cortex, except the final 5-minute centrifugation can be omitted. Doing so reduces the change of loosing material, which is limited in comparison to cortex.

14. Following dissociation, plate the spinal cord cell suspension in medium and distribute in poly-L-lysine-coated T-75 flasks, 5 spinal cords per flask.

15. For harvesting microglia, we find that spinal cord mixed glia cultures mature more slowly than do the cortical cultures. As such, optimal recovery of microglia is achieved after ~10 days in vitro. Isolation of separate populations of microglia and astrocytes is carried out as described for cortical cultures.

16. Spinal cord astrocytes are seeded onto a collagen substratum (cells will clump and detach if poly-lysine is used).

2.1.10. Preparation of cerebellar microglia

1. Cerebellar tissue from 7-8-day-old rat pups was used. The procedure followed is described in detail in our earlier publication (Facci and Skaper, 2012a), and makes use of a commercially available papain dissociation kit (Worthington, Lakewood, New Jersey, USA).

2. To favor the growth of glia, the culture medium used was that described above for cortical and spinal cord glia.

2.2. Real-time polymerase chain reaction (RT-PCR)

Microglia and astrocytes were seeded in poly-lysine-coated 12-well plates (collagen-coated for spinal cord astrocytes) at a density of 500,000 cells per well, using glia cell growth medium and allowed to adhere overnight. Cells were then incubated with LPS for 6 h, and total RNA was extracted from cells by TRIzol (Invitrogen), according to the manufacturer’s instructions. RNA
integrity and quantity were determined by RNA 6000 Nano assay in an Agilent BioAnalyser. RT was performed with Superscript III reverse transcriptase (Invitrogen). The Real-time RT-PCR reaction was performed in a MX 3000P thermal cycler in a final volume of 12.5 µL, containing 100 nM of each primer and 1X SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich). The PCR cycling conditions were 4 min of denaturation at 94°C, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, followed by a dissociation thermal profile of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The following primer pairs were used:

**GAPDH**, 5’-CAAGGTCATCCATGACAACCTTTG-3’ F and 5’-GGGCCATCCACAGTCTTCTG-3’ R (89 bp);

**IL-1β**, 5’-TGTGGCAGCTACCTATGTCT-3’ F and 5’-GGGAACATCACACTAGCA-3’ R (146 bp);

**iNOS** ((inducible nitric oxide synthase or NOS2), 5’-CACACAGCGCTACAACATCC-3’ F and 5’-CCATGATGGTCACATTCTGC-3’ R (149 bp);

**TNF-α** (tumor necrosis factor-α), 5’-CATCTTTCTCAAAACTCGAGTGACAA-3’ F and 5’-TGGGAGTAGATAAGGTACAGCCC-3’ R (174 bp);

**IL-6**, 5’-TCACAGAAGGAGTGCTAAGG-3’ F and 5’-GCTTAGGCATAGCAGCACACTAGG-3’ R (115 bp);

**Iba1**, 5’-AAGCTGGAGCCTCAGACG-3’ F and 5’-AACCCCAAGTTTCTCCAGCA-3’ R (101 bp)

Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. Dissociation curves were generated for each primer pair, showing single product amplification.
2.3. Aβ peptide toxicity to cortical neurons

Aβ(1-42) was prepared by dissolving the peptide in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma) to a concentration of 2.5 mg/ml and left at room temperature for 10 min. The 1,1,1,3,3,3-hexafluoro-2-propanol was then evaporated under a stream of air, and the peptide film was redissolved in dimethylsulfoxide (containing 0.0625% trifluoroacetic acid) to a concentration of 5 mM. Serum-free medium was pre-warmed to 37°C and added to dilute the 5 mM peptide solution to 80 µM, before adding to cells to give a final concentration of 20 µM Aβ(1-42) (rat sequence, kindly provided by Prof. Vincenzo De Filippis, Scienze del Farmaco). Aβ(25-35) (Sigma) was prepared as a 10 mM stock solution in 0.1% trifluoroacetic acid, and added to the culture medium to achieve the indicated final concentrations.

2.4. Immunofluorescence

Cortical enriched astrocytes or purified spinal cord microglia were seeded on poly-L-lysine-coated 12-mm diameter coverglasses (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) placed in the wells of a 24 well multiwall plate, at a density of 15,000 or 30,000 cells per well, respectively, in culture medium and allowed to adhere overnight. Two days later the cells were fixed with 4% paraformaldehyde for 30 min at 4°C, and washed 4 x 5 min with PBS/0.05% Triton X-100 (PBS-T), and blocked with PBS/10% FCS for 1 h at room temperature. The fixed cell monolayers were incubated overnight at 4°C with one of the following primary antibodies: Iba1 (Wako, rabbit polyclonal affinity purified, 1:500), GFAP (mouse monoclonal, 1:1000, Sigma-Aldrich, Milan, Italy), P2X7R (rabbit monoclonal, 1:500, Abcam, Cambridge, UK). Cells were then washed 5 x 5 min with PBS, and incubated for 1 h at room temperature with anti-mouse-AlexaFluor555 or anti-rabbit-AlexaFluor488 secondary antibody (1:500, Invitrogen). Nuclei where visualized by incubating 2 min with 100 ng/ml 4’-6’-diamidino-2-phenylindole (DAPI) (Boehringer-Mannheim, Germany). Cover glasses were mounted onto glass slides using Fluoromount-G (Southern Biotech, USA), and images were acquired on a Leica DMI4000 B microscope equipped
for immunofluorescence (Leica Microsystems GmbH, Wetzlar, Germany) using a Leica DFC 480 digital camera (Leica Microsystems GmbH, Wetzlar, Germany).

2.5. Mediator release

Microglia and astrocytes were plated in wells of a 96-well plate (poly-L-lysine coated, or collagen for spinal cord astrocytes) at a density of 100,000 or 50,000 cells per well, respectively, using glia cell growth medium and allowed to adhere overnight. Cells were stimulated to release pro-inflammatory mediators in medium containing the indicated concentration of LPS. In some cases cells were pretreated with LPS ('priming') for 2 h in serum-free medium prior to stimulation with ATP for 1 h.

2.5.1. Nitric oxide assay

Nitric oxide (NO) has a relatively short half-life in aqueous solution. Hence, quantitative assessment of NO production has generally relied on the indirect measurement of its oxidized products, nitrite and nitrate, which are regarded as suitable markers of NO generation. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This kit employs the NADH-dependent enzyme nitrate reductase for conversion of nitrate to nitrite prior to quantitation of nitrate using Griess reagent — thus providing for accurate determination of total NO production.

Equal volumes of cell culture medium and Griess reagent (Sigma-Aldrich) were incubated for 15 min, and the amount of nitrite (as evidenced by the purple azo reaction product) quantified using a standard curve of sodium nitrite at O.D. 540 nm. Figure 8 shows a typical standard curve.
2.5.2. Cytokine ELISA assays

Cells were stimulated to release pro-inflammatory mediators in medium containing the indicated concentration of LPS or other agent. Cell supernatants were harvested after 24 hours and cytokine release was assayed by sandwich ELISA (Enzyme-Linked Immunosorbent Assay) according to the manufacturer’s instructions (Antigenx America, Huntington Station, New York). The assay principle is illustrated below:

(1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.
Figure 9 shows a typical standard curve for IL-1β (similar curves are obtained for TNFα).

![Figure 9](image)

2.6. Cell vitality assays

2.6.1. MTT

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g. dimethylsulfoxide) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is generally a measure of cell viability (Mosmann, 1983).

Remove the culture medium from the 96-well plate with a multichannel pipettor. Add to each well 50 μL of serum- and phenol red-free DMEM containing 0.15 mg/mL MTT (which represents a 1:20 dilution from the 3 mg/mL stock in PBS). Incubate the MTT-containing cultures for 1 h at 37°C. Examination of the cultures under a light microscope will show that viable cells contain blue crystals. This is the formazan reaction product. Remove the culture medium by again using the
multichannel pipettor and add 50 μL of dimethyl sulfoxide per well. This is to dissolve the reaction product. Gently tap the microwell plate from underneath to mix the blue reaction product uniformly with the solvent. Read the plate using a SpectraMax M2 microplate reader (Molecular Devices). For optimal results, the plate should be read at a wavelength of 570 nm (test wavelength), followed by 630 nm (reference wavelength). The difference \((A_{570} - A_{630})\) is used as the final absorbance value generated by the sample. Correcting for optical imperfections in the microplates by subtracting \(A_{630}\) is recommended, but is not an essential procedure.

2.6.2. Lactate dehydrogenase (LDH) release assay

The CytoTox 96® nonradioactive cytotoxicity assay (Promega) measures spectrophotometrically the release of LDH into the culture medium, and is an index of loss of plasma membrane integrity. The assay was performed following the manufacturer’s instructions, and is also detailed in Facci and Skaper (2012b), and will not be repeated here.

2.7. Statistics

Data are given as mean ± SEM. Statistical analyses to determine group differences were performed either by two-sample equal variance Student’s \(t\) test, or by one-way analysis of variance, followed by Dunnett’s or Bonferroni’s post-hoc tests for comparisons involving more than two data groups.
3. RESULTS

Part 1

3.1. Cellular, molecular and pharmacological characterization of glia cell Populations from cortex, spinal cord and cerebellum, and responsiveness to ATP

3.1.1. Morphological characterization

Microglia and astrocytes prepared using the methods described here result in cell populations which are ≥ 99% and ≥ 95% pure, respectively (Rosin et al., 2004). Immunostaining of cortical astrocytes shows a confluent carpet of GFAP+ cells, interspersed with a few Iba1+ immunoreactive cells (microglia) (Figure 10, right panels). Purified microglia (spinal cord) show virtually all cells labelled for Iba1 (Figure 10, left panels). In these images, nuclei are colored blue with DAPI, which forms fluorescent complexes with natural double-stranded DNA.
Figure 10. Immunostaining of purified microglia and enriched astrocytes with the cell-type specific markers Iba1 and GFAP, respectively.
Figure 11. Immunostaining of purified microglia and enriched astrocytes with the cell-type specific markers Iba1 and GFAP, respectively.
3.1.2. Molecular characterization

Cortical microglia expressed high levels of mRNA for the macrophage/microglia protein Iba1 (ionized calcium binding adaptor molecule) relative to astrocyte-enriched cultures (Figure 12), expressed as a ratio to GFAP. Similar results were obtained for spinal cord microglia (data not shown).

**Figure 12.** Cortical microglia express high levels of Iba1 mRNA relative to astrocytes

![Graph showing Iba1/GFAP levels for microglia (MO) and astrocytes (AG) with different LPS concentrations](image)

Purified microglia (MO) and enriched astrocyte (AG) cell populations prepared from neonatal rat cortex were incubated with the indicated concentrations of LPS for 6 h, and then processed for RT-PCR analysis. Data, given as means ± SEM (duplicate culture wells) are normalized to GFAP levels, and are representative of 3 experiments. Qualitatively similar results were obtained with spinal cord microglia and astrocytes. CTR, no-LPS control.

Microglia (Block et al., 2007) and astrocytes (van Noort and Bsibsi, 2009) express toll-like receptors (TLRs), a family of pattern-recognition receptors in the innate immune system. TLRs are key players in the activation and maintenance of pain and in certain neuropathologies: for example, microglial TLR4 has been implicated in oligodendrocyte injury (Lehnardt et al., 2002), neurodegeneration (Lehnardt et al., 2003), and apoptosis of activated microglia (Jung et al., 2005), while activation of astrocyte-expressed TLRs can lead to the release of pro-inflammatory molecules.
van Noort and Bsibsi, 2009) and modulate features of the immune response (Dong and Benveniste, 2001; Farina et al., 2007). Intracerebral (Lehnardt et al., 2002; Pang et al., 2010) or systemic (Lehnardt et al., 2003; Qin et al., 2007) delivery of LPS the major component of Gram-negative bacterial walls and a ligand for TLR4, leads to microglia activation and neural cell injury, and is used as a model for CNS inflammation (Belarbi et al., 2012; Kellom et al., 2012; Machado et al., 2011; Qin et al., 2007).

Indeed, LPS concentration-dependently up-regulated expression of mRNA for iNOS, IL-1β and TNF-α in purified cortical microglia and enriched astrocytes (Figure 13) although the relative degree of induction and LPS sensitivity appeared to be more robust in microglia. It is interesting to note that LPS also up-regulated expression of Iba1 mRNA as well (Figure 12).

**Figure 13.** LPS induces pro-inflammatory gene expression in cortical microglia and astrocytes

Purified microglia (MO) and enriched astrocyte (AG) cell populations prepared from neonatal rat cortex were incubated with the indicated concentrations of LPS for 6 h, and then processed for RT-PCR analysis of: iNOS (left panel), IL-1β (middle panel), and TNF-α (right panel) mRNA. Data are means ± SEM (duplicate culture wells) normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels, and are representative of 3 experiments.
3.1.3. Biochemical characterization

LPS concentration-dependently up-regulated expression/output of IL-1β protein, as determined by ELISA, from both purified cortical microglia and enriched astrocytes (Figure 14). Again, the relative degree of induction and LPS sensitivity appeared to be more robust in microglia. Spinal cord glia exhibited qualitatively similar behaviors (data not shown).

Figure 14. LPS induces IL-1β production/output from cortical microglia and astrocytes

Purified microglia (■) and enriched astrocyte (▲) cell populations prepared from neonatal rat cortex were seeded in 96 well plates (50,000 and 100,000 cells, respectively), and incubated the following day with the indicated concentrations of LPS for 24 h. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

Further, BzATP, but not ATP (the former a more potent P2X7R agonist) (Donnelly-Roberts et al., 2009) increased (albeit modestly) IL-1β output from cortical microglia over a 48-h period (Figure 15) – in contrast to the robust response elicited by LPS. In the case of cortical astrocytes only LPS treatment led to IL-1β release into the culture medium (Figure 16), and this response was again more modest in comparison to that of microglia.
**Figure 15.** BzATP, but not ATP induces IL-1β production/output from cortical microglia

Purified microglia prepared from neonatal rat cortex were seeded in 96 well plates (100,000 cells), and incubated the following day with the indicated concentrations of LPS, ATP, or BzATP for 24 h. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). The increase elicited by 1 mM BzATP differed significantly (**p<0.01, ***p<0.001) from control values at all time points.

**Figure 16.** Neither BzATP nor ATP induces IL-1β production/output from cortical astrocytes

Enriched astrocytes prepared from neonatal rat cortex were seeded in 96 well plates (50,000 cells), and incubated the following day with the indicated concentrations of LPS, ATP, or BzATP for 24 h. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).
Biological activity of IL-1 is the result of transcription of two related gene products: IL-1α and IL-1β. Both IL-1α and IL-1β are synthesized as 31–34-kDa procytokines and are then converted into the mature 17-kDa form; however, although IL-1α is biologically active in both the pro and mature form, IL-1β is only active in the mature form (Beuscher et al., 1990). Conversion of pro into mature IL-1β is catalyzed by a cysteine protease known as IL-1β-converting enzyme (Dinarello, 1998) that is the prototypical member of the caspase family (caspase 1) (Thornberry et al., 1992). Proteolytical maturation is also linked via a poorly known mechanism to IL-1β release into the extracellular space. Likewise, peritoneal macrophages, microglial cells, and dendritic cells respond weakly to LPS stimulation, thus suggesting that a second stimulus may be needed to elicit IL-1β secretion from these cell types. Endotoxin (LPS)-dependent release of IL-1β from rodent microglial cells is a very inefficient process, and secreted IL-1β is mostly in the procytokine unprocessed form. Among stimuli reported to enhance and accelerate IL-1β release from mononuclear phagocytes (cytolytic T cells, K⁺ ionophores, bacterial exotoxins, and ATP) (Bhakdi et al., 1990; Hogquist et al., 1991b; Perregaux and Gabel, 1994), ATP is one of the most interesting because this nucleotide is present at a concentration of 5-10 mM in the cytosol of most cells, thus it can be released in large amounts following plasma membrane damage or acute cell death. Furthermore, it is becoming increasingly clear that ATP is released by several cell types via nonlytic pathways in response to stimulation with many different agonists, among which is LPS itself (Cotrina et al., 1998; Ferrari et al., 1998; Sugita et al., 1998). ATP, via activation of P2X7R, is one of the most powerful stimuli for secretion of IL-1β in its mature form (Ferrari et al., 1996, 1997c; Hogquist et al., 1991a; Perregaux and Gabel, 1994, 1998). Intriguingly, ATP is an efficient stimulus for IL-1β secretion only after the cells have undergone a short 'priming' with endotoxin, suggesting that this nucleotide is unable to trigger transcription of the IL-1β gene, but rather acts post-translationally, probably accelerating the step(s) involved in the proteolytical maturation of this cytokine.
To verify this priming behavior, cultures of purified cortical microglia and enriched astrocytes were first incubated with different concentrations of LPS for 2 h, followed by addition of 5 mM ATP and incubation continued for another 60 minutes. This concentration of ATP was chosen based on the earlier studies of Sanz and Di Virgilio (2000). Culture medium was then collected and analyzed for IL-1β content (Figure 17).

**Figure 17.** Addition of extracellular ATP to LPS-primed cortical microglia and astrocytes augments the release of IL-1β

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Purified neonatal rat cortical microglia (panel A) and enriched astrocytes (panel B) were seeded in 96 well plates (50,000 and 100,000 cells, respectively), and incubated the following day with the indicated concentrations of LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).
The increase in IL-1β release elicited by ATP was concentration-dependent (Figure 18). The 'background' increase in IL-1β output from cortical microglia and astrocytes exposed to 1 μg/ml LPS for 3 h was variable, and ATP (up to 5 mM) on its own produced little or no IL-1β release over the time-course of the priming experiment.

**Figure 18.** Addition of extracellular ATP to LPS-primed cortical microglia and astrocytes augments the release of IL-1β

Purified neonatal rat cortical microglia (left panel) and enriched astrocytes (right panel) were seeded in 96 well plates (50,000 and 100,000 cells, respectively), and incubated the following day with 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). *p<0.05 and ***p<0.001 vs LPS alone.

Addition of extracellular ATP to LPS-primed spinal cord microglia also produced a robust release of IL-1β (Figure 19), and in spinal cord astrocytes (to be described later). The priming response was found to occur as well in cerebellar-derived microglia and astrocytes (Figure 20). The latter observations are novel, in that priming has not been reported previously for glia of either spinal cord or cerebellar origin.
**Figure 19.** Addition of extracellular ATP to LPS-primed spinal cord microglia augments the release of IL-1β

Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000), and incubated the following day with 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

**Figure 20.** Addition of extracellular ATP to LPS-primed cerebellar microglia and astrocytes augments the release of IL-1β

Purified microglia (left panel) and enriched astrocytes (right panel) cultured from 7-day postnatal rat cerebellum were seeded in 96 well plates (50,000 and 100,000 cells, respectively), and incubated the following day with 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).
3.1.4. Pharmacological characterization

In order to confirm the involvement of P2X7R in the ATP-dependent augmentation of IL-1β release from LPS-primed cells, the above experiments were repeated in the presence of a series of P2X7R antagonists differing in chemical structure and target affinity. A-740003 is a potent (pIC\textsubscript{50} 7.0 for rat) and selective P2X7R antagonist (Honore et al., 2006) (Figure 21):

Figure 21. A-740003 is a highly selective P2X\textsubscript{7} antagonist

A-740003 selectively blocks P2X\textsubscript{7} receptors and not other P2 subtypes. ATP (4 μM) was used to activate P2X\textsubscript{1}, P2X\textsubscript{4}, and P2Y\textsubscript{1} receptors, and ATP (10 μM) was used for P2X\textsubscript{2a} and P2X\textsubscript{2a/3} receptors. UTP (1 μM) was used to activate P2Y\textsubscript{2} receptors. BzATP (5 μM) was used for human P2X\textsubscript{7} receptors. (Taken from Honore et al. J. Pharmacol. Exp. Ther. 2006)

A-740003 (10 μM) completely abolished IL-1β release which occurred over and above that obtained with LPS (1 μg/ml) alone, in both cortical purified microglia and enriched astrocytes (Figure 22, left and right panels, respectively).
Figure 22. The P2X7R antagonist A-740003 blocks the rise in IL-1β release caused by addition of extracellular ATP to LPS-primed cortical microglia and astrocytes

Purified neonatal rat cortical microglia (left panel) and enriched astrocytes (right panel) were seeded in 96 well plates (50,000 and 100,000 cells, respectively), and incubated the following day with: A-740003 (10 µM) for 30 minutes, followed by additional of 1 µg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures, where indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

A concentration-response comparison of A-740003 with another P2X7R antagonist, Brilliant Blue G (pIC<sub>50</sub> 6.0 for rat) (Donnelly-Roberts et al., 2009) showed that these two compounds blocked the ATP-dependent component of IL-1β release from LPS-primed cortical astrocytes in keeping with their reported target affinities (Figure 23). The structurally unrelated, potent and selective P2X7R antagonist AZ10606120 (pIC<sub>50</sub> 6.5 for rat) (Michel et al., 2008) which binds in a positive cooperative manner to sites distinct from, but coupled to, the ATP binding site also abolished the ATP-dependent component of IL-1β release from LPS-primed cortical astrocytes (Figure 24), as did the competitive P2X7R antagonist A-438079 (Nelson et al., 2006) and the irreversible antagonist periodate-oxidized ATP (ATP-P) (pIC<sub>50</sub> ~4.5) (Figure 25).
Figure 23. The P2X7R antagonists A-740003 and Brilliant Blue G concentration-dependently block the rise in IL-1β release caused by addition of extracellular ATP to LPS-primed cortical astrocytes.

Rat cortical enriched astrocytes were seeded in 96 well plates (50,000 cells), and incubated the following day with: A-740003 or Brilliant Blue G at the concentrations indicated for 30 minutes, followed by additional of 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

Figure 24. The P2X7R antagonist AZ10606120 concentration-dependently blocks the rise in IL-1β release caused by addition of extracellular ATP to LPS-primed cortical astrocytes.

Rat cortical enriched astrocytes were seeded in 96 well plates (50,000 cells), and incubated the following day with: AZ10606120 at the concentrations indicated for 30 minutes, followed by additional of 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).
Figure 25. The P2X7R antagonists A-438079 and periodate-oxidized ATP (ATP-P) concentration-dependently block the rise in IL-1β release caused by addition of extracellular ATP to LPS-primed cortical astrocytes.

Rat cortical enriched astrocytes were seeded in 96 well plates (50,000 cells), and incubated the following day with: A-438079 or ATP-P at the concentrations indicated for 30 minutes, followed by additional of 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

In the dorsal horn of spinal cord, pharmacological inhibition of P2X7R with A-438079 prevents LPS-induced release of IL-1β; cytokine release is also absent in spinal cord slices taken from P2X7R knock-out mice (Clark et al., 2010). Application of ATP did not evoke release of IL-1β from the dorsal horn unless preceded by an LPS priming stimulus, and this release was dependent on P2X7R activation. Extensive phosphorylation of p38 MAPK in microglial cells in the dorsal horn was found to correlate with IL-1β secretion following both LPS and ATP. When cortical and spinal cord microglia were pretreated for 30 minutes with the selective p38 inhibitor SB-202190, IL-1β output from ATP-challenged and LPS-primed was reduced (Figure 26). Similar results were obtained with a second, structurally different p38 inhibitor (SB-239063) (not shown).
Figure 26. The p38 MAPK inhibitor SB-202190 reduces the rise in IL-1β release caused by addition of extracellular ATP to LPS-primed cortical and spinal cord microglia

Purified neonatal rat cortical (left panel) and spinal cord (right panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: SB-202190 (10 μM) for 30 minutes, followed by additional of 1 μg/ml LPS for 2 h. ATP (5 mM) was then added to the cultures, where indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). ***p<0.001 vs LPS+ATP.

Unexpectedly, the p38 MAPK inhibitor SB-202190 caused a marked increase in IL-1β release from both cortical and spinal cord microglia incubated with LPS for 24 h (Figure 27). However, SB-202190 augmented IL-1β release from these long-term LPS-treated cortical and spinal cord microglia in a manner that was influenced by the concentration of FBS in the culture medium (Figure 28). The reason for the effect of FBS on this behavior is not known, but may account for the lack of increase in IL-1β in p38-treated microglia subjected to a priming stimulus (Figure 26).

This effect of the p38 inhibitor was not general, as neither the LPS-stimulated rise in IL-6 nor NO release was augmented; SB-202190 actually decreased NO production (Figure 29). Noradrenaline, which has been reported to inhibit IL-1β and NO release from LPS-stimulated microglia (Dello Russo et al., 2004), did so in both spinal cord (Figure 30) and cortical (not shown) microglia. However, noradrenaline did not affect IL-1β release in the priming response (see Part 3).
Figure 27. The p38 MAPK inhibitor SB-202190 strongly increases IL-1β release from long-term LPS-treated cortical microglia and astrocytes

Purified neonatal rat cortical (left panel) and spinal cord (right panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: SB-202190 (10 µM) for 30 minutes, followed by addition of 10 ng/ml LPS for 24 h. The IL-1β content of the culture medium was then determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). ***p<0.001 vs LPS alone.

Figure 28. The p38 MAPK inhibitor SB-202190 augments IL-1β release from long-term LPS-treated cortical and spinal cord microglia: influence of FBS concentration.

Purified neonatal rat cortical (left panel) and spinal cord (right panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: SB-202190 (10 µM) for 30 minutes, followed by addition of 10 ng/ml LPS for 24 h. The IL-1β content of the culture medium was then determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). *p<0.05, **p<0.01 and ***p<0.001 vs the corresponding LPS-only cultures at that concentration of FBS.
Figure 29. The p38 MAPK inhibitor SB-202190 augments IL-1β release, but not IL-6 or NO, from long-term LPS-treated spinal cord microglia

Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: SB-202190 (10 µM) for 30 minutes, followed by addition of 10 ng/ml LPS for 24 h. The IL-1β, IL-6 and NO contents of the culture medium were then determined as described under Methods.

Figure 30. Norepinephrine inhibits IL-1β and NO release from spinal cord microglia treated 24 h with LPS

Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: the indicated concentrations of norepinephrine for 30 minutes, followed by addition of 10 ng/ml LPS for 24 h. The IL-1β and NO contents of the culture medium were then determined as described under Methods.
3.2. The role of astrocytes in the P2X7R priming response with ATP

As demonstrated in Part 1 (Figure 22) not only microglia, but also astrocytes appear to release IL-1β following addition of extracellular ATP to LPS-primed cells. Astrocytes are the predominant cell type in the CNS; hence, confirming their ability to display P2X7R-dependent release of the pro-inflammatory cytokine IL-1β is an important question. The elucidation of astrocyte function has benefited from the ability to study these glial cells under defined conditions in vitro. Although astrocyte cultures are relatively easy to establish, these cultures can be contaminated by microglia, which have been shown to modify astrocyte responses under certain circumstances (Brown et al., Ciccarelli et al., 2000; Xiong et al., 1999). Furthermore, because pro-inflammatory stimuli can affect the expression of similar genes in microglia and astrocytes, interpretation of results using astrocyte cultures can be confounded by the presence of contaminating microglia (Saura et al., 2003; Solà et al., 2002). Anti-mitotic agents have been employed to inhibit microglial growth in primary astrocyte cultures (Hewett, 1999; Swanson et al., 1997). However, such an approach can be applied only on high-density monolayers after the astrocytes have entered a non-proliferative state induced by cell–cell contact. This allows a period of microglial cell growth prior to mitotic inhibition; thus, such cultures can still have a considerable number of microglia present.

L-leucyl-L-leucine methyl ester (L-LME) (Figure 31) is a lysosomotropic agent originally used to selectively destroy macrophages (Thiele et al., 1983). It was later found that brief incubation with L-LME in general eliminates cells with cytotoxic potential (for example, cytotoxic T cells, natural killer cells), and has been employed to deplete microglia from neural cultures including astrocytes (Giulian et al., 1993; Guillemín et al., 1997) and oligodendrocytes (Hewett et al., 1999). L-LME-mediated toxicity is dependent on the conversion of membranolytic products of structure (Leu-
Leu)$_n$-OMe ($n \geq 3$) by the acyltransferase activity of the enzyme dipeptidyl peptidase I, a lysosomal thiol protease also known as cathepsin C (Thiele and Lipsky, 1990).

Figure 31

Hamby et al. (2006) recently demonstrated that exposing confluent cortical astrocytes to 50-75 mM L-LME for 60-90 minutes effectively depleted microglia from the high-density astrocyte monolayers, as evidenced by the selective depletion of microglial-specific markers. Purified monolayers appeared morphologically normal 24 h after L-LME treatment and expressed iNOS and cyclooxygenase-2 proteins upon stimulation with LPS plus IFN-$\gamma$, albeit (and not surprisingly) to a lower level than unpurified monolayers. L-LME treatment did not alter global protein synthesis and a reactive phenotype could be induced in the purified monolayers upon treatment with cyclic AMP-elevating agents.

To verify the effect of L-LME treatment in our cultures enriched cortical and spinal cord astrocytes were first incubated 60 minutes with 50 mM L-LME, and then stimulated 48 h later with LPS for 6 h and processed for Iba-1 gene expression by RT-PCR. In addition, and to control for a microglia-specific expression of the Iba-1 signal, some cultures received varying numbers of purified microglia 24 h after L-LME. The latter were left for 24 h to allow microglia to attached, and were then treated with LPS for 6 h. As Figure 32 shows, L-LME treatment effectively eliminated Iba-1 mRNA expression from both cortical and spinal cord astrocytes. Further, the re-addition of purified microglia to L-LME-treated astrocytes resulted in the reappearance of Iba-1 gene expression, as a function of microglia cell number introduced.
Enriched astrocyte cell populations prepared from either tissue were treated for 60 minutes with 50 mM L-LME, and returned to fresh culture medium for 24 h. After this time purified cortical or spinal cord microglia were added to the astrocyte cultures, at the densities indicated, and incubation continued for a further 24 h. Cultures were then challenged with LPS (10 ng/ml) and processed for Iba1 mRNA expression by RT-PCR after 6 h. Data are means ± SEM (duplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments.

The reintroduction of purified microglia to L-LME-treated astrocytes resulted in the recovery of functional LPS responsiveness, as well. An example of this is shown in Figure 33. Under these conditions L-LME (first three sets of columns from the left in each figure): eliminated the LPS induction of IL-1β mRNA in cortical and spinal cord astrocytes (Panel A); reduced, but did not fully abolish the LPS-induced rise in IL-6 (Panel B) and TNF-α (Panel C) mRNA; abolished in spinal cord, but did not cortical astrocytes the LPS-induced rise in iNOS mRNA expression (Panel D). This last observation is consistent with the reported expression by rodent cortical astrocytes of iNOS in vitro (Akama et al., 2000; Buskila et al., 2005) even after treatment with L-LME (Hamby et al., 2006) and in vivo (Murphy, 2000). A lower L-LME concentration (5mM) was ineffective while incubating purified microglia with 50 mM (but not 5 mM) L-LME for 60 minutes resulted in complete cell destruction after 24 h, assessed morphologically and with the MTT vitality assay. Astrocytes treated with 50 mM L-LME did not evidence any overt morphological changes or loss of MTT reactivity or GFAP immunostaining (data not shown).
Enriched astrocyte cell populations prepared from either tissue were treated for 60 minutes with 50 mM L-LME, and returned to fresh culture medium for 24 h. After this time the indicated numbers of purified cortical or spinal cord microglia were added to the astrocyte cultures, and incubation continued for a further 24 h. Cultures were then challenged with LPS (10 ng/ml) and processed for mRNA expression by RT-PCR after 6 h: IL-1β (panel A), IL-6 (panel B), TNF-α (panel C), iNOS (panel D). Data are means ± SEM (duplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Culture wells seeded with the same numbers of microglia contained too little material to reliably process.

Reintroduction of microglia (second three sets of columns in each panel) restored LPS responsiveness for IL-1β (Figure 33A) and IL-6 (Figure 33B) release; the effect was more pronounced in spinal cord glia co-cultures. In the latter, expression levels clearly exceeded those for L-LME-treated astrocytes already at the lowest microglia number (9,000), and approached values of LPS-stimulated (but L-LME untreated) astrocytes with 36,000 microglia. For TNF-α, microglia addition to L-LME-depleted astrocytes also restored LPS responsiveness, but in this case cortical-
derived glia appeared more sensitive to the presence of microglia in comparison to spinal cord (Figure 33C). Addition of spinal cord microglia to L-LME-treated astrocytes fully restored iNOS gene induction by LPS, becoming evident already with the lowest number of microglia added (Figure 33D). The effect of microglia addition was less evident for cortical astrocytes, as iNOS mRNA expression remained relatively high even after treatment with L-LME.

The ability to reconstitute LPS responsiveness in microglia/astrocyte co-cultures was also observed in terms of mediator output. Addition of microglia to L-LME-treated cortical astrocytes resulted in NO release in relation to the number of microglia added, becoming already detectable with less than 1000 cells per culture well (Figure 34, left panel). In contrast, the same numbers of microglia, in the absence of astrocytes failed to produce NO upon incubation with LPS, except at the highest number (Figure 34, right panel). A similar behaviour was observed in the case of IL-6 (not shown).

**Figure 34.** Co-culture of cortical microglia and L-LME-treated cortical astrocytes restores LPS-induced NO release

Left panel: Enriched cortical astrocytes cultured in a 96 well microplate were treated with 50 mM L-LME for 60 minutes, and returned to fresh culture medium for 24 h. After this time purified cortical microglia were added, at the numbers indicated, to the astrocyte cultures and incubation continued for a further 24 h. Cultures were then challenged with LPS (10 ng/ml) and the culture medium collected after 24 h for analysis of NO content.

Right panel: The same numbers of microglia were cultured in a parallel plate, treated with LPS as above and medium analyzed for NO content. Data are means ± SEM (n=3). Cultures derived from spinal cord microglia and astrocytes behaved in a qualitatively similar fashion (not shown).
Addition of cortical microglia to L-LME-treated cortical astrocytes restored the priming response to LPS (IL-β release), an effect proportionate to the numbers of microglia (Figure 35, left panel). As for the case of NO release in LPS-stimulated astrocyte-microglia co-cultures, equivalent numbers of purified microglia cultured in the absence of astrocytes failed to generate appreciable amounts of IL-β when incubated with LPS, except at the highest cell number (Figure 35, right panel). Qualitatively similar results were obtained using cerebellar-derived astrocytes and microglia (Figure 36).

**Figure 35.** Co-culture of cortical microglia and L-LME-treated cortical astrocytes restores LPS sensitization to ATP-induced release of IL-1β

*Left panel:* Enriched cortical astrocytes cultured in a 96 well plate were treated with 50 mM L-LME for 60 minutes, and returned to fresh culture medium for 24 h. After this time purified cortical microglia were added, at the numbers indicated, to the astrocyte cultures and incubation continued for a further 24 h. The cultures were then incubated in serum-free medium with 1 µg/ml LPS for 2 h followed by addition of ATP to a final concentration of 5 mM. After a further 60 minutes incubation culture medium was collected for IL-1β analysis.

*Right panel:* The same numbers of microglia were cultured in a parallel plate, treated with LPS and ATP as above and medium analyzed for IL-1β content. Data are means ± SEM (n=3). Cultures derived from spinal cord microglia and astrocytes behaved in a qualitatively similar fashion (not shown).
Figure 36. Co-culture of cerebellar microglia and L-LME-treated cerebellar astrocytes restores LPS sensitization to ATP-induced release of IL-1β

Left panel: Enriched cerebellar astrocytes cultured in a 96 well plate were treated with 50 mM L-LME for 60 minutes, and returned to fresh culture medium for 24 h. After this time purified cerebellar microglia were added, at the numbers indicated, to the astrocyte cultures and incubation continued for a further 24 h. The cultures were then incubated in serum-free medium with 1 µg/ml LPS for 2 h followed by addition of ATP to a final concentration of 5 mM. After a further 60 minutes incubation culture medium was collected for IL-1β analysis.

Right panel: The same numbers of microglia were cultured in a parallel plate, treated with LPS and ATP as above and medium analyzed for IL-1β content. Data are means ± SEM (n=3).

The increased release of mediators in microglia-astrocyte co-cultures could be due to soluble factors derived from microglia. For example, IL-1β can activate astrocytes (Guo et al., 2004; Wu et al., 2008) and increase their production of NO (Chao et al., 1996). However, addition of IL-1β to microglia-depleted astrocytes, up to amounts produced by the equivalent of 100,000 microglia in a 96-well culture, failed to cause release of NO (unpublished observations). This question was next addressed by examining the effect of conditioned medium from L-LME-treated and LPS-stimulated astrocytes on mediator output from purified microglia. The experimental design was similar to that in Figure 33, except that parallel sets of cultures with a range of microglia numbers received either
LPS or medium conditioned over astrocytes incubated with LPS for 24 h. Cortical astrocytes treated with L-LME, as expected, failed to produce NO upon incubation with LPS (10 ng/ml) (Figure 37A). Addition of cortical microglia to L-LME-treated astrocytes, followed by stimulation with LPS resulted in the recovery of LPS responsiveness in a microglia cell number-dependent manner. Again, and consistent with Figure 34, the same numbers of microglia alone (625 – 20,000) gave little or no signal upon LPS exposure for 24 h (Figure 37B). When a parallel plate of microglia was incubated with medium from astrocytes treated with LPS (10 ng/ml) for 24 h, NO release levels were very low and well below values achieved for astrocyte-microglia co-cultures (Figure 37C). A similar experiment was not carried out for the priming response, as the relatively short (2 h) incubation time with LPS prior to ATP addition is unlikely to have resulted in the de novo synthesis of mediator(s).

**Figure 37: A-B-C.** Culture medium from microglia-depleted, LPS-treated cortical astrocytes is not sufficient to provoke NO release from microglia alone.

*Panel A:* Enriched cortical astrocytes cultured in 96 well microplates were treated with 50 mM L-LME for 60 min, and returned to fresh culture medium for 24 h. After this time purified cortical microglia were added to one plate of astrocytes, at the numbers indicated, and incubation continued for a further 24 h. Cultures were then challenged with LPS (10 ng/ml) and the culture medium collected after 24 h for analysis of NO content. The far right column (100,000 microglia) did not receive LPS. In this experiment culture wells seeded with 100,000 microglia were included as a 'reference point', as this is the density normally used in our 96 well format. The second plate of LPS-treated astrocytes (no microglia added) was used to generate conditioned medium (see panel C).

*Panel B:* The same numbers of microglia as in panel A were cultured alone in a parallel plate, treated with LPS as above and medium analyzed for NO content after 24 h.

*Panel C:* A second plate of microglia (same numbers as in panel B) received conditioned medium from the LPS-treated astrocytes (see panel A). NO release was analyzed after 24 h. All data are means ± SEM (n=3).
Figure 37: A-B-C. Culture medium from microglia-depleted, LPS-treated cortical astrocytes is not sufficient to provoke NO release from microglia alone.
3.3. Is the P2X7R priming response strictly dependent on ATP, LPS and TLR4?

3.3.1. Amyloid peptide

As discussed above, extracellular ATP is a mediator of intercellular communication and a danger signal. Release of this and other nucleotides modulates microglia responses via P2Y and P2X receptors, among which the P2X7 subtype stands out for its pro-inflammatory activity. For example, increased expression of P2X7R mRNA has been described in AD-derived microglia compared to non-demented brain, along with prominent P2X7R protein immunoreactivity in association with Aβ plaques and localized to HLA-DR-immunoreactive microglia (McLarnon et al., 2006) as well as in a mouse model of AD (Parvathenani et al., 2003). Despite unequivocal evidence for Aβ-mediated activation of microglia, the plasma membrane receptors involved are largely unknown. Different receptors, among which CD36 (El Khoury et al., 1996), α6β1 integrins, CD14, the formyl peptide receptor-like protein, and TLR2 (Chen et al., 2006) are ligated by Aβ, thus leading to microglia activation, generation of inflammatory mediators and Aβ internalization. Sanz et al. (2009) reported that the Aβ fragment (25-35) triggered increases IL-1β secretion in LPS-primed microglia from wild-type but not from P2X7R-deleted mice. However, the Aβ(25-35) fragment does not occur in nature; rather, Aβ(1-42) is the peptide that is initially deposited within the extracellular plaques of AD patients and is highly prone to aggregation. Soluble oligomers of Aβ(1-42) are now believed to be the principal pathological form and are synaptotoxic (Lacor et al., 2007).

To examine further the ability of Aβ to trigger IL-1β release from LPS-primed purified microglia and enriched astrocytes, these two glial cell populations were first incubated with 1 μg/ml LPS for 2 h, followed by addition of either Aβ(25-35) or Aβ(1-42) (20 μM), or 5 mM ATP. As
**Figure 38** shows, neither Aβ peptide was able to provoke IL-1β release, while ATP functioned as expected. The action of ATP was blocked by 10 µM A740003.

**Figure 38.** Aβ peptides are unable to mimic the effect of ATP in enhancing IL-1β release from LPS-primed cortical microglia

Purified neonatal rat cortical microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: A740003 (10 µM) where indicated for 30 minutes, followed by addition of 1 µg/ml LPS for 2 h. ATP (5 mM), Aβ(25-35) (20 µM) or Aβ(1-42) (20 µM) was then added to the cultures, as indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells).

### 3.3.2. Ethanol intoxication

The brain is one of the major target organs of ethanol actions, and its chronic and acute intoxication results in significant alterations in brain structure and function, and in some cases to neurodegeneration. Ethanol has immunomodulatory effects and induces specific alterations in the TLRs response in many tissues. These actions depend on the cell type, ethanol dose and treatment duration, as well as the concomitant presence of pathogens and their characteristics. Recent findings indicate that low concentrations of ethanol (10 mM) promote inflammatory processes in brain and in glial cells by up-regulating cytokines and inflammatory mediators (iNOS, NO, COX-2), and by activating signaling pathways (MAPKs) and transcriptional factors (NF-κB) implicated in
inflammatory injury (Blanco and Guerri, 2007). TLR4/IL-1RI receptors may be involved in 
etanol-mediated inflammatory signaling, since pharmacologically blocking or genetic deletion 
these receptors abolishes the production of ethanol-induced inflammatory mediators and cell death 
(Blanco and Guerri, 2007; Fernandez-Lizarbe et al., 2009). In summary, these studies suggest that 
TLR4/ IL-1RI are important targets of ethanol-induced inflammatory brain damage.

To investigate the possibility that ethanol could activate microglia and replace LPS in the 
priming response to ATP, spinal cord microglia were first incubated with 10-100 mM ethanol for 24 
and 48 h (following the conditions described by Fernandez-Lizarbe et al. (2009). While LPS (50 
ng/ml) incubation resulted in the expected production of NO, none of the ethanol concentrations 
tested were effective (Figure 39). The TLR4 antagonist polymyxin B (10 µg/ml) fully blocked the 
activity of LPS. The measurement of NO was chosen, as its release was the most robust response to 
ethanol (equivalent to LPS) as reported by Fernandez-Lizarbe et al. (2009). Comparable results 
were obtained using cortical microglia (not shown).

Figure 39. Ethanol does not stimulate NO production from spinal cord microglia

Purified neonatal rat spinal cord were seeded in 96 well plates (100,000 cells), and incubated the following 
day with: 50 ng/ml LPS, or 10-30-100 mM ethanol (EtOH) for 24 h and 48 h, after which time the culture 
medium collected for analysis of NO content. Data are means ± SEM (triplicate culture wells).
Cortical and spinal cord microglia were then incubated with 100 mM ethanol or 1 µg/ml LPS for 2 h, followed by challenge with 5 mM ATP for 1 h. Measurement of IL-1β release into the culture medium revealed the expected priming effect of LPS, while ATP was ineffective in ethanol 'primed' cells (Figure 40). Interestingly, adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), a non-hydrolyzable ATP analog which competitively inhibits ATP-dependent enzyme systems was also efficacious in the priming response (Figure 40).

Figure 40. Ethanol cannot stimulate IL-1β release from LPS-primed microglia

Purified neonatal rat cortical (upper panel) and spinal cord (lower panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with 1 µg/ml LPS for 2 h. ATP (5 mM), EtOH (100 mM) or AMP-PNP (5 mM) was then added to the cultures as indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). **p<0.01 vs LPS.
3.3.3. TLR2 and TLR3

Focus has recently shifted to TLR signaling pathways in neurodegenerative disorders (Campbell, 2004; Jin et al., 2008; Okun et al., 2009), including motor neuron disease (Casula et al., 2011), brain injury (Campbell, 2004) and neuropathic pain (Christianson et al., 2011). While the TLRs were originally described according to their ability to respond to exogenous microbial products there is sufficient literature to suggest endogenous products activating TLRs during sterile inflammation. In addition to TLR4, microglia also express functional TLR2 (Kim et al., 2007; Shi et al., 2011) and TLR3 (Obata et al., 2008; Mei et al., 2011) whose activation participates in nerve injury-induced neuropathic pain. However, it is not known if agonists for TLR2 or TLR3 are able to prime microglia for ATP-dependent IL-1β release.

To investigate this question, the response of microglia to TLR2 and TLR3 agonists was examined. Zymosan is a desiccated preparation of yeast cell wall commonly employed to stimulate the activity of the reactive oxygen species-producing enzyme NADPH oxidase in immune cells (DeChatelet et al., 1975), and also activates microglia (Harrigan et al., 2008) by direct binding to TLR2 resulting in NF-κB activation and TNF-α secretion (Sato et al., 2003). To first test microglia responsiveness to zymosan (Sigma), cells were incubated with different concentrations for 24 h and release IL-1β analyzed. As Figure 41 shows, zymosan concentration-dependently stimulated the release of IL-1β from both cortical and spinal cord microglia. A concentration of 3 µg/ml zymosan was chosen for the remaining experiments.
Figure 41. Zymosan concentration-dependently stimulates release from cortical and spinal cord microglia

Purified neonatal rat cortical (left panel) and spinal cord (right panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with 10 ng/ml LPS or the indicated concentrations of zymosan ('Zy') for 24 h. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). ***p<0.001 vs LPS.

Next, microglia were incubated for 2 h with 3 µg/ml zymosan or 1 µg/ml LPS, followed by a further incubation with 5 mM ATP for 1 h. Under these conditions, zymosan proved to be efficacious as LPS in augmenting IL-1β release (Figure 42).
Zymosan is effective like LPS in priming cortical and spinal cord microglia to ATP-dependent IL-1β release.

Purified neonatal rat cortical (upper panel) and spinal cord (lower panel) microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with 1 µg/ml LPS or 3 µg/ml zymosan for 2 h. ATP (5 mM) was then added to the cultures as indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). ***p<0.001 vs LPS.
TLR3 is a key pattern recognition receptor for double-stranded RNA and the synthetic double-stranded RNA poly inosinic:polycytidylic acid (poly I:C) (Alexopoulou et al., 2001), both of which potently induce the type I interferons α and β and other inflammatory cytokines (Jacobs and Langland, 1996; Matsumoto and Seya, 2008). Microglia recognize double-stranded RNA via TLR3 (Town et al., 2006). A 2-h incubation of spinal cord microglia to poly(I:C) (Sigma) primed the cells to ATP augmentation of IL-1β release, although the effect did not appear to be as robust as with LPS or zymosan (Figure 43).

**Figure 43.** Poly(I:C) is capable of priming spinal cord microglia to ATP-dependent IL-1β release.

Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000 cells), and incubated the following day for 2 h with: 1 µg/ml LPS; 3 µg/ml zymosan; 0.5-50 µg/ml poly(I:C) (‘P’). ATP (5 mM) was then added to the cultures as indicated, and incubation continued for a further 60 minutes. The IL-1β content of the culture medium was determined by sandwich ELISA. Data are means ± SEM (triplicate culture wells). *p<0.05 or ***p<0.001 vs the same concentration of poly(I:C) alone.
As reported in Part 1, the p38 MAPK inhibitor SB-202190 markedly raised IL-1\(\beta\) release from cells incubated with LPS for 24 h (Figure 27), while norepinephrine reduced IL-1\(\beta\) release (Figure 30). SB-202190 and norepinephrine showed similar behaviors also for zymosan and poly(I:C) stimulated spinal cord microglia (Figure 44, compare panel A with panels B and C)).

**Figure 44.** The p38 MAPK inhibitor SB-202190 markedly elevates IL-1\(\beta\) release from LPS-, zymosan- and poly(I:C)-treated spinal cord microglia

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Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: 10 \(\mu\)M SB-202190 ('SB') or 10 \(\mu\)M norepinephrine ('NE') for 30 minutes, followed by addition of: (A) 10 ng/ml LPS, (B) 3 \(\mu\)g/ml zymosan ('Zy'), (C) 50 \(\mu\)g/ml poly(I:C) for 24 h. The IL-1\(\beta\) content of the culture medium was then determined by ELISA. Data are means ± SEM (triplicate culture wells). *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) vs compound alone.
In contrast, SB-202190 reduced the rise in IL-1β release caused by addition of extracellular ATP to LPS-, zymosan- or poly(I:C)-primed spinal cord microglia (Figure 45), while norepinephrine was without effect.

**Figure 45.** The p38 MAPK inhibitor SB-202190 reduces the rise in IL-1β release caused by addition of extracellular ATP to LPS-, zymosan or poly(I:C) primed spinal cord microglia

Purified neonatal rat spinal cord microglia were seeded in 96 well plates (100,000 cells), and incubated the following day with: 10 µM SB-202190 ('SB') or 10 µM norepinephrine ('NE') for 30 minutes, followed by addition of: (A) 10 ng/ml LPS, (B) 3 µg/ml zymosan ('Zy'), (C) 50 µg/ml poly(I:C) ('Poly') for 2 h, and then 5 mM ATP for 1 h. IL-1β content of the culture medium was then determined by ELISA. Data are means ± SEM (triplicate culture wells). *p<0.05, ***p<0.001 vs the same conditions without SB-202190.
4. DISCUSSION

Although inflammation is a protective cellular response aimed at removing injurious stimuli and initiating the healing process, prolonged inflammation, known as chronic inflammation, goes beyond physiological control, and eventually destructive effects override the beneficial effects. Nowadays, persistent inflammation is considered as an underlying contributor to virtually every chronic disease, including neuropathic pain. Recent studies demonstrate that chronic inflammation in the CNS is also a hallmark of various neurodegenerative disorders in which progressive loss of structure and function of neurons and neuronal death are observed (Barnum and Tansey, 2010; Dauer and Przedborski, 2003; Gao et al., 2003; Giovannini et al., 2002; Jantaratnotai et al., 2003).

For example, the concentration of nitrite, a metabolite of NO, increases in the cerebrospinal fluid of patients with PD and AD in comparison with age-matched controls (Qureshi et al., 1995). Consistently, the ablation of iNOS in mutant mice significantly protects dopaminergic neurons from MPTP neurotoxicity, indicating that iNOS is essential in MPTP-induced substantia nigra pars compacta dopaminergic neurodegeneration (Dehmer et al., 2000). A variety of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, eicosanoids, and other immune neurotoxins, are found in either cerebrospinal fluid or affected brain regions of patients with neurodegenerative disorders (Nagatsu et al., 2000). Finally, NF-κB, a transcription factor required for the transcription of most proinflammatory molecules, is activated in the substantia nigra pars compacta of PD patients and MPTP-intoxicated mice and monkeys, and selective inhibition of NF-κB in mice and monkeys by NF-κB essential modifier-binding domain peptides protects dopaminergic neurons from MPTP toxicity (Ghosh et al., 2008; Mondal et al., 2012). Therefore, inflammation is an important target for neuronal protection in neurodegenerative disorders and neuropathic pain, the latter which typically develops when peripheral nerves are damaged due to surgery, bone compression in cancer, diabetes or infection.

Activation of glial cells (microglia and astroglia) has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including AD, PD, Creutzfeldt-Jacob disease, HIV-107
associated dementia, stroke, and multiple sclerosis (Dauer and Przedborski, 2003; Ghosh et al., 2008; González-Scarano and Baltuch, 1999). Glia act also as a link between neuroinflammation and neuropathic pain (Jha et al., 2012). Activated microglia and astroglia accumulate at sites of injury or plaques in neurodegenerative CNS (González-Scarano and Baltuch, 1999; Carson, 2002). Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival and activated astrocytes may have important beneficial effects in the recovery of injured CNS by actively monitoring and controlling the extracellular water, pH, and ion homeostasis, it is believed that severe activation of these brain cells causes various autoimmune responses leading to neuronal death and brain injury (Dauer and Przedborski, 2003; González-Scarano and Baltuch, 1999; Carson, 2002). During activation, microglia and astroglia express various genes related to inflammation, such as proinflammatory cytokines, proinflammatory enzymes, and proinflammatory adhesion molecules. Therefore, characterization of signaling pathways required for the activation of glial cells is an active area of investigation because compounds capable of antagonizing such signaling steps may have therapeutic benefit in neurodegenerative disorders and neuropathic pain.

It is well-known that ATP can serve as an important chemical gliotransmitter that mediates a broad range of physiological and pathological processes in the nervous system (Burnstock, 2007). ATP acts as a short-term signalling molecule in neurotransmission, neuromodulation and secretion and has long-term (trophic) roles in cell proliferation, differentiation and death in development and regeneration. Three subclasses of purine and pyrimidine receptors have been identified, P1 adenosine receptors, P2X ionotropic nucleotide receptors and P2Y metabotropic receptors. ATP is released physiologically by many cell types by mechanical deformation and, after release, ATP undergoes ectonucleotidase degradation. Purinergic receptors appeared early in evolution and have a widespread distribution on many non-neuronal cells and neurons. Purinergic signalling is involved in embryonic and stem cell development. There is a rapidly growing literature dealing with the pathophysiology of purinergic signalling, including therapeutic developments for diseases both
outside (for example, thrombosis, osteoporosis, kidney failure, cystic fibrosis, and cancer) and within the nervous system (cerebral ischemia, chronic neurodegenerative disorders, pain) (Burnstock, 2011).

ATP is a key neurotransmitter in the development and maintenance of neuropathic pain (Burnstock, 2009; Inoue and Tsuda, 2009; Tsuda et al., 2010). Both P2Y and P2X purinoceptors are widely distributed in the sensory nervous system and exhibit various effects both at neuronal and glial cells (Burnstock and Knight, 2004; Ruan et al., 2005; Zeng et al., 2009). Microglia are activated in response to a diversity of stimuli, ranging from peripheral inflammation to CNS injury, which is necessary and sufficient to induce neuropathic pain. There is abundant evidence that microglia are activated in the dorsal horn in a wide variety of nerve injury models (Gwak et al., 2012; Itoh et al., 2011). Following nerve injury, there are many molecular and cellular changes of the peripheral and central nervous systems, among which ATP and its purine receptors have been widely studied. The signal(s) for activating microglia in these models of neuropathic pain is unclear, but it has been proven that microglia are exquisitely responsive to extracellular ATP, which is released by cellular damage, nearby astrocytes or neurons from either synaptic or non-synaptic regions (Davalos et al., 2005; Di Virgilio, 2006).

The P2X7R is a unique member of the P2X receptor family, because it is activated only by high concentrations of ATP (>100 μM) and its prolonged exposure to ATP has been shown to form a much larger pore than any other P2X channel (North, 2002). Numerous studies indicate that P2X7R is linked to responses associated to spinal cord damage and inflammation being primary mediators of pain sensation (Broom et al., 2008; Chessell et al., 2005; Honore et al., 2006; McGaraughty et al., 2006). P2X7R−/− mice do not develop either mechanical allodynia or thermal hyperalgesia in a partial nerve ligation model (Chessell et al., 2005). P2X7R is primarily expressed in microglia and peripheral macrophages. A recent study has demonstrated that P2X7R is required for the activation and proliferation of microglia, suggesting that it regulates immune function and inflammatory responses (Monif et al., 2009). Activation of P2X7R can promote the release of some cytokines
such as IL-1β and IL-17, as well as superoxide products, all of which play important roles in the generation or maintenance of pain. Further, functional P2X7Rs have been demonstrated in peripheral glial cells in rat dorsal root ganglion, and this may participate also in peripheral sensory transduction of pain perception (Zhang et al., 2005).

In this study, a LPS-primed, P2X7R-dependent response (IL-1β release) has been characterized in purified microglia cultured from rat cortex, spinal cord and cerebellum. While confirming the behavior of cortical microglia, this is the first report of a priming response in cells from spinal cord and cerebellum. Here, P2X7R dependence was demonstrated by the use of a series of receptor-selective agonists of varying potency, which blocked in a concentration-dependent manner the ATP- but not LPS-dependent component of IL-1β release. These same antagonists were initially described using cell lines over-expressing the recombinant P2X7R, in terms of calcium flux (Donnelly-Roberts and Jarvis, 2007); however, there efficiency versus priming-dependent IL-1β release was not mentioned. These findings are relevant in terms of the importance ascribed to spinal cord microglia in neuropathic pain (He et al., 2012). For example, in the dorsal horn of spinal cord, pharmacological inhibition of the P2X7R prevents LPS-induced release of IL-1β; cytokine release is also absent in spinal cord slices taken from P2X7R knock-out mice (Clark et al., 2010). Application of ATP did not evoke release of IL-1β from the dorsal horn unless preceded by an LPS priming stimulus, and this release was dependent on P2X7R activation. Further, activation of cerebellar microglia has recently been reported to play a heretofore unknown role in cerebellar function, including release of pro-inflammatory cytokines such as IL-1β (Cutando et al., 2012).

Extensive phosphorylation of p38 MAPK in microglial cells in the dorsal horn was found to correlate with IL-1β secretion following both LPS and ATP (Clark et al., 2010). In addition, activation of Src family kinases in spinal microglia contributes to formalin-induced persistent pain through the p38-MAPK pathway (Tan et al., 2012). In accord with the latter observations, when cortical and spinal cord microglia were pretreated for 30 minutes with selective p38 inhibitors (SB-
IL-1β output from ATP-challenged and LPS-primed spinal cord and cortical microglia was reduced. Rather unexpectedly, when SB-202190-treated microglia were incubated with LPS for 24 hours IL-1β release was markedly increased over that for LPS alone. Conceivably, this could represent a form of positive feedback control, although there is no published evidence for such a phenomenon. This effect appeared to be specific for IL-1β release mediated by long-term LPS stimulation of microglia, as the p38 inhibitor did not augment release of either IL-6 or NO (output of NO actually decreased). The β-adrenergic receptor agonist noradrenaline concentration-dependently decreased NO and IL-1β production from 24-hour LPS-stimulated microglia, as described by others (Dello Russo et al., 2004), but did not alter the IL-1β release LPS-primed, ATP-treated cells. The form(s) of IL-1β released (precursor versus mature) under these different conditions remains to be determined.

Until now there have been no reports of a P2X7R-dependent priming response in astrocytes. Astrocytes are the predominant cell type in the CNS, and such would be an important factor in neuroinflammation. Indeed, the first set of experiments described here suggested that astrocytes also are able to release IL-1β following addition of extracellular ATP to LPS-primed cells. It is not uncommon for astrocyte cultures to be contaminated by microglia (as shown here), and this can alter astrocyte responses (Brown et al., 1996; Ciccarelli et al., 2000; Xiong et al., 1999). Pro-inflammatory stimuli can affect the expression of similar genes in microglia and astrocytes, and interpretation of results using astrocyte cultures can be confounded by the presence of contaminating microglia (Saura et al., 2003; Solà et al., 2002). To obviate this potential problem, the enriched astrocyte cultures were depleted of residual microglia using the lysosomotropic agent L-LME. L-LME accumulates in the lysosomes of lytic cells such as cytotoxic T cells, macrophages and microglia, producing membranolytic products of structure (Leu-Leu),-OMe (n≥3) by cathepsin C (a lysosomal thiol protease). Molecular biological, immunocytochemical, and biochemical methods clearly showed that L-LME-treated astrocytes were devoid of detectable numbers of
microglia. Further, these purified astrocytes (cortical, spinal cord) failed to respond to LPS, in terms of mRNA induction and NO and cytokine output. When astrocyte/microglia co-cultures were reconstituted by adding defined numbers of microglia to purified astrocytes, LPS responsiveness was restored. It is important to note here that LPS sensitivity of the astrocyte/microglia co-cultures far exceeded that of equivalent numbers of microglia alone.

Whether or not microglia are the main producers of the genes/products studied is a key point in this study. Factors released from microglia can affect astrocyte responses to LPS (Holm et al., 2012). Further, IL-1β can activate astrocytes (Guo et al., 2004) leading, e.g. to increased production of NO (Chao et al., 1996). Addition of IL-1β to microglia-depleted astrocytes, up to amounts produced by the equivalent of 100,000 microglia in a 96-well culture did not lead to release of NO. Analyzing the gene products such as cytokines in these cultures is technically difficult, as the cytokines are largely released into the culture medium. At least in the case of NO production, this phenomenon did not depend on soluble factors released from LPS-treated astrocytes, as conditioned medium from 24-h LPS-treated purified astrocytes failed to induce a greater output of NO from microglia than direct LPS addition to the latter. Moreover, a heightened responsiveness of the astrocyte/microglia co-cultures was also observed for LPS/ATP priming. That mediator output in the microglia/astrocyte co-cultures was far in excess of that found for the same numbers of microglia cultured alone suggests that these two glia cell types influence their collective response to LPS. These observations may explain, in part, the perceived role of astrocytes in pain (Chiang et al., 2012). Astrocytes can produce microglial mitogens (Shafit-Zagardo et al., 1993); however, these are unlikely to have influenced the astrocyte/microglia co-culture behaviors, given the relatively short time span of the experiments. To avoid potential effects of mitogenic factors, the co-cultures were not used at longer times. Thus, one cannot determine if prolonged co-culture incubation time before challenge with LPS would have resulted in even more robust responses.
Many studies have shown that P2X7R immunoreactivity is increased in activated microglia of many models of diseases, such as AD, multiple sclerosis, amyotrophic lateral sclerosis and focal cerebral ischemia (McLarnon et al., 2006; Parvathenani et al., 2003; Yanagisawa et al., 2008; Yiangou et al., 2006), while increased expression of P2X7R mRNA has been described in AD-derived microglia compared to non-demented brain (McLarnon et al., 2006) - suggesting that microglial P2X7R might be a general mediator of stress during pathological states. Despite unequivocal evidence for Aβ-mediated activation of microglia, the plasma membrane receptors involved are largely unknown. Sanz et al. (2009) reported that the Aβ fragment (25-35) triggered increases IL-1β secretion in LPS-primed microglia from wild-type but not from P2X7R-deleted mice. To examine further the ability of Aβ to trigger IL-1β release from LPS-primed purified microglia and enriched astrocytes, these two glial cell populations were first incubated with 1 µg/ml LPS for 2 h, followed by addition of either Aβ(25-35) or Aβ(1-42) (20 µM), or 5 mM ATP. Aβ(1-42) is the peptide that is initially deposited within the extracellular plaques of AD patients and is highly prone to aggregation. Soluble oligomers of Aβ(1-42) are now believed to be the principal pathological form and are synaptotoxic (Lacor et al., 2007). However, neither Aβ peptide was able to mimic the effect of ATP in enhancing IL-1β release from LPS-primed cortical microglia.

Chronic and acute ethanol intoxication alters brain structure and function, and in some cases can lead to neurodegeneration. Concentrations of ethanol found in drinkers (10 mM) promote inflammatory processes in brain and in glial cells by up-regulating cytokines and inflammatory mediators (iNOS, NO, COX-2) and transcription factors implicated in inflammatory injury (Blanco and Guerri, 2007). Pharmacological block or genetic deletion of TLRs abolishes the production of ethanol-induced inflammatory mediators and cell death (Blanco and Guerri, 2007; Fernandez-Lizarbe et al., 2009). However, 10-100 mM ethanol failed to provoke production of NO from spinal cord and cortical microglia – unlike LPS. When cortical and spinal cord microglia were incubated
with 100 mM ethanol or 1 µg/ml LPS for 2 h, followed by challenge with 5 mM ATP for 1 h, IL-1β was released into the culture medium with LPS, while ATP was ineffective in ethanol 'primed' cells.

Here we show for the first time that agonists for TLR2 (zymosan) or TLR3 (poly(I:C)) are also capable of priming microglia for ATP-dependent IL-1β release. As with LPS, the p38 MAPK inhibitor SB-202190 reduced the rise in IL-1β release caused by addition of extracellular ATP to zymosan or poly(I:C) primed microglia, while norepinephrine was without effect. In contrast, but in keeping with the data for microglia subjected to a 24-h incubation with LPS, SB-202190 markedly raised IL-1β release from cells incubated with zymosan and poly(I:C) for 24 h, while norepinephrine reduced IL-1β release. These findings broaden considerably the potential role of P2X7R in inflammation-associated neuropathologies, and support the idea that a better understanding of astrocyte/microglia interactions may improve our understanding in how these cells respond to CNS injury or inflammation, in particular where TLRs are involved.

Increasing emphasis is now being placed on TLR signaling pathways in neurodegenerative disorders (Campbell, 2004; Jin et al., 2008; Okun et al., 2009), including motor neuron disease (Liu et al., 2009; Casula et al., 2011), brain injury (Campbell, 2004) and neuropathic pain (Christianson et al., 2011). Systemic bacterial insults accelerate disease progression in animals and in patients with AD (Cunnungham et al., 2005). There are reports of exacerbation of chronic CNS pathology by systemic gram-negative bacterial stimulation in a model of prion disease and in animal models of PD (Godoy et al., 2008), amyotrophic lateral sclerosis (Nguyen et al., 2004), AD (Sly et al., 2001) and ageing (Godbout et al., 2005). In addition to exogenous microbial products like LPS, endogenous products can activate TLRs during sterile inflammation. Besides TLR4, microglia also express functional TLR2 (Kim et al., 2007; Shi et al., 2011) and TLR3 (Obata et al., 2008; Mei et al., 2011) proteins, whose activation participates in nerve injury-induced neuropathic pain. A recent report also demonstrated that systemic challenge with the TLR3 agonist poly(I:C) induces amplified
IFNα/β and IL-1β responses in the diseased brain and exacerbates chronic neurodegeneration (Field et al., 2010).

**Epilogue**

It is now generally accepted that high levels of extracellular nucleotides such as ATP may be released under pathological conditions such as inflammation, trauma, and stress. Recent findings suggest that increased P2X7R numbers drive microglial activation, rather than P2X7R over-expression being a consequence of microglial activation (Monif et al., 2009). Signaling via P2X7Rs may thus allow cells to sense and respond to events occurring in the extracellular environment, modulate the transcription of genes involved in cellular inflammatory processes and to thus regulate cytokine responses. The therapeutic exploitation of P2X7Rs is now under way because of their potential role, not only in such disorders as AD, spinal cord injury, and sensory neuropathies but also in multiple sclerosis, neuropathic pain, and rheumatoid arthritis, (Broom et al., 2008; Romagnoli et al., 2008; Volonté et al., 2012) as well as depressive illness. There is growing appreciation for the role of P2X7R modulation of pro-inflammatory IL-1β processing, and the analgesic activity of P2X7R antagonists. At the same time, recent findings (Chen et al., 2008) suggest that alternative therapeutic strategies for controlling pain may be to target P2X7R-associated proteins such as PanX1 hemichannels, which are directly involved in the damaging effects of P2X7R activation (Pelegrin and Surprenant, 2006). P2X7Rs are expressed with some selectivity on different types of cells in the cardiovascular system, and drugs affecting P2X7R signaling may have promise as antihypertensive and anti-thrombotic agents (Ralevic and Burnstock, 2003). Further investigation of the P2X7R with receptor sub-selective antagonists in preclinical studies as well as in disease-specific clinical trials will be critical to evaluating this target’s potential therapeutic use.

Looking beyond the data presented here, it would be of interest to investigate a role for P2X7R in glia-mediated injury to dopaminergic neurons. Glia/neuron co-cultures can be prepared from the
mesencephalic area, which contain a minor population of dopaminergic neurons. Subjecting such cultures to LPS as an inflammatory stimulus has been reported to result in the selective loss of dopaminergic neurons (Peng et al., 2005), which is microglia-mediated. Using the experience developed here, it should be possible to selective manipulate the glial cell contribution in such cultures. Another line of investigation worth pursuing would be that of looking at the potential for one TLR to regulate expression of others, for example, would a TLR4 agonist influence TLR2 and/or TLR3 expression, and vice versa. This could be extended also to examine whether or not TLR activation can influence expression of nuclear receptors, whose activation is known to have anti-inflammatory actions. Finally, preliminary experiments indicate that the observed astrocyte/microglia co-culture behaviors with LPS occur also when using agents which activate TLR2 and TLR3. Future experiments may explore both the generally of this astrocyte-microglia interaction, utilizing astrocyte/microglia co-cultures and other TLR agonists, in terms of long-term activation and the ATP priming response. In the latter case, mediator release assessment could be extended to include, besides IL-1β, also IL-18 (released as the mature polypeptide following P2X7R activation) which contributes to neuroinflammation and neuropathic pain following peripheral nerve injury in mice (Kim Kim and Moalem-Taylor, 2011).
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6. Publications


