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Astrocyte-microglia interaction in the expression of a pro-inflammatory or pain-related phenotype: molecular and cellular aspects

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ABSTRACT

In the central nervous system (CNS), glial cells not only serve supportive and nutritive roles for neurons, but also respond to protracted stress and insults by up-regulating inflammatory processes. Reactive astrocytes and microglia have been detected in animal models of neuronal injury such as ischemia, axotomy, and neurotoxic insult, and in the human brain in neurodegenerative diseases. Reactive glial cells produce a wide array of pro-inflammatory molecules, including nitric oxide, cytokines and chemokines. The complexity of studying glial activation in vivo has led to the widespread adoption of in vitro approaches, for example the use of the bacterial toxin lipopolysaccharide (LPS, a ligand for toll-like receptor 4 (TLR4)) as an experimental model of glial activation. In the latter, however, the contribution of microglia, if any, to the response by astrocytes remains an open question, as such astrocyte cultures frequently contain minor numbers of microglia. In the present study, we set up a *in vitro* model to evaluate the interaction between astrocytes and microglia in the CNS. At first we used mixed glial cultures from neonatal rat cortex and spinal cord, and we control for the presence of other cell types (endothelium, oligodendrocytes, and neurons). Then we purified microglia and enriched astrocyte cultures in order to evaluate the response to inflammatory (LPS), or pain stimuli (substance P, vasoactive intestinal peptide, calcitonin gene related peptide). Under our experimental conditions, we demonstrated that enriched astrocyte cultures respond to LPS for longer times than purified microglia, but glial cultures lack response to peptides involved in neuronal pain transmission.

Subsequently, enriched ($\leq$5% microglia) astrocytes cultured from neonatal rat cortex and spinal cord were treated with the lysosomotropic agent L-leucine methyl ester to eliminate residual microglia, as confirmed by loss of microglia-specific marker genes. L-Leucine methyl ester treatment lend to a loss of LPS responsiveness, in terms of nitric oxide and cytokine gene up-regulation and mediator output into the culture medium. Astrocyte responsiveness could be reconstituted by re-addition of increasing numbers of purified microglia which, by themselves, yielded little or no signal upon LPS exposure. Given that astrocytes greatly outnumber microglia in the CNS, these data suggest that a similar 'cross-talk' between microglia and astrocytes in vivo may be an important element in the evolution of an inflammatory pathology. In the absence of pathogens, TLR signaling can be activated by molecules called damage associated molecular patterns, released by the injured tissue. The astrocyte/microglia co-culture paradigm described here may provide a useful starting point to

elucidate the molecular mechanisms underlying astrocyte- and microglia-specific responses pertaining to, although not limited to, CNS inflammation, especially where TLR activation plays a role.

RIASSUNTO

Nel sistema nervosa centrale (SNC), le cellule gliali non svolgono solamente una funzione di supporto ai neuroni, ma rispondono “attivandosi” a rilevanti stress e insulti chimici. Astrociti e microglia attivati sono stati individuati in modelli animali in cui è stata indotta una lesione del SNC, ischemia, assotomia, neurotossicità da tossine neurotossiche, e, nel caso dell’uomo, in malattie neurodegenerative. Cellule gliali attivate possono rilasciare diversi fattori pro-infiammatori, come l’ossido nitrico, citochine e varie chemochine, agenti, questi, tutti coinvolti nei processi infiammatori. Tuttavia, poiché questo fenomeno risulta piuttosto complesso *in vivo*, numerosi studi hanno cercato di allestire dei modelli *in vitro* in grado di comprendere il contributo delle varie cellule gliali nell’infiammazione, nel dolore cronico e in quello neuropatico.

Classici modelli di infiammazione a livello del SNC, coinvolgono i recettori dell’immunità innata della famiglia Toll-like (TLR), in particolare il recettore TLR4. *In vitro* e *in vivo*, trattando con lipopolisaccaridi (LPS) batterici colture cellulari da tessuto nervoso o animali, si induce uno stato di attivazione della microglia e degli astrociti simile a quello presente nell’infiammazione. Tuttavia, nonostante in numerosi studi sia stato dimostrato il coinvolgimento della microglia nel fenomeno infiammatorio ed algico, resta ancora poco chiaro il preciso ruolo degli astrociti, poiché le colture di quest’ultimi contengono spesso delle contaminanti di microglia.

In questo studio, abbiamo allestito un modello *in vitro* utile per valutare le interazioni tra astrociti e microglia nei fenomeni infiammatori ed algici. Inizialmente abbiamo utilizzato colture gliali miste ottenute da corteccia e midollo spinale di ratto neonato di due giorni. Abbiamo valutato la presenza nella coltura cellule oligodendrocitarie, endoteliali e neuronali, evidenziando come sostanzialmente la coltura fosse composta quasi esclusivamente da cellule microgliali e astrocitarie. Successivamente, separando la microglia dagli astrociti, abbiamo ottenuto colture arricchite in astrociti, le quali evidenziano una contaminante di microglia inferiore al 5%. Queste colture sono state quindi stimolate con LPS, o con peptidi coinvolti nella genesi o nel mantenimento dello stimolo algico a livello neuronale (quali sostanza P, peptide intestinale vasoattivo, peptide correlato al gene della calcitonina, ecc.). Abbiamo quindi dimostrato che, nelle nostre condizioni sperimentali, le colture arricchite in astrociti rispondono, al trattamento con LPS, per periodi più prolungati rispetto alla microglia purificata. Tuttavia, entrambe le colture gliali non evidenziano nessuna risposta pro-infiammatoria quando trattate con i tre neuro-peptidi testè elencati.

Successivamente, le colture arricchite in astrociti sono state trattate con l’agente lisosomotropico L-Leucina metil estere (L-LME), un composto che, come dimostrato nelle nostre colture, è in grado di

causare la morte della microglia e la conseguente scomparsa dei suoi marcatori genici specifici. Dopo trattamento con L-LME, la coltura pura di astrociti evidenzia una perdita di risposta all'LPS sia in termini di assenza di induzione trascrizionale dei geni che codificano l'enzima inducibile nitrico sintasi e alcune citochine infiammatorie, come pure di un mancato rilascio di questi fattori nel mezzo di coltura. La risposta degli astrociti all'LPS può essere ripristinata mediante riaggiunta alle colture astrocitarie di quantità crescenti di cellule microgliali. Tuttavia se le stesse quantità di cellule di microglia utilizzate nella riaggiunta, vengono coltivate in assenza di astrociti, esse evidenziano una risposta all'LPS significativamente minore. Poiché nel SNC gli astrociti sono presenti in numero molto superiore alla microglia, questi dati suggeriscono che come *in vitro*, anche *in vivo*, potrebbe esistere una cooperazione tra astrociti e microglia e ciò potrebbe risultare di vitale importante nella genesi e nel mantenimento dell'infiammazione e del dolore patologico. Infatti, anche in assenza di patogeni nel SNC, i recettori TLR dell'immunità innata, possono essere attivati da fattori della famiglia delle proteine associate a danni molecolari (damage associated molecular patterns, DAMP) che vengono attivati da danni cellulari che non implicano una risposta infiammatoria da tossine batteriche.

La co-coltura di astrociti e microglia descritta nel presente studio può rappresentare, quindi, un punto di partenza per chiarire il meccanismo molecolare che sottintende alla specifica risposta infiammatoria mediata da astrociti e microglia, causata dall'attivazione dei recettori della famiglia TLR.

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List of abbreviations

5HT	5-hydroxytryptamine
AA	Arachidonic acid
AD	Alzheimer’s disease
ALS	Amyotrophic lateral sclerosis (ALS
AQP4	Aquaporin 4
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
β-ACT	β-actin
β-AR	β-adrenoceptors
β-III tub	β-III tubulin
catS	Cathepsin S
CB1	Cannabinoid receptor 1
CCL	chemoattractant cytokine (chemokine) ligand
CCR	chemoattractant cytokine (chemokine) receptor
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CTR	Control
Cx	Connexin
DAMPs	Danger-associated molecular patterns
DAPI	4'-6'-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
dNTP	Deoxyribonucleotide triphosphates
DRG	Dorsal root ganglion
E	Embrional
EAE	Autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EP	Prostaglandin E2 receptor
ET-1	Cndothelin receptor 1
FCS	Fetal calf serum
GABA	γ-aminobutyric acic
galC	Galactosylceramidase (galactocerebrosidase)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
GFAP	glial fibrillary acid protein
GFP	Green fluorescent protein
GLT	Clutamate transporters
Glu	Glutamate
GMP	Granulocytic-myeloid progenitors
gp 130	Glycoprotein 130
GR	Clucocorticoid receptor
GRK2	Protein-coupled receptor kinase 2

GSK	Glycogen synthase kinase
HD	Huntington's disease
HIV-1	Human immunodeficiency virus 1
HSC	hematopoietic stem cell
HSP	Heat shock protein
Iba1	Ionized calcium binding adaptor molecule 1
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory transcription factor
ITGAM	integrin alpha M
JNK1	c-Jun N-terminal kinase 1
KMO	Kynurenine 3-monooxygenase
L-LME	L-leucine methylester
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
Mal	MyD88 adapter-like
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
Mcam	Melanoma cell adhesion molecule
MCP-1	Monocyte chemotactic protein-1
MD-2	Myeloid differentiation protein-2
MDP	Monocyte–dendritic cell progenitors
mGluR	Metabotropic glutamate receptor
MHC	Major histocompatibility complex
M-MLV	Moloney Murine Leukemia Virus
MOR	μ-opioid receptor
MS	Multiple sclerosis (MS
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
NF-kB	Nuclear factor-kB
NGF	Nerve growth factor
NO	Nitric oxide
NRG	Neuregulin
NSC	Neural stem cells
NT	Neurotrophin
P2X	Purin receptor 2X
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission tomography
PGE	Prostaglandin
PGN	Peptidoglycan
PI3K	Phosphatidylinositol 3-kinases

PKCε Protein kinase C epsilon
RANTES Regulated upon Activation, Normal T-cell Expressed, and Secreted
ROS Reactive oxygen species
Rox 6-carboxy-X-rhodamine
RT Retrotranscription
SARM Sterile-α-and armadillo-motif-containing protein
SOD Cu,Zn-superoxide dismutase
SP substance P
TGFβ-1 Transforming growth factor β-1
TLR Toll-like receptor
TLR Toll-like receptor
TMT Trimethyltin
TNF-α Tumor necrosis factor-α
TRAF Tumour necrosis factor-receptor-associated factor adaptor protein
TRAF TNF-receptor-associated factor
TRIF TIR-domain-containing adapter-inducing interferon-β
TrkB Tyrosine kinase B receptor
TRPV Transient receptor potential vanilloid
VIP vasoactive intestinal peptide
vWF von Willebrand factor
YFP Yellow fluorescent protein

1. INTRODUCTION

1.1 INFLAMMATION AND CENTRAL NERVOUS SYSTEM NEUROPATHOLOGIES

The immune system is essential for the maintenance of tissue homeostasis and the response to infection and injury. Inflammatory stimuli may induce beneficial effects such as phagocytosis of debris and apoptotic cells, and initiate repair processes, but uncontrolled inflammation can result in production of neurotoxic factors that exacerbate neurodegenerative pathology. The inflammatory response involves a delicate balance between the innate and adaptive immune systems to deal with inflammatory stimuli. As a consequence, genes that are critical to amplification of inflammatory responses are normally repressed under physiological conditions, and are only induced when cells are stressed. Inflammatory responses are initiated by pattern recognition receptors, which include the Toll-like receptors (TLRs) that recognize "invading" pathogen-associated molecules (**Table 1.1**, Takeuchi and Akira, 2010). For example, TLR4 recognizes lipopolysaccharide (LPS) associated with gram-negative bacteria, and TLR3 recognizes viral double-stranded RNA. TLRs are highly expressed on macrophages and microglia and may respond to endogenously derived molecules, such as protein aggregates or signals released from apoptotic cells. TLR2 and TLR4 are implicated in chronic inflammation in animal models, and specific TLR4 polymorphisms are associated with several human diseases, including atherosclerosis, type 2 diabetes and rheumatoid arthritis, raising the possibility of involvement of these receptors in neurodegeneration (Balistreri et al., 2009). In addition to TLRs, purinergic receptors (e.g., $P2X_7$) are also expressed on microglia and astrocytes and can respond to ATP released from apoptotic cells (Di Virgilio et al., 2009). Microglia and astrocytes respond to cell signaling via so-called "scavenger receptors" that are involved in the phagocytosis of oxidized proteins, lipids, and apoptotic cells (Husemann et al., 2002).

Inflammatory responses are typically localized and involve communication between immune and other central nervous system (CNS) cells. CNS resident microglia exhibit a deactivated phenotype (ramified) in the healthy brain and maintain tissue homeostasis through communication with astrocytes and neurons (Lumeng et al., 2007). The phenotype of resident macrophages is considered activated and designated M1 or "classical activation", which describes the pro-inflammatory phenotypic response. M2 or "alternative activation" describes

phenotypic responses to cytokines, such as interleukin (IL)-4 and IL-13 (Nathan and Ding, 2010). Therefore, transition

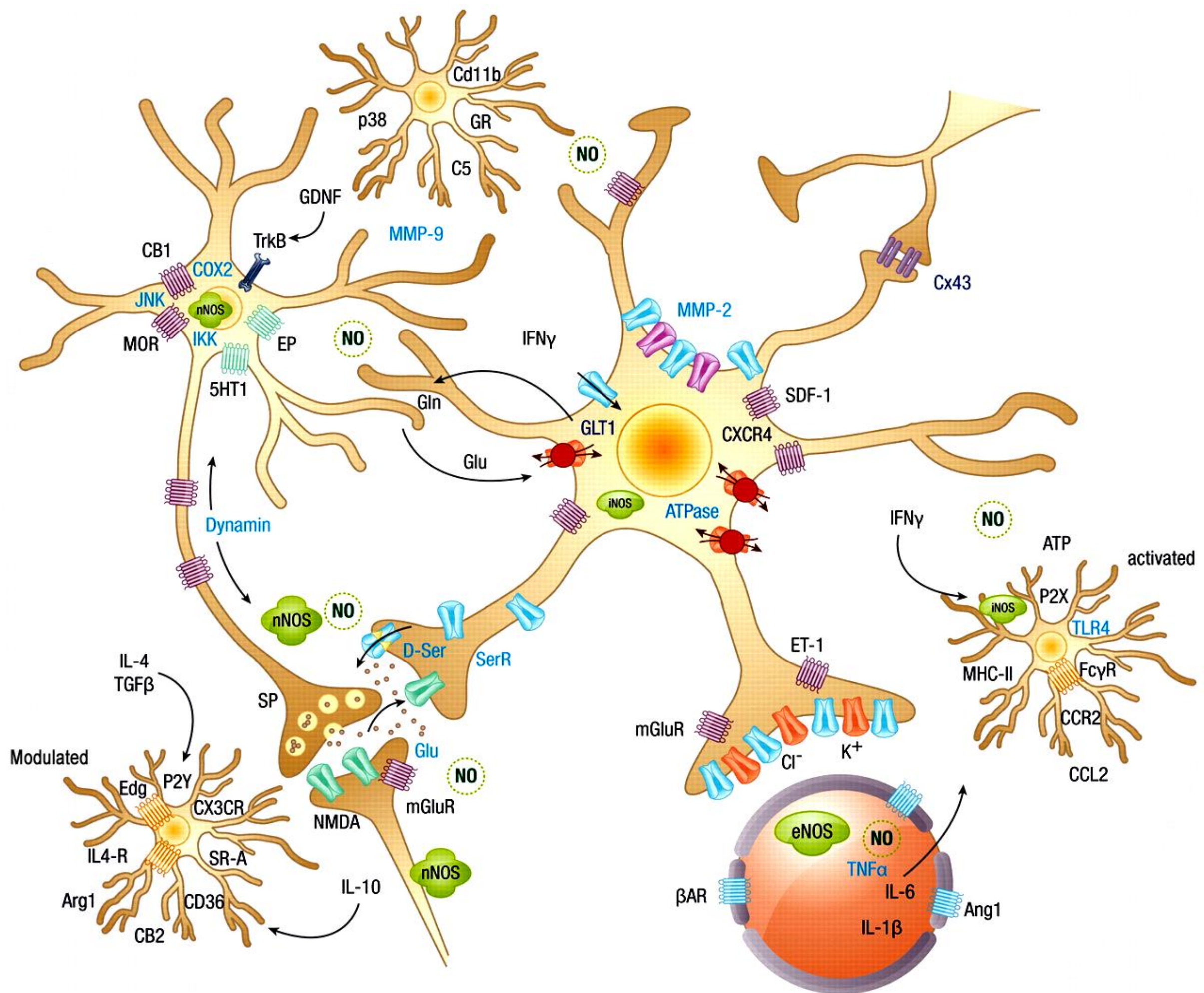
Table 1.1 Expression of mouse TLRs among the principal cell types in the CNS

	TLRs		
	Low expression	Intermediate expression	High expression
Microglia			**1, 2, 3, 4, 5, 6, 7, 8, 9**
Astrocytes/Oligodendrocytes	**2, 4**	**5, 9**	
Neurons	**1, 3, 6, 7, 8**	**2, 4**	**5, 9**

Taken from Tang et al., 2007; Crack and Bray, 2007

of macrophages from the M1 to the M2 phenotype is generally indicative of inflammatory pathology. The CNS is an immunologically privileged site and circulating immune cells normally do not have access to it in the absence of inflammation or injury. Dendritic cells with specialized antigen-presenting capabilities are not present under normal conditions, but when microglia sense danger through TLR4, they secrete inflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β to act on astrocytes and induce secondary inflammatory responses (Saijo et al., 2009). The initiation of an immune response involves the development of adaptive immunity. Inflammatory markers include cytokines (e.g., TNF-α, IL-1β, IL-6) that amplify inflammation; and chemokines like monocyte chemotactic protein-1 (MCP-1) that recruit additional immune cells. In addition, inflammation induces genes that encode proteins with antimicrobial activities such as inducible nitric oxide synthase (iNOS) and genes that modulate substrate metabolism, protein synthesis, cell motility, phagocytosis, and antigen presentation. However, inflammatory responses may induce collateral damage such as the generation of reactive oxygen species (ROS). Furthermore, induction of proteins that inhibit signal transduction pathways such as suppressor of cytokine signaling proteins, transcriptional repressors like activating transcription factor-3, nuclear receptor related protein-1, anti-inflammatory molecules (e.g. IL-10), transforming growth factor β-1 and ligands for TAM (Tyro3, Axl and Mer) receptors are mechanisms that may resolve inflammation.

Fig. 1.1 A model of inflammation in the CNS



Upon neuronal injury, ischemia, inflammation, or pain, neurons and glia can release nitric oxide (NO), acting on neighboring cells to modify their functions. When microglia and astrocytes are activated, they enhance NO production by inducible NO synthase (iNOS). Activated microglia up-regulate iNOS gene, mainly via interferon γ (IFNγ) or TNF-α stimulation (but also by IL-1β and IL-6). By direct S-nitrosylation of Toll-like receptor (TLR)-coupling proteins, matrix metalloproteinases (MMPs) and stress enzymes, such as c-Jun N-terminal kinase (JNK), NO may promote inflammatory processes. Direct S-nitrosylation of cyclooxygenase-2 (COX-2) contributes to N-methyl-D-aspartate (NMDA) receptor-mediated glutamate toxicity, which is further controlled by S-nitrosylation-mediated d-serine production in astrocytes and glutamate reuptake by glutamate transporters (GLT1). NO produced in endothelial cells by eNOS modifies eNOS activity, functions of β-adrenoceptors (β-AR), and potassium channels by direct S-nitrosylation. The effect of NO depends on the site and timing of its production and the redox status in its close proximity. Microglia as well as astrocytes can release IL-1β, IL-6 and TNF-α to improve inflammation or pain. However, neurons or glia can release IL-1, IL-10, or TGFβ to modulate the inflammatory response.

Direct S-nitrosylation targets are in blue text. 5HT, 5-hydroxytryptamine (serotonin); CB1, cannabinoid receptor 1; CCL, chemokine ligand; CCR, chemokine receptor; EP, prostaglandin E_2 receptor; ET-1, endothelin receptor 1; GDNF, glial cell line-derived neurotrophic factor; GR, glucocorticoid receptor; IL, interleukin; mGluR, metabotropic glutamate receptor; MHC, major histocompatibility complex; MOR, μ-opioid receptor; TGFβ, transforming growth factor β; TrkB, tyrosine kinase B receptor (modified from Tegeder et al., 2011).

1.1.1 The failure of anti-inflammatory therapies for CNS injuries and diseases

There is significant evidence that innate immunity may be detrimental to neurons and oligodendrocytes, in stark contrast with other observations that inflammation is beneficial to recovery after CNS injuries. These opposing effects may largely depend on the time or phasic progression of the disease, i.e. early vs. late stage. On the beneficial side, microglia release neurotrophic factors that can induce neuroprotection and contribute to repair after injury. Microglia may also clear cell debris and toxic proteins, preventing their accumulation. Such beneficial effects of innate immune cells are an integral part of planning human clinical trials using anti-inflammatory drugs for CNS diseases. On the harmful side, microglia and other innate immune cells can produce inflammatory markers and induce apoptosis, providing a formidable challenge to balance between beneficial and harmful immune responses and fine-tuning immune cell function. Such a dual effect of the immune reaction to injury and disease raises serious concerns about anti-inflammatory human clinical trials, which to date have been failures. In the context of stroke or ischemia for example, pro-inflammatory cytokines, microglia activation and leukocyte infiltration are key to determine whether a stroke will lead to reversible ischemic deficits or permanent damage. Inhibition of TNF-α and IL-1, which mediate post-ischemic mechanisms, is neuroprotective in animal models of stroke (Allan and Rothwell, 2001; Allan et al., 2005). IL-1 and TNF-α may modulate the post-ischemic response either directly, by damaging endothelial cells, neurons and glial cells, or indirectly, via leukocyte attraction to the site of injury. The recruitment of monocytes, neutrophils and lymphocytes depends on the early release of pro-inflammatory molecules by resident cells (Wang et al., 2007), so anti-inflammatory intervention is time-sensitive. However, recruitment of immune cells can have protective effects, as depletion of T-regulating (T-reg) cells, which can suppress TNF-α and IFN-γ, dramatically ameliorates delayed brain damage (Liesz et al., 2009). T-reg cells also play an important role in reversing Th17 cell-mediated neurodegeneration in a Parkinson’s disease mouse model (Reynolds et al., 2010). Considering that inflammation is a common denominator in CNS diseases, targeting the correct timing of an immune response is pivotal to successful designs of human clinical trials.

1.2 MICROGLIA

1.2.1 Origin of microglia

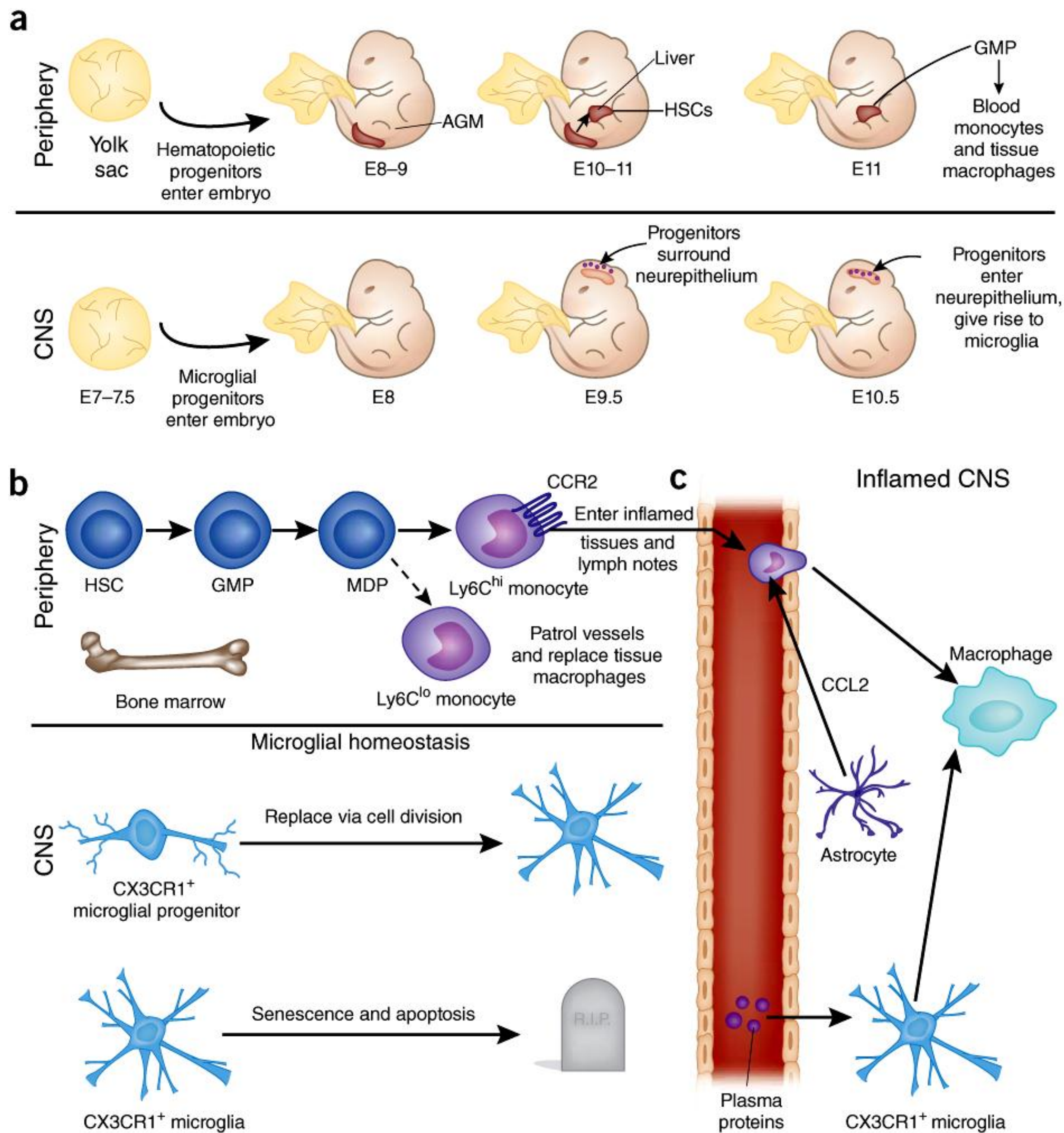
Microglial precursors begin to colonize the embryonic human CNS during the first trimester, around 6.5 to 8.5 weeks of gestation, leading to a well-established microglial population in the second trimester (Ashwell et al., 1991; Hutchins et al., 1990; Esiri et al., 1991; Geny et al., 1995; Rezaie and Male, 1999). At this early time point, the first immature macrophages can already be detected in the yolk sac (Alliot et al., 1999). These macrophages may act as precursors of microglial cells, which then develop through a non-monocyte pathway. At embryonic day 13.5 (E13.5), when the fetal liver is the primary hematopoietic organ and the main site of hematopoietic stem cell (HSC) expansion and differentiation, microglial precursors can be detected in substantial numbers in the ventricular lining of the fourth ventricle (Lichanska et al., 2000). However, whether organs of definitive haematopoiesis, such as the fetal liver, are a substantial source of adult microglia in the brain remains unclear. Notably, a significant increase in the number of CD11b+ F4/80+ microglia can be observed during the early postnatal periods in rodents (Alliot et al., 1999). The developing CNS is colonized by microglia from specific sites of predilection, described as "fountains of microglia" by del Rio-Hortega. These include the superior tela choroidea and the pia mater covering the cerebral peduncules. From here, the cells migrate into the corpus callosum, the cerebral trigonum (fornix) and the optic thalami (thalami), and then into additional areas of the brain. In the cerebellum, the primary site for microglial entry is the inferior tela choroidea of the bulbocerebellar fold of the pia mater (del Rio-Hortega, 1932, 1939). However, it has long been uncertain whether the increase in microglia cell number is a result of the proliferation of embryonic microglial precursors, a phenomenon that is frequently observed in the developing brain (Cuadros et al., 1998), or whether a new recruitment of monocyte-derived microglial precursors occurs. The latter hypothesis is indirectly supported by the observation that the absence of microglia in mice deficient for the transcription factor PU.1 can be rescued by the injection of wild-type bone marrow cells into newborns, leading to a complete repopulation of the CNS by donor-derived microglia (Beers et al., 2006).

A recent study has now shed light on the mysterious origins of microglia (**Fig. 1.2**). By inducing Cre recombinase activity through injections of tamoxifen into pregnant mice between days E7.00 and E7.50 after conception, when embryonic haematopoiesis is restricted to the yolk sac, the authors identified immature yolk sac macrophages as the predominant source of microglia (Ginhoux et al., 2010). Notably, myeloid progenitors from the blood after

birth did not significantly contribute to the pool of adult microglia, which is at odds with the results of previous studies, and strongly suggests that the expansion of microglial numbers in the postnatal period depends on proliferation of the resident microglia population. Thus, the vast majority of adult microglia appeared to be yolk sac-derived (from a remarkably restricted time period during early embryogenesis). It remains open whether adult microglia could also be derived in part from the embryonic liver or other hematopoietic organs during embryogenesis, such as aortic-gonadal-mesonephros. One limitation of this seminal work is that only one third of yolk sac macrophages could be labeled genetically.

Other studies in rats used cell transplantation techniques, and revealed that perivascular macrophages, but not cells with ramified microglia characteristics, were present in the CNS parenchyma after irradiation and bone marrow transplantation. Similar results were obtained in humans, when women who underwent sex-mismatched bone marrow transplantation were examined for the engraftment of Y chromosome–positive microglial cells (Hickey et al., 1992). Notably, all of these studies were based on immuno-histochemical approaches and therefore lacked the sensitivity of cell transfer experiments with genetically labeled cells. A later study used retroviral transduction of hematopoietic cells with green fluorescent protein (GFP) to examine the long-term fate of myeloid cells in the murine CNS after bone marrow transplantation, in an experimental setting including whole body irradiation (Cuadros et al., 1998). This study found GFP-expressing parenchymal microglia deep in the mouse cerebellum, striatum and hippocampus several weeks after transplantation. Despite the differences with the earlier studies mentioned above, the concept of bone marrow–derived phagocytes in the CNS was firmly established. Subsequently, many publications demonstrated an infiltration of bone marrow–derived phagocytes in animal models without obvious blood-brain barrier damage, such as amyotrophic lateral sclerosis (Solomon et al., 2006), Alzheimer's disease (AD) (Malm et al., 2005) and scrapie (Priller et al., 2006), among others (Priller et al., 2001; Djukic et al., 2006). All of these studies used irradiation of the recipients followed by whole bone marrow transplantation to discriminate between the progeny of donor-derived, labeled hematopoietic cells and host resident microglia. To elucidate the effect of irradiation on the engraftment of myeloid cells in the CNS, the heads of the recipient mice were protected from irradiation by shielding. Notably, *de novo* generation of bone marrow–derived phagocytes from the circulation was strongly diminished in the brains of mice that were not irradiated before transplantation (Mildner et al., 2007). Another study provided complementary data

Fig. 1.2 Origin of microglia and monocytes in rodents



(**a**) Embryological origins of monocytes and microglia. Monocytes (top) trace to migration of hematopoietic progenitors from yolk sac to the aorta-gonad-mesonephros region at E8. There, hematopoietic stem cells are generated and accumulate in the fetal liver beginning at E11, where they differentiate into granulocytic-myeloid progenitors (GMPs), then to blood monocytes and tissue macrophages. Microglial progenitors (bottom) arise in the yolk sac between E7–7.5, enter the embryo at E8 and surround the neuroepithelium by E9.5, between the surface ectoderm and brain rudiment. At E10.5 the earliest microglia are found in the neuroepithelium. (**b**) Steady-state maintenance of monocytes and microglia. In adult life, monocytes (top) are generated in the bone marrow from hematopoietic stem cells that differentiate to GMPs and then to monocyte–dendritic cell progenitors (MDPs). Mature Ly6C^{hi}, CCR2^{+} 'inflammatory' monocytes are released into the circulation, from which they are destined to enter inflamed tissues or lymph nodes. MDPs are also proposed to give rise to Ly6C^{low}, CCR2^{-} 'resident' monocytes, which patrol blood vessel walls and also replace tissue macrophages. Microglia (bottom) may senesce and undergo apoptosis at a low rate and then be replaced by cell division from CNS-resident CX3CR1^{+} progenitors. (**c**) Both monocytes and microglia can give rise to macrophages during neuroinflammation. Reactive astrocytes produce the chemoattractant cytokine (chemokine) CCL2, which signals to receptor CCR2 on Ly6C^{hi} monocytes, aiding their entry into the CNS parenchyma. Local cytokine and tissue signals can then induce transformation of monocytes to macrophages. Concurrently, inflammation can disrupt the blood-brain barrier, allowing plasma proteins to enter and activate microglia to adopt a macrophage phenotype (Ramsohoff, 2011)

from investigations of the recruitment of peripheral myeloid precursors into the CNS using parabiosis (in which the circulations of mice are joined for a period of time to allow for example, cranial irradiation),and intravenous transfer of femoral bone marrow enriched for a population of the circulation of a parabiotic recipient with labeled cells. This study convincingly showed that there were no bone marrow–derived phagocytes in the CNS of the GFP-negative partner under any tested condition, including total-body irradiation of the parabiotic recipient (Ajami et al., 2007).

These findings indicate that the engraftment of bone marrow–derived myeloid cells in the CNS is an extremely rare event, which is strongly influenced by the experimental design (for hematopoietic progenitors and stem cells). Furthermore, these results underscore the fact that endogenous microglia are of yolk sac origin and exhibit a high potential for self-renewal and proliferation.

1.2.2 Macrophages versus microglia

Expression of a bone marrow marker (such as GFP) and myeloid markers (such as CD11b or Iba1) are typically used to identify immigrating bone marrow cells as “microglia” in the CNS parenchyma of bone marrow chimeric mice. These cells exhibit morphologies ranging from round amoeboid to ramified (Vallieres and Sawchenko, 2003; Wirenfeldt et al., 2007). *In vitro* studies have shown that macrophages can develop a ramified morphology and microglia an amoeboid morphology (Sievers et al., 1994; Wilms et al., 1997; Bohatschek et al., 2001), suggesting that morphology alone may not discriminate these populations. In 1991, Sedgwick and colleagues (Sedgwick et al., 1991) used flow cytometry to demonstrate that the resident $CD11b^{+}$ microglial population in un-manipulated rat brain express low levels of CD45 ($CD45^{dim}$). Analysis of CD45 levels in bone marrow-chimeric rats confirmed that $CD45^{dim}$ cells were radiation-resistant parenchymal microglial cells, and that $CD45^{high}$ cells were other CNS macrophages and some T lymphocytes (Ford et al., 1995). CD45 levels have also been used to discriminate resident from infiltrating myeloid cells in mouse brain (Renno et al., 1995; Carson et al., 1998). Flow cytometric evaluation of CD45 levels as a means of discriminating macrophages from microglia has been widely employed in CNS studies ranging from autoimmune inflammation and viral infection to injury and ischemia (Sedgwick et al., 1991; Renno et al., 1995; Katz-Levy et al., 1999; Mcmahon et al., 2002; Babcock et al., 2003, 2006, 2008; Greter et al., 2005; Wirenfeldt et al., 2005; Lambertsen et al., 2009). Although bone marrow chimeras are widely used for histological analyses, and CD45 levels

are commonly used for evaluation by flow cytometry, few studies have compared macrophage/microglial immigration by combining CD45 analysis with bone marrow chimera technology. Overall, these studies demonstrate significant increases in bone marrow-derived $CD45^{high}$ macrophages and $CD45^{dim}$ microglia in experimental models. The proportion of $CD45^{high}$ cells expressing bone marrow markers (e.g. GFP) generally matches the degree of reconstitution, which indicates that this does reflect an immigrating population. The proportion of $CD45^{dim}$ cells expressing bone marrow markers is also significant, but generally much lower, especially after an acute CNS insult (Wirenfeldt et al., 2005, 2007; Remington et al., 2007; Clausen et al., 2008; Lambertsen et al., 2009). In acute injury models, e.g. perforant pathway transection, this can reach 0.6-13% of $CD45^{dim}$ microglia depending on the length of time between lesion and death and post-transplantation survival time (Wirenfeldt et al., 2005, 2007). Higher proportions (up to 29%) of bone marrow-derived microglia have been reported in mice with cuprizone-induced demyelination, a model in which there is relatively little involvement of $CD45^{high}$ macrophages (Remington et al., 2007), but in which a previous histological study demonstrated immigrating bone marrow-derived cells such as peripheral macrophages (Mcmahon et al., 2002). Taken together, these data indicate that the majority of $CD45^{high}$ cells are reliably infiltrating macrophages, and that the majority of $CD45^{dim}$ cells form the resident microglial population, although a significant subset of $CD45^{dim}$ microglia are recently derived from the bone marrow. It is not known whether these cells enter the CNS with intermediate CD45 levels or as $CD45^{high}$ cells that then down-regulate CD45 expression. The exact nature of the bone marrow-derived precursor cells that differentiate into microglia and macrophages is not entirely known. Recent studies, however, suggest precursors of adult murine microglia to be a subpopulation of monocytes that display a Ly-6ChiGr-1+CCR2+CX3CR1lo phenotype. Ly-6ChiCCR2+ cells have been demonstrated to accumulate in CNS lesions and differentiate into microglia (Mildner et al., 2007).

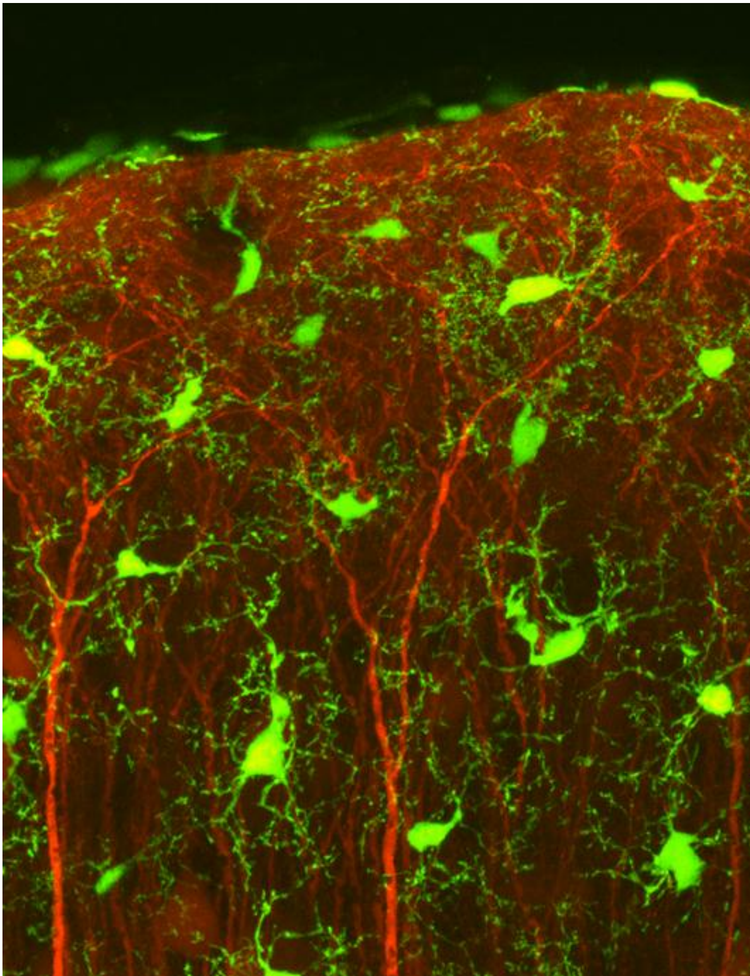
1.2.3 Microglia in the mature CNS

In the fully developed CNS microglia are numerous and distributed throughout the parenchyma. In the healthy, undisturbed CNS, microglia can be referred to as "resting\surveying microglia" (Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007; Kettenmann and Verkhratsky, 2008). Microglial reactivity is a transition from this highly active surveying state towards an even more reactive state, where microglia focus their activity on a pathological event and respond with morphological and functional changes to the disturbance present. Recent experiments suggest that microglia also support and monitor

synaptic function (Tremblay et al., 2010), control synaptogenesis (Roumier et al., 2004) and induce *in vitro* developmental apoptosis of Purkinje cells (Marín-Teva et al., 2004). Microglia are thus important for CNS development and maintenance.

Microglia exhibit “functional plasticity”, meaning that they adapt their level of reactivity to the activating stimulus and in that way respond properly to pathological processes in the CNS parenchyma. Intercellular distances between “surveying microglia” are generally 50-60 µm under healthy conditions, with their somata remaining in a fairly fixed position. In contrast, microglial processes are very dynamic and highly motile with new processes constantly being formed, and others being retracted (Nimmerjahn et al., 2005). Therefore, the 'classical' image of a microglial cell with its small elongated soma and long slender processes should be viewed as a snapshot of that cell at that point in time, a morphology which could change within a matter of a few minutes. The fate of microglia after activation and after the activating stimulus has subsided is not clear. The surveying activity of microglia in the normal brain has been elegantly demonstrated with two-photon microscopy (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglia can, however, also be visualized *in vivo* in humans by positron emission tomography (PET) imaging. The normal CNS expresses a very low level of the peripheral benzodiazepine binding site, to which the synthetic ligand PK11195 can bind and serve as a PET ligand when ^{11}C-labeled. Activated microglia up-regulate the peripheral benzodiazepine binding site and therefore increase their binding of PK11195. *In vivo* activated microglia are the primary source of PK11195 binding, and increased PK11195 binding can be utilized for visualizing microglial reactivity *in vivo* in humans by PET-scanning, e.g. in dementia (Banati et al., 1997; Cagnin et al., 2001). The intensity of PK11195 may be related to the level of microglial activation (as demonstrated in a rat model) rather than the number of activated microglia (Ito et al., 2010). Activating stimuli for microglia can be very diverse. Two interesting regulatory mechanisms known to influence microglial activity are ATP and the CD200-CD200 receptor (CD200R) complex. ATP is a potent regulator of microglial surveying activity and microglial processes display strong chemotaxis towards extracellular ATP (Honda et al., 2001; Davalos et al., 2005). Two-photon microscopy imaging studies have emphasized how highly responsive microglia are to injury and pathology in the CNS, directing their processes towards damage in the parenchyma within minutes (Davalos et al., 2005). This rapid and directed response by microglial processes adds to the traditional panel of criteria used to classify microglial activation, including changes in morphology,

Fig. 1.3 Microglia in the adult CNS



GFP⁺ Microglia and YFP⁺ Neurons in P12 mouse hippocampus. Transverse section of mouse P12 hippocampus. Microglia were labelled by costitutive expression of GFP transfected protein (green); neurons are labelled by constitutive expression of YFP protein (red). (L. Fuller and M. Dailey, 2010). (http://www.biology.uiowa.edu/daileylab/image-gallery.html).

proliferation, antigen expression, and cytokine production (Streit et al., 2004b; Ladeby et al., 2005). For microglia to react as swiftly as demonstrated in two-photon microscopy studies, these cells must accordingly be very closely regulated. In the CNS, CD200 is expressed on neurons and CD200R on microglia (Webb and Barclay, 1984; Hoek et al., 2000; Wright et al., 2000). The CD200-CD200R complex is involved in regulation of myeloid cell activation, and microglia spontaneously displayed a more activated phenotype in mice lacking the CD200 molecule (Hoek et al., 2000). Increased expression of CD200 has, on the contrary, been demonstrated to protect neurons against microglial-induced damage (Chitnis et al., 2007). These results demonstrate a key role for the CD200-CD200R complex in regulating microglial activity in the CNS.

1.2.4 Microglial involvement in human diseases

Pathology or damage to the CNS parenchyma can often elicit a microglial response. In certain CNS diseases microglia are likely to play a central role in disease pathogenesis itself. Following are some examples of well-known human pathologies in which microglia are involved.

1.2.4.1 Alzheimer's disease

AD is a neurodegenerative disorder for which there is presently no treatment that ameliorates disease outcome (Querfurt et al., 2010). The brains of individuals with AD contain senile plaques composed of extracellular deposits of amyloid peptides (collectively termed Aβ) derived from amyloid precursor protein. In addition, neurons in affected regions contain intracellular aggregates (designated neurofibrillary tangles) comprised of hyperphosphorylated forms of the microtubule-associated protein tau. During the development of AD, these two processes interact in vicious succession (LaFerla et al., 2010).
Genetic association studies have identified susceptibility-linked gene variants in inflammatory pathways, such as complement (complement receptor 1/CR1, complement inhibitor clusterin) and chemokine CXCL8 (http://www.alzgene.org/default.asp). Subsequently, the clusterin- and CR1-associated single-nucleotide polymorphisms were identified in a large genome-wide association study and verified (Lambert et al., 2009; Corneveaux et al., 2010). Moreover, epidemiological investigations have consistently demonstrated a protective effect for mid-life exposure to non-steroidal anti-inflammatory agents against later development of AD (in t' Veld et al., 2001), even if administration of these agents had no effect on the progression of established AD in several clinical trials (Firuzi et al., 2006).
The exact manner in which microglia are involved in the pathology of AD, and to what extent the reactive microgliosis observed in AD is beneficial or may even be detrimental to the brain is still not resolved. Fibrillar Aß1-42 plays an important role in activating microglia through TLR2 in AD (Jana et al., 2008). It has been hypothesized that the subsequent microglial production of potential neurotoxic molecules, such as pro-inflammatory cytokines and ROS, could mediate some of the neurodegenerative pathology observed in AD (Akiyama et al., 2000). This hypothesis is supported by accumulation of activated microglia around brain amyloid deposits (Itagaki et al., 1989; Perlmutter et al., 1990) and observations that Aß can activate microglia (Jana et al., 2008). However, an alternative hypothesis has been suggested, namely that microglial senescence and dysfunction might be involved in the pathogenesis and progression of AD (Streit, 2004a; Flanary et al., 2007). Microglia dysfunction and loss of

normal homeostatic and neuroprotective functions could compromise their ability to clear Aß at a sufficient rate, resulting in the development of plaque acceleration. Furthermore, activation may shorten the lifespan of microglia, leading to increased microglial cell death and concomitant release of neurotoxic substances, thereby facilitating neurodegeneration (Streit, 2004a).

The neuropathological characterization of AD tissues revealed the presence of numerous mediators of innate immunity, including complement components and chemokine system elements such as CXCL8 and its receptor CXCR2 (Heneka et al., 2010). These factors are also present early in the course of several animal models of AD, such as those that induce pathology via over-production of Aβ, or through transgenic over-expression of mutant tau species that cause frontotemporal dementia (Schwab et al., 2010).

In tissue from individuals with AD, the inflammatory cellular reaction is comprised of myeloid cells (microglia, monocytes and perivascular macrophages) and astrocytes, arguing against the involvement of adaptive immunity. A study in a mouse model of AD suggested that the recruitment of monocytes infiltrating from the bloodstream to plaques could be abrogated as a result of CCR2 deletion, a receptor specific for the monocyte chemoattractant protein family of chemokines (El Khoury et al., 2007). These data suggest that bone marrow–derived phagocytes can enter AD tissues where they are attracted to nascent plaques, and that infiltrating peripheral myeloid cells are far more efficient at amyloid clearance than resident CNS microglia.

Recently it was reported that CCR2 deficiency markedly impairs the ability of perivascular myeloid cells to clear Aβ leading to amplified vascular Aβ deposition, whereas parenchymal plaque deposition remained unaffected. Taken together, these data advocate selective functions of CCR2-expressing myeloid subsets in AD (Mildner et al., 2011). One recent pertinent observation was that eliminating perivascular macrophages with toxic liposome infusions caused increased accumulation of vascular amyloid (Hawkes et al., 2009). Notably, microglia might mediate amyloid clearance or inhibit amyloidogenesis by means other than phagocytosis, including production of proteolytic enzymes (Mueller-Steiner et al., 2006; Sun et al., 2008). Alternatively, microglia or monocytes might induce the expression of Aβ-degrading enzymes in other CNS cells.

1.2.4.2 Parkinson's disease (PD)

PD is the second most common neurodegenerative disorder after AD, with an age-related increase in incidence. It is characterized by motor symptoms, such as tremor, rigidity, postural instability and bradykinesia. The disease course is often complicated by behavioral and psychiatric symptoms and by cognitive impairment. The pathological hallmarks of PD are the loss of dopaminergic neurons and the presence of eosinophilic inclusions called Lewy bodies and dystrophic neurites in the substantia nigra pars compacta in the midbrain. However, neuronal cell loss is not confined to the substantia nigra or the dopaminergic system, but also occurs in other brain regions. PD is characterized by the accumulation of reactive MHC class II–positive microglia in the substantia nigra (McGeer et al., 1988; Hunot et al., 1999). PET studies using [^{11}C](R)-PK11195 revealed microglial activation in pons, basal ganglia, and frontal and temporal cortical regions of individuals with PD, starting early in the disease process without significant longitudinal changes (Gerhard et al., 2006). Notably, post-mortem samples of individuals with PD also showed infiltration of CD4+ and CD8+ T cells in the substantia nigra, suggesting a pathogenic role of neuroinflammation (Hunot et al., 1999). Nevertheless, the contribution of microglia to the pathogenesis of PD is far from clear.

Direct evidence for a neurotoxic function of microglia comes from animal models of PD. Methyl-4-phenyl-1,2,3,6-tetrahydropyridine induces parkinsonian-like symptoms in humans, primates and mice after oxidation to the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium in the brain. As a result of mitochondrial complex I inhibition, neuronal metabolism is compromised and ROS accumulate. Alpha-synuclein, a protein that is mutated in rare familial forms of PD (Polymeropoulos et al., 1997) is misfolded and forms aggregates, as is the case in the Lewy bodies of PD. Notably, the cytotoxicity induced by misfolded alpha-synuclein appears to be non–cell autonomous and to involve myeloid cells. Thus, normal and misfolded alpha-synuclein are secreted from dopaminergic neurons and phago-cytosed by microglia, which activate NADPH oxidase and produce ROS (Zhang et al., 2005). Along these lines, mice deficient in inducible NOS or defective in NADPH oxidase exhibit less neuronal cell loss in the methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of PD (Liberatore et al., 1999; Wu et al., 2003). In rats, 6-hydroxydopamine administration leads to parkinsonian-like symptoms as a result of selective uptake of the toxin into dopaminergic neurons and subsequent cell death via the generation of ROS. Activated microglia are present in the brains of 6-hydroxydopamine–lesioned rats and pharmacological neutralization of the pro-inflammatory cytokine soluble TNF significantly reduces dopaminergic cell death, suggesting a pathogenic role of inflammation in neurodegeneration (McCoy et al., 2006). A

recent study indicated that the chemokine receptor CX3CR1 is involved in controlling microglial neurotoxicity (Cardona et al., 2006). Mice deficient in CX3CR1 show increased microglial activation and enhanced dopaminergic cell loss in the substantia nigra after systemic administration of LPS. Overall, inflammation may have a sensitizing function in nigrostriatal pathway degeneration, which is consistent with the epidemiological finding of decreased incidence of PD in chronic users of the non-steroidal anti-inflammatory drug ibuprofen (Chen et al., 2005), and the delayed occurrence of post-encephalitic parkinsonism after viral infection (Soji et al., 1993).

1.2.4.3 Amyotrophic lateral sclerosis (ALS)

Microglia are also involved in ALS, a progressive neurodegenerative disorder that specifically affects the upper and lower motor neurons in the adult, leading to atrophy of skeletal muscles, spasticity, paresis and subsequently to death in 4–6 years. Twenty percent of familial ALS cases are caused by a mutation in the gene for the free radical-scavenging metalloenzyme Cu,Zn-superoxide dismutase (SOD1). The fact that microglia are actively involved in this disease was shown in SOD mutant mice (Beers et al., 2006). In this study, mice with ALS symptoms caused by the expression of mutant SOD1 gene (SOD1G93A) were bred to transcription factor *PU.1*–deficient mice, which are characterized by a lack of myeloid cells (McKercher et al., 1996). Because both *PU.1−/−* (also known as SPI1) and *PU.1−/−*; SOD1G93A mice die shortly after birth, intraperitoneal bone marrow transfer into newborns was performed. *PU.1*−/−; SOD1G93A newborns received either wild-type or SOD1G93A bone marrow cells, and survival and motor neuron loss were both analyzed. Notably, transplanted *PU.1*−/−; SOD1G93A mice showed full reconstitution of the CNS with donor-derived myeloid cells, although recipient mice did not receive prior irradiation. Notably, *PU.1*−/−; SOD1G93A pups that were reconstituted with wild-type bone marrow showed a substantially longer survival and decreased motor neuron loss, indicating a pathology-promoting role of SOD1G93A microglial cells (Beers et al., 2006). Similar results were obtained by using a different approach: inactivation of mutant SODG37R specifically in CD11b+ microglia extended the survival of mice significantly, particularly during the late phase of disease (Boillée et al., 2006). Indeed, the neurotoxic nature of mutant SOD1-expressing microglia was shown directly, and additional activation of microglial cells in ALS mice by macrophage-colony stimulating factor treatment resulted in exacerbated symptoms (Gowing et al., 2009). This treatment led to increased proliferation and an altered morphology of microglial cells, and enhanced expression of pro-inflammatory cytokines such as IL-1β and

TNF-α. Notably, and in contrast with the long-term reconstitution experiments in neonates (Beers et al., 2006), bone marrow transplantation of wild-type bone marrow cells in adult 6-week-old SOD1G93A mice (Solomon et al., 2006) or even allogenic bone marrow transplantation in individuals with sporadic ALS did not result in any beneficial outcome (Appel et al., 2008). This discrepancy could be a result of inefficient engraftment of the CNS by bone marrow–derived elements in adults, as was shown previously (Ajami et al., 2007), which could lead to an insufficient replacement of mutant SOD1–expressing microglia by wild-type bone marrow–derived cells.

1.2.4.4 Huntington's disease (HD)

HD is a monogenic autosomal-dominant neurodegenerative condition that is caused by an increased CAG repeat length in exon one of the gene encoding huntingtin. CAG encodes glutamine and a polyglutamine tract in excess of 39 (with healthy individuals showing between 6 and 26) is invariably associated with HD. Symptoms and signs of HD include choreic movements, cognitive impairment, personality change, and weight loss. Both neuropathology and imaging studies have demonstrated profound striatal atrophy, along with predominant loss of medium spiny neurons.

Establishment of the gene defect in HD led to the development of mouse models, beginning with transgenic over-expression of greatly expanded CAG repeats. These models progressed to the use of knock-in gene targeting to place the mutant huntingtin gene, carrying repeat numbers typical of human disease, under the control of the endogenous mouse locus. Because the mutant gene product is widely expressed, it is important to consider whether neurotoxicity in HD is cell-autonomous or requires interactions among CNS cells types, with mutant huntingtin causing different patterns of dysfunction in different cells, whose pathological interactions culminate in the HD phenotype.

Recent results have implicated microglia in HD pathology (Giorgini et al., 2005). Metabolic derivatives of tryptophan include both neurotoxic moieties, such as 3-hydroxykynurenine and quinolinic acid, as well as the neuroprotectant l-kynurenine, produced in a branched catabolic pathway involving both astrocytes and microglia. The neurotoxic effects of 3-hydroxykynurenine and quinolinic acid involve their serving as mimics of excitotoxic neurotransmitters and increasing the production of ROS. The enzyme kynurenine 3-monooxygenase (KMO), expressed virtually only in microglia among CNS cells, lies at a critical branch point favoring production of the tryptophan-derived neurotoxins and shows increased expression in HD, as well as in mouse models of HD (Thevandavakkam et al.,

2010). Genetic or pharmacological inhibition of KMO ameliorated HD–like pathology in mice (Giorgini et al., 2005). It has been proposed that microglia respond to the presence of mutant huntingtin with elevated expression of KMO, leading to neurotoxicity. Arguing in favor of altered myeloid cell function in individuals with HD, the circulating monocytes of affected individuals were shown to over-express inflammatory cytokines (Björkqvist et al., 2008). These results point to a previously unknown pathway by whereby microglia are implicated in neurodegeneration.

1.2.4.5 Multiple sclerosis (MS)

The exact role of microglia in the complex pathogenesis of MS is not well known. There are, however, well described microglial functions known to be involved in MS pathology. For example, activated microglia can acquire the ability to stimulate Th1 and Th2 CD4+ T-cell lines and present antigens, as demonstrated *in vitro* (Aloisi et al., 1999, 2001). Based on the autoimmune nature of MS, bone marrow transplantation has been attempted as treatment for this disease, based on the rationale that autoimmunity could be attenuated by myeloablative treatment and immune function restored by subsequent bone marrow transplantation (Burt et al., 1997). This treatment has, however, thus far not been effective in patients with progressive disease and high pre-transplantation disability scores (Burt et al., 2003; Samijn et al., 2006).

1.2.4.6 HIV-1 infection

Microglia are the primary target cells for HIV-1 in the CNS. Microglia express CD4 receptors and chemokine receptors such as CCR3 and CCR5 that enable HIV-1 to bind to and infect microglia (He et al., 1997). Monocytes infected with HIV outside the CNS can act as Trojan horses and carry the virus through the blood-brain barrier into the parenchyma, where the infection can spread to microglia (Williams and Blakemore, 1990). HIV-1 infection of the brain can eventually lead to HIV-1 dementia (Navia et al., 1986a, b; Garden, 2002).

1.2.4.7 Rasmussen's encephalitis

Microglia are implicated in this rare but very disabling disease which causes intractable epilepsy in childhood (Rasmussen et al., 1958). The etiology of Rasmussen's encephalitis is unknown, but very striking neuropathological signs can be observed in the brains of these patients including almost exclusive unilateral involvement of the brain, and often severe inflammatory changes with T-cell infiltration and microglial activation in the involved

cerebral hemisphere (Vining et al., 1993; Pardo et al., 2004). Microglial reactivity is often scattered but can be very evident (Wirenfeldt et al., 2009). Despite intense research it is still unresolved whether the reactive microgliosis observed in Rasmussen's encephalitis is a primary phenomenon or secondary to a yet undiscovered etiology.

1.3 ASTROCYTES

1.3.1 Origin and roles in development

Astrocytes develop from embryonic neural tissue, after an initial production of neurons in many CNS regions. Nevertheless, astrocytes exert a number of important functions during development of both gray and white matter, like guiding the migration of developing axons and certain neuroblasts (Powell et al., 1999). In addition, astrocytes are essential for the formation and function of developing synapses by releasing molecular signals such as thrombospondin (Barres et al., 2008; Christopherson et al., 2005; Ullian et al., 2001). Astrocytes appear also to influence developmental synaptic pruning by releasing signals that induce expression of complement C1q in synapses and thereby tag them for elimination by microglia (Barres et al., 2008; (Stevens et al., 2007). As regards the development of white matter, the loss or dysfunction of astrocyte connexins and gap junctions leads to dysmelination (Lutz et al., 2009).

1.3.2 Astrocytes in the mature CNS

Astrocytes function as supportive cells in neural tissue, and are responsible for a wide variety of complex and essential functions in the healthy CNS, including development, synaptic transmission and information processing by neural circuit functions. The potential loss of normal astrocyte functions or gain of abnormal effects can contribute to, or play primary roles in disease processes. There are numerous examples of astrocyte contributions to clinical and pathological mechanisms (Takano et al., 2009; Barres, 2008; De Keyser et al., 2008; Seifert et al., 2006; Sofroniew, 2009, 2005, 2000).

A prototypical marker for immunohistochemical identification of astrocytes is glial fibrillary acid protein (GFAP), a member of the family of intermediate filament proteins, which include vimentin, nestin, and others, that serve largely cyto-architectural functions (Perny and Pekna, 2004). However, GFAP expression can be regarded as a sensitive and reliable marker that labels most, but not all, CNS astrocytes. In addition it is expressed in other CNS cell types, e.g. some neural progenitor/stem cells (Bramanti et al., 2010). Glutamine synthetase and S100b have also been used for immunohistochemical identification of astrocytes and reactive astrocytes (Goncalves et al., 2008; Noremberg, 1979), but these molecules are not entirely exclusive to astrocytes. Studies conducted on the analysis of the astrocyte transcriptome in rodents and humans have

Fig. 1.4 Astrocytes in the mature CNS

Confocal sagittal thin section of rat cerebellum labeled with rabbit anti-β-III-tubulin antibodies (green, Alexa Fluor 488), GFAP (red, Alexa Fluor 568), and nuclei counterstained with DRAQ5 (pseudocolored cyan). Images were recorded with a 10x objective using a zoom factor of 1.4 and sequential scanning with the 488-nanometer spectral line of an argon-ion laser, the 543-nanometer line from a green helium-neon laser, and the 633-nanometer line of a red helium-neon laser. (Olympus Imaging, Japan)

identified numerous molecules enriched in astrocytes as compared with other neural cells, such as neurons and oligodendrocytes (Cahov et al., 2008; Lovatt et al., 2007). In addition, such studies are identifying potential candidates for additional, and better, molecular markers with which to identify astrocytes and to study their functions (Barres, 2008).

Astrocytes in the mature CNS can be divided into 2 main subtypes, protoplasmic or fibrous, on the basis of differences in their morphology and anatomical location (Ramon Y Cajal, 1909). Protoplasmic astrocytes are found throughout all gray matter, and exhibit several stem branches that give rise to many finely branching processes in a uniform globoid distribution. Fibrous astrocytes are found throughout all white matter and exhibit many long fiber-like processes (Ramon Y Cajal, 1909). Classical and modern neuroanatomical studies also indicate that both astrocyte subtypes make extensive contacts with blood vessels, envelop synapses, form gap junctions between distal processes of neighboring astrocytes, and processes of fibrous astrocytes contact nodes of Ranvier (Peters et al., 1991).

In general, astrocytes form a contiguous and essentially non-overlapping orderly and well organized tissue network. There are no CNS regions devoid of astrocytes or closely related cells. In the healthy CNS, individual protoplasmic astrocytes have essentially non-overlapping domains in gray matter, such that only the most distal tips of processes from individual astrocytes interdigitate with one another and thereby provide the substrate for formation of

gap junctions detected at the ultrastructural level (Halassa et al., 2007; Nedergaard et al., 2003; Bushong et al., 2002; Ogata et al., 2002). Similar individual astrocyte domains appear likely to exist in white matter, but this has not yet been as extensively reported on. Individual protoplasmic astrocytes typically extend from five to ten main stem branches, each of which gives rise to many finely branching processes that are evenly distributed throughout the astrocyte domain in the gray matter. In hippocampus or cortex many finely branching processes from a single astrocyte are estimated to contact several hundred dendrites from multiple neurons and to envelope 100,000 or more synapses (Halassa et al., 2007; Bushong et al., 2002; Ogata et al., 2002). It is also noteworthy that human astrocytes are larger, structurally more complex, and more diverse than astrocytes in rodents (Oberheim et al., 2009).

All astrocytes express potassium and sodium channels and can exhibit evoked inward currents, but unlike neurons, astrocytes do not propagate action potentials along their processes (Seifert et al., 2006; Nedergaard et al., 2003). However, they exhibit regulated increases in intracellular calcium concentration ($[Ca^{2+}]i$) that represent a form of astrocyte excitability (Charles et al., 1991; Cornell-Bell et al., 1990) of particular significance in astrocyte–astrocyte as well as in astrocyte–neuron intercellular communication. Astrocyte $[Ca^{2+}]i$ elevations can occur as intrinsic oscillations resulting from Ca^{2+} released from intracellular stores, or be triggered by neurotransmitters (including glutamate and purines) released during neuronal activity, or elicit the release from astrocytes of transmitters such as glutamate into the extracellular space and thereby trigger receptor-mediated currents in neurons. Moreover, the release of Ca^{2+} can be propagated to neighboring astrocytes (Perea et al., 2009; Shigetomi et al., 2008; Halassa et al., 2007; Volterra et al., 2005; Nedergaard et al., 2003). It is noteworthy that astrocytes can couple to neighboring astrocytes through gap junctions formed by connexins, and that the gap junctional coupling of astrocytes into multicellular networks may play a role in both normal function and CNS disorders (Seifert et al., 2006; Nedergaard et al., 2003).

1.3.3 Astrocyte heterogeneity

The increasing awareness of the complexity, importance, and diversity of astrocyte functions is giving rise to a growing interest in the potential for specialization and heterogeneity among astrocytes (Hewett, 2009). There is a long-standing recognition of an extended family of astroglial cells that share similarities with, but also exhibit differences to, protoplasmic and fibrous astrocytes, including Müller glia in the retina, Bergmann glia of the cerebellum,

tanycytes at the base of the third ventricle, pituicytes in the neurohypophysis, cribrosocytes at the optic nerve head, and others. These different cell types express various astrocyte-related molecules such as GFAP, S100b, glutamine synthetase and others, and exert functions similar to astrocytes in manners specialized to their locations. In addition, these different types of astroglial cells share with astrocytes the ability to become reactive in response to CNS insults and have the potential to play important roles in pathological changes in their specific locations. As regards protoplasmic and fibrous astrocytes, there appears to be considerable molecular, structural, and potentially functional diversity of astrocytes at both the regional and local levels, although such investigations are at an early stage (Hewett, 2009; Bachoo et al., 2004). In this context it is interesting to note that the number, complexity, and diversity of astroglial cells associated with neurons has increased considerably with evolution, such that the ratio of astrocytes to neurons is 1:6 in worms, 1:3 in rodent cortex, and 1.4:1 in human cortex, implying that astrocyte roles increase in importance with sophistication of neural tissue (Nedergaard et al., 2003). The human cerebral neocortex contains multiple subtypes of astrocytes, including those that do not appear to exist in rodent cortex (Oberheim et al., 2009).

Recent studies have identified specific morphological and biochemical phenotypes of neural stem cells (NSC) in embryonic, juvenile, and adult brain. In the embryonic brain, it is now clear that radial cells are NSC that give rise to cortical pyramidal neurons (Kriegstein and Alvarez-Buylla, 2009; Malatesta et al., 2003; Noctor et al., 2001). As these radial cells mature, they adopt GFAP expression and some give rise to GFAP-expressing radial NSC that persist in juvenile and adult forebrain, while others become astrocytes (Kriegstein and Alvarez-Buylla, 2009; Merkle et al., 2004; Levitt and Rakic, 1980). Some GFAP-expressing radial NSC remain constitutively active throughout life in the subependymal (or subventricular) zone of the lateral ventricles and in the subgranular zone of the hippocampal dentate gyrus, where they are the predominant source of adult neurogenesis (Garcia et al., 2004; Imura et al., 2003; Seri et al., 2001; Doetsch et al., 1999). Based almost entirely on the observation that these adult NSC express GFAP, they have been referred to by some authors as a ‘sub-type’ of astrocyte (Kriegstein and Alvarez-Buylla, 2009; Doetsch et al., 1999), but it is not clear that such a nomenclature is either warranted or useful (Barres, 2003; Goldman, 2003; Morest and Silver, 2003).

There is ample evidence that mature adult astrocytes are not neurogenic (Imura et al., 2006). Even after CNS trauma, proliferating reactive astrocytes give rise only to other astrocytes and do not spontaneously exhibit multipotent neurogenic potential unless they are reprogrammed genetically or by exposure to high doses of specific growth factors in vitro (Buffo et al., 2008,

2005). In addition, GFAP-expressing NSC differ in morphology (radial phenotype), biochemical phenotype (LeX and nestin expression), and physiological characteristics from protoplasmic and fibrous astrocytes (Imura et al., 2006; Liu et al., 2006; Garcia et al., 2004).

1.3.4 Involvement of astrocytes in physiological processes

1.3.4.1 Regulation of blood flow

Astrocytes make extensive contacts with and have multiple bidirectional interactions with blood vessels, and regulate the local CNS blood flow. Recent findings show that astrocytes produce and release various molecular mediators, such NO, prostaglandins (PGE), and arachidonic acid (AA), that can increase or decrease CNS blood vessel diameter and blood flow in a coordinated manner (Gordon et al., 2007; Iadecola et al., 2007). Moreover, because astrocyte processes contact both blood vessels and synapses, they may be primary mediators of changes in local CNS blood flow in response to changes in neuronal activity (Koheler et al., 2009), and titrate blood flow in relation to levels of synaptic activity (Schummers et al., 2008; Wolf and Kirchhoff, 2008).

1.3.4.2 Fluid, ion, pH, and transmitter homeostasis

Astrocyte processes envelop essentially all synapses (Brown and Ransom 2007; Peters et al., 1991) and exert essential functions in maintaining the fluid, ion, pH, and transmitter homeostasis of the synaptic interstitial fluid in a manner that is critical for healthy synaptic transmission. Astrocyte processes are rich in the aquaporin 4 (AQP4) water channel and in transporters for the uptake of K^+ (Zador et al., 2009; Seifert et al., 2006; Simard et al., 2004). Astrocyte membranes have different means of proton shuttling, including the Na^+/H^+ exchanger, bicarbonate transporters, monocarboxylic acid transporters, and the vacuolar-type proton ATPase (Obara et al., 2008). AQP4 water channels are densely clustered along astrocyte processes that contact blood vessels and play a critical role in regulating fluid homeostasis in healthy CNS, and play roles also in vasogenic and cytotoxic edema. Astrocyte processes at synapses are essential in neurotransmitter homeostasis by expressing high levels of transporters for glutamate, γ-aminobutyric acic (GABA), and glycine that serve to clear the latter from the synaptic space (Schummers et al., 2008; Sattler et al., 2006). After uptake into astrocytes, the transmitters are converted enzymatically into precursors such as glutamine and recycled back to synapses for reconversion into active transmitters. Networks of astrocytes linked together by gap junctions are thought to be able to rapidly dissipate small molecules

such as potassium and glutamate and prevent their potentially detrimental accumulation (Schummers et al., 2008).

1.3.4.3 Roles in synapse function

There is strong evidence that astrocytes play direct roles in synaptic transmission through the regulated release of synaptically active molecules including glutamate, purines (ATP and adenosine), GABA, and D-serine (Perea et al., 2009; Shigetomi et al., 2008; Halassa et al., 2007; Nedergaard et al., 2003). The release of such gliotransmitters occurs in response to changes in neuronal synaptic activity, involves astrocyte excitability as reflected by increases in astrocyte $[Ca^{2+}]i$, and can alter neuronal excitability (,Perea et al., 2009; Shigetomi et al., 2008; Halassa et al., 2007; Nedergaard et al., 2003). Such evidence has given rise to the 'tripartite synapse' hypothesis (Perea et al., 2009; Halassa et al., 2007). In this hypothesis the synapse comprises three elements: the presynaptic terminal, the postsynaptic terminal, and an astrocyte between them (Halassa et al., 2007; Perea et al., 2009; Parea and Araque, 2005; Araque et al., 1999). The localization and spatial distribution of these elements of the tripartite synapse vary according to brain region. In addition to having direct effects on synaptic activity via gliotransmitter release, astrocytes have the potential to exert powerful and long-term influences on synaptic function via release of growth factors and related molecules. Some molecular mechanisms have been identified by which astrocytes play a role in the formation, maintenance, and pruning of synapses during development (Barres et al., 2008; Christopherson et al., 2005; Stevens et al., 2007). Such mechanisms may also provide astrocytes with the means of exerting powerful influences on synaptic remodeling and pruning in the healthy adult CNS or in response to CNS disorders (Barres et al., 2008). Cytokines such as TNF-α influence homeostatic synaptic scaling by inducing the insertion of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors at post-synaptic membranes (Stellwagen et al., 2006). Although it is not certain whether astrocytes or microglia are primary sources of TNF-α in the CNS in vivo, the effects on synaptic function of astrocyte-derived growth factors and cytokines warrant further study. Astrocytes are also sources of neuroactive steroids (neurosteroids), including estradiol, progesterone and various intermediaries and metabolites that can have synaptic effects, particularly at $GABA_A$ receptors (Garcia-Segura et al., 2006).

1.3.4.4 Energy and metabolism

Astrocytes contribute importantly to CNS metabolism. Astrocytes, whose processes on the one hand contact blood vessels and on the other contact neuronal perikarya, axons (at nodes of Ranvier), and synapses, are well-positioned to take up glucose from blood vessels and furnish energy metabolites to different neural elements in gray and white matter. Astrocytes are the principal storage sites of glycogen granules in the CNS, and the greatest accumulation of astrocytic glycogen occurs in areas of high synaptic density (Peters et al., 1991; Phelps, 1972). Their glycogen utilization can sustain neuronal activity during hypoglycemia and during periods of high neuronal activity (Brown and Ramson, 2007; Suh et al., 2007), and it can be modulated by transmitters such as glutamate (Brown and Ramson, 2007). During these periods, glycogen is broken down to lactate that is transferred to adjacent neural elements (both synapses in gray matter and axons in white matter) where it is used aerobically as fuel (Brown and Ramson, 2007; Pellerin et al., 2007; Brown et al., 2004; Voutsinos-Porche et al., 2003). In addition, computer-based modeling studies suggest that during periods of high neuronal activity, inhibition of phosphofructokinase leads to impairment of neuronal glycolysis, with the consequence that lactate effluxed from astrocytes becomes the preferred energy substrate for neurons (Occhipinti et al., 2009).

1.4 MICROGLIA-ASTROCYTE INTERACTIONS

1.4.1 Microglia are activated earlier than astrocytes

Activation of microglia and astrocytes occurs at different stages in several neurodegenerative diseases. In experimental autoimmune encephalomyelitis (EAE), microglia proliferated at the initial stage while astrocytes started to respond markedly at the late recovery stage (Matsumoto et al., 1992). In AD, activation of astrocytes also occurred subsequent to microglial activation (Gatan and Overmier, 1999). Similarly, astrocytes were activated following microglial activation when human-derived dense-core amyloid plaques (typical for AD) were injected into rat brain (Frautschy et al., 1998). Trimethyltin (TMT), a triorganotin compound, is employed as a well-established neurodegeneration model. In TMT-treated rats, significant increases in the expression of GFAP typically occurred after microglial activation (McCann et al., 1996). Moreover, microglial responses occurred with TMT concentrations at or below the neurotoxicity limit, whereas astrocyte activation required a high concentration (Reali et al., 2005), confirming that microglia respond to TMT more quickly than astrocytes. In neuropathic pain microglia are again activated earlier than astrocytes. For instance, astrocytes have been reported to respond to spinal nerve injury more slowly than microglia (Colburn et al., 1997). Similarly, intrathecal ATP administration caused spinal microglial activation within 1 day, while astrocytic activation peaked at 1–3 days. Furthermore, minocycline (a microglial inhibitor) attenuated the induction but not the early and late phase of maintenance, while fluorocitrate (a glial metabolic inhibitor) attenuated the induction and the early phase but not the late phase of maintenance (Nakagawa et al., 2007). Considering that fluorocitrate disrupts the function of both astrocytes and microglia while minocycline only targets microglia, it was suspected that microglia was activated earlier than astrocytes.

Activated microglia promote astrocytic activation. Pro-inflammatory cytokines, which are closely involved in various diseases (including trauma, ischemia, AD, epilepsy, and others), play important roles in the facilitation of activated microglia for astrocytic activation (secretion of pro-inflammatory cytokines from microglia is characteristic of activation, so most studies have focused on them.). Among various cytokines, IL-1 is a pivotal mediator, not only because of its fast release in these pathological conditions, but also for its ability to up-regulate other inflammatory cytokines, such as IL-6 and TNF-α (John et al. 2005). In a model of CNS trauma (corticectomy injury), IL-1-positive cells entirely overlapped with ionized calcium binding adaptor molecule 1 (Iba1, a microglial marker) positive microglia

surrounding the lesion site while GFAP immunoreactive cells were not present, indicating microglia to be the only source of IL-1 (Herx et al., 2000; Herx and Yong, 2001). Increased IL-1 expression has been detected in reactive microglia surrounding amyloid plaques in AD (Shaftel et al., 2007) Moreover, IL-1 receptor antagonist is neuroprotective in cerebral ischemia, as deletion of genes encoding for both agonists IL-1α and IL-1β in mice reduced ischemic brain damage by 80% (Boutin et al., 2001). Exogenous administration or over-expression of IL-1 promotes astrocytic activation, which leads to astrogliosis (John et al., 2004). IL-1 injection into brain resulted in astrocytic activation indicated by GFAP up-regulation (Balasingam et al., 1994; Lee et al., 2010). Moreover, IL-1 induces nuclear hypertrophy and intercellular adhesion molecule-1 expression in astrocytes (Albrecht et al., 2002; Kyrkanides et al., 1999). Consistent with these reports, astrocytic activation was delayed in mice lacking IL-1 receptor (Herx et al., 2000). IL-1 mediates neurotoxicity of activated astrocytes. Large amounts of NO can be induced from primary human astrocytes, which was totally blocked by IL-1 receptor agonist protein (Chao et al., 1996), while an increased level of glutamate could lead to excytotoxicity (Hu et al., 2000; Jing et al., 2010). In addition, free radicals are released from IL-1-activated astrocytes (Thornton et al., 2006). On the other hand, IL-1 also contributes to the protective role of activated astrocytes. Connexin 43 (Cx43) is the main constitutive gap junction protein, and is a target for deleterious molecules released from injured astrocytes. Inhibition of Cx43 in astrocytes is thus generally considered to be neuroprotective. It has been reported that expression of Cx43 and gap junctional communication among astrocytes were inhibited in microglia/astrocyte co-cultures (Rouachet al., 2002b). This inhibition was mimicked by treating astrocyte cultures with conditioned medium from activated microglia. IL-1β and TNF-α were identified as the main factors responsible for this conditioned medium-mediated activity (Rouach et al., 2002a). In addition, IL-1 up-regulated nerve growth factor (NGF) and TGF-β in astrocytes at both the gene and protein level (da Cunha et al., 1993; Jauneau et al., 2006), both beneficial for CNS recovery. In addition, IL-18 may play an important role in activation of microglia/astrocytes, as nerve injury induced a striking increase in IL-18 and IL-18 receptor (R) expression in the dorsal horn, and IL-18 and IL-18R were up-regulated in hyperactive microglia and astrocytes, respectively. Inhibition of IL-18 signaling pathways not only suppressed injury-induced tactile allodynia, but also decreased the phosphorylation of nuclear factor-kB (NF-κB) in spinal astrocytes and the induction of astroglial markers (Miyoshi et al., 2008). Prostaglandin (PG)D2 also contributes to activation of microglia/astrocytes. In the genetic demyelination mouse twitcher (a model of human Krabbe's disease), activated microglia expressed

hematopoietic PGD synthase (HPGDS) and activated astrocytes expressed the DP1 receptor for PGD2. Cultured microglia produce PGD2 by the action of HPGDS, while cultured astrocytes express two types of PGD2 receptor (DP1 and DP2) and show enhanced GFAP production after stimulation of either receptor with its respective agonist. Blockade of the HPGDS/PGD2/DP signaling pathway using HPGDS- or DP1-null twitcher mice or treating with an HPGDS inhibitor resulted in remarkable suppression of astrogliosis and demyelination (Mohri et al., 2006).

1.4.2 Activated astrocytes facilitate distant microglial activation

Astrocyte activation results in cytosolic Ca^{2+} increase and its propagation among astrocytes. ATP is the second messenger mainly responsible for Ca^{2+} wave propagation within the astrocytic network (Cotrina et al., 1998). Purinergic receptors are highly expressed by microglia, and it is probable that ATP mediates astrocyte-to-microglia communication (Honda et al., 2001; Norenberg et al., 1997; Shigemoto-Mogami et al., 2001; Suzuki et al., 2004). In response to local injury ATP is released from astrocytes and activates local microglia, indicating that the astrocytic Ca^{2+} wave network can also spread to microglia (Davalos et al., 2005; Schipke et al., 2002; Verderio and Matteoli, 2001). ATP induces rapid changes in microglial morphology and migration, causing rapid microglia proliferation and migration towards the site of injury (Davalos et al., 2005; Haynes et al., 2006). Formation and shedding of membrane vesicles are important for phagocytosis and secretion. Astrocyte-derived ATP could induce formation of vesicles in nearby microglia facilitating microglial phagocytosis, while the ATP-degrading enzyme apyrase and $P2X_7$ receptor antagonists inhibited this process (Fang et al., 2009). Purinergic $P2Y_{12}$ and $P2Y_6$ receptors on microglia have been reported to be critical for movement and phagocytosis, respectively (Koizumi et al., 2007; Sasaki et al., 2003). Microglia also respond to ATP by triggering potassium currents (Boucsein et al., 2003), and secretion of cytokines (Bianco et al., 2005; Hide et al., 2000) and plasminogen (Hide et al., 2000; Inoue et al., 1998), all of which are closely involved in pathological cascades. By propagating astrocytic calcium waves, the distant microglia could be activated. This is significant, especially in the expansion of infarct volume in focal ischemia and spreading of pain. Ischemia could evolve to an infarct core which is surrounded by ischemic penumbra (ischemia border zone). Microglia distant from the infarct core can be activated without signs of tissue damage (Lehrmann et al., 1997). The astrocytic Ca^{2+} signaling and in particular the ability of astrocytes to propagate long-distance Ca^{2+} waves probably contribute to the distant microglial activation (Nedergaard and Dirnagl, 2005). The

activated microglia and astrocytes in ischemic penumbra would thus cause a delayed expansion of the infarct volume if not properly treated. Similarly, pain is recognized not only at the injury site, but also some distance from the original wound. Conceivably, Ca^{2+} waves propagated among activated astrocytes can spread to distant areas, leading to activation of microglia removed from the site of injury producing pro-inflammatory cytokines are capable of over-activating pain-transmitting neuronal tracts (Hansson, 2006; Milligan et al., 2003; Watkins and Maier, 2003).

1.4.3 Activated astrocytes can inhibit microglia

Activated astrocytes can exert inhibitory effects on microglia. For example, they can decrease microglial production of NO, ROS and TNF-α (von Bernhardi and Eugenín, 2004; Smits et al., 2001; Tichauer et al., 2007). The ability of microglia to phagocytose and remove senile plaques was markedly suppressed when cocultured with astrocytes, while microglia in the presence of astrocytes showed reduced capacity to phagocytose latex beads (Dewitt et al., 1998). These effects can be mediated by transforming growth factor β (TGF-β), which is mainly produced by astrocytes (Ramírez et al., 2005). This cytokine deactivated microglia by down-regulating the expression of molecules associated with antigen presentation and production of pro-inflammatory cytokines, NO, and oxygen free radicals (Herrera-Molina and von Bernhardi, 2005). Moreover, TGF-β dramatically diminished the clustering of BV-2 cells (a mouse microglial cell line) and attenuated their chemotactic migration towards Aβ aggregates (Huang et al., 2010). Pre-treatment with TGF-β attenuated activation of NF-kB and up-regulation of IL-1 mRNA levels, reducing production and release of pro-inflammatory cytokines (Chen and Wahl, 2002; Hu et al., 1999).

1.5 ASTROCTYTES AND MICROGLIA IN PATHOLOGICAL PAIN

1.5.1 Pathological pain and glia

Pain is an unpleasant sensory experience induced by noxious stimuli. Physiological pain is important for animals to avoid potential injury, while pathological pain is unpleasant, lasts for an extended period of time after injury and is characterized by a heightened responsiveness to both noxious and non-noxious stimuli (hyperalgesia and allodynia, respectively). One type of pathological pain is neuropathic pain, generally defined as a chronic pain state resulting from peripheral or central nerve injury due either to acute events (e.g. amputation, spinal cord injury) or systemic disease (e.g. diabetes, viral infection and cancer). Chronic pain costs approximate $100 billion annually in healthcare and lost productivity in the United States alone (McCarberg et al., 2006). Currently available treatments for neuropathic pain, including tricyclic antidepressants and gabapentin, typically show limited efficacy in the majority of cases (Childers and Baudy, 2007). Better treatment for neuropathic pain requires a comprehensive understanding of its pathogenesis. Chronic pain (such as inflammatory and neuropathic) is believed to be caused by aberrant neuronal responses along the pain transmission pathway from dorsal root ganglion (DRG) to spinal cord, thalamus and cortex. Both peripheral and central origins are likely to be involved in chronic pain, although their contribution may be different depending on the various forms of chronic pain. For example, the sensitization of nociceptors after tissue injury by inflammatory factors leads to primary hyperalgesia and inflammatory pain. Similarly, central sensitization and synaptic plasticity in the CNS contribute significantly to neuropathic pain. Therefore, targeting neuronal plasticity changes in somatosensory pathways is a major direction for identifying pain-relieving medications. However, in chronic pain states, neurons are not the only cell type involved. Glial cells, including astrocytes and microglia, are additional players in the initiation and maintenance of neuropathic and inflammatory pain. These glial cells have close interactions with neurons and thus modulate pain transmission particularly under pathological conditions (Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Milligan and Watkins, 2009).

1.5.2 Activation of glial cells after peripheral nerve injury

Activation of microglia and astrocytes can occur following physiological changes in the body, such as trauma in the CNS, ischemia, inflammation, and infection. The activation of these glial cells is most often implicated in the development, spread, and potentiation of chronic and neuropathic pain (Coull et al., 2005; Raghavendra et al., 2004; Watkins et al., 2001; Milligan et al., 2000; Chacur et al., 2001; Meller et al., 1994; Colburn et al., 1997; Garrison et al., 1991). Microglia and astrocytes are generally activated in the dorsal horn following peripheral nerve injury. After receiving a pain stimulus, peripheral neurons transmit pain signals to the dorsal horn of the spinal cord, releasing neurotransmitters such as calcitonin gene-related protein (CGRP), substance P, glutamate, GABA, serotonin (released from descending pain pathways), and ATP. These neurotransmitters initiate the activation of glial cells in the area of the synapse, further sensitizing postsynaptic neurons. Various mechanisms by which glial cells are activated have been suggested. These include a mechanism via biochemical mediators, including substance P, CGRP, NO, purinergic agents (such as ATP), glutamate, and endogenous opioid peptides, which are released at the time of injury and travel through or between afferent neurons, not only affecting synaptic transmission but also activating glial cells (Guo et al., 2007; Ikeda et al., 2007; Sun et al., 2007; Jourdain et al., 2007; Fields et al., 2006; Holguin et al., 2004). ATP released by afferent neurons causes migration and activation of microglia within a range of 50 to 100 μm, producing an intracellular increase in Ca^{2+} and brain-derived neurotrophic factor (BDNF), which results in the activation and translocation of NF-kB to the nucleus, thereby initiating expression of numerous pro-inflammatory agents. NO acts similarly upon NF-kB in astrocytes affecting gene expression and inducing activation (Guo et al., 2007; Brahmachari et al., 2006; Holguin et al., 2004; Meller and Gebhart, 1993). Sensory neurons undergoing painful stimuli release substance P (or via NO-stimulated production of substance P) which activates CNS glia via neurokinin-1 receptors. Other biochemical mediators, e.g. purinergic agonists, glutamate, and opioid peptides, induce activation of glial cells by direct interaction with specific membrane receptors.

Another mechanism involves glial cell activation via shifts in intracellular and extracellular ion concentrations. An increase in afferent neuronal input causing an elevation of extracellular K^+ leads to increased K^+ uptake by astrocytes, resulting in membrane depolarization, morphologic changes, and possibly activation (Hansson et al., 2006). Furthermore, K^+ induces microglial activation in rat hippocampal tissue *in vitro* (Abraham et al., 2001). Similarly, an influx of Ca^{2+} results in the activation of astrocytes and microglia, with concomitant changes in morphology and cellular function. Pro-inflammatory agents generated and released by

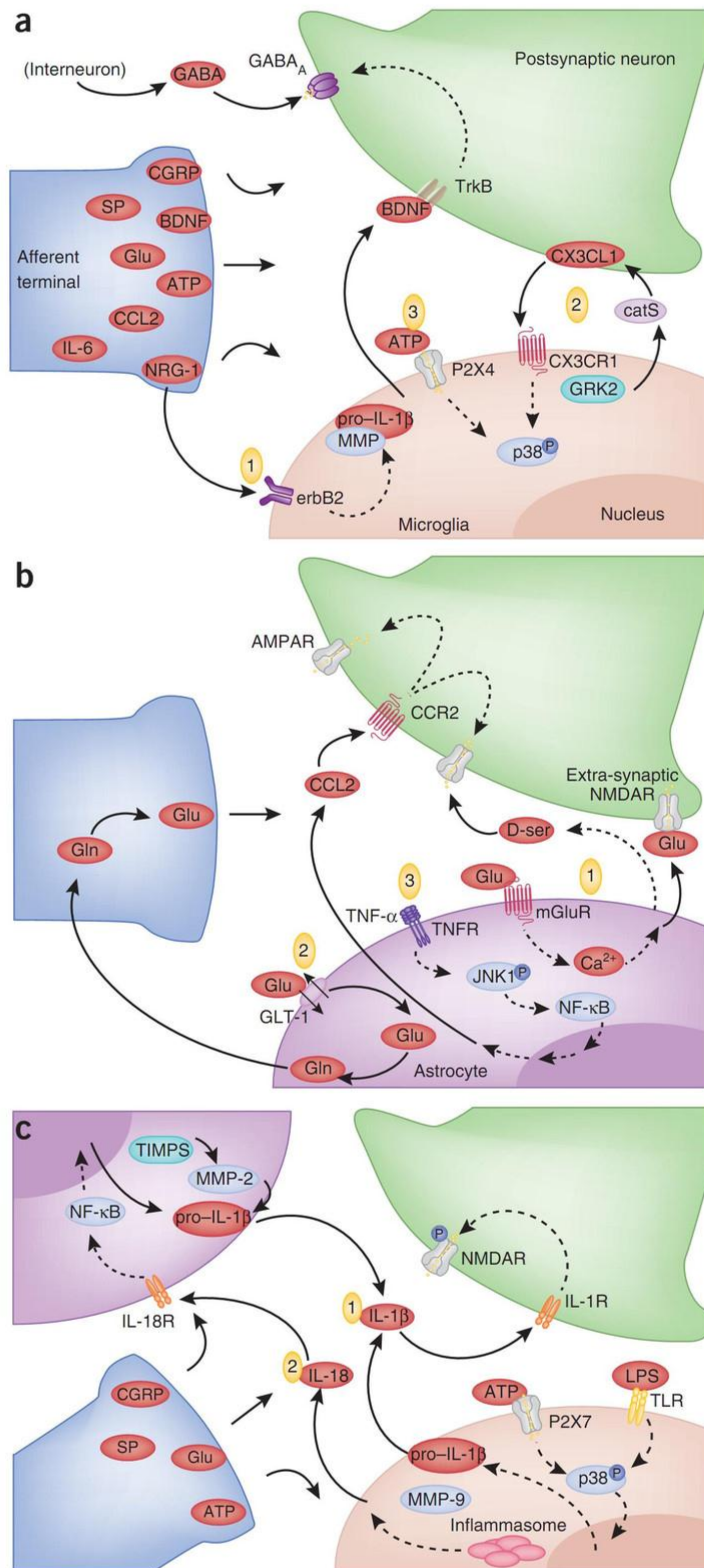
activated glial cells can further activate neighbouring glial cells. In addition, peripheral injury results in astrocyte activation in the trigeminal complex of the brain stem (Wang et al., 2007; Cao and DeLeo, 2008). Interestingly, proximal blockade of primary afferent input following a peripheral nerve injury fails to inhibit glial activation at both the spinal and supraspinal levels. These studies suggest that supraspinal-activated astrocytic cells may potentially modulate neuropathic pain by further activation of glial cells in the spinal cord via descending pathways (Wei et al., 2007).

Peripheral nerve injury can lead to increased blood-brain barrier permeability, allowing peripheral macrophages to migrate, proliferate, and differentiate into activated glial cells in the brain (Cao and DeLeo, 2008; Wei et al., 2007; Gordh et al., 2006; Gou et al., 2005). In addition, peripherally generated inflammatory agents, outside of the neuronal afferent pathway, can activate glial cells in the CNS. For example, a proximal anesthetic block fails to inhibit either spinal cyclooxygenase gene expression or PGE2 release into the cerebrospinal fluid. Interestingly, acute pain, such as a paper cut or a needle prick, will not activate glial cells (Holguin et al., 2004). However, following a more serious injury, glial cells exhibit dynamic plasticity and switch from a resting state to become active in the modulation of neuronal activity (Rent and Dubner, 2008). Once activated, glial cells change their morphology, via hypertrophy and retraction of the processes, and synthesize specific cell markers and kinases, some having an active role in initiating and potentiating an immune response.

1.5.3 Role of glial activation in neuropathic pain

Microglia and astrocytes are both involved in neuropathic pain pathways. After a threshold stimulus, activated glial cells release inflammatory stimulants such as cytokines, prostaglandins, neurotrophic factors, ATP, NO, D-serine, and glutamate (Cao and De Leo, 2008; Sun et al., 2007; Bhangoo et al., 2007; Werry et al., 2006; Hamke et al., 2006; Ji et al., 2006; Clark et al., 2006; Garrison et al., 1991). These inflammatory agents play a critical role in the development and maintenance of central sensitization and hyperalgesia (Holguin et al., 2004) by altering the polarization characteristics of the afferent neurons and thus modulate the transmission of painful stimuli to the CNS (Perea and Araque, 2007; Parpura et al., 1994). For example, astrocyte activation leads to increasing intracellular Ca^{2+} which stimulates calcium-dependent glutamate release, resulting in an inward current produced in adjacent neurons (Parpura and Haydon, 2000). Current research suggests that microglia are involved in the

Fig. 1.5 Activation of neurons and glia in chronic pain



Activation of glia and neurons in the dorsal horn of the spinal cord after peripheral injury. (a) Microglia-neuron interactions. Upon activation, afferent nerve terminals release neurotransmitters, substance P (SP), CGRP, glutamate (Glu), ATP and BDNF, as well as inflammatory mediators including IL-6 and CCL2 and the growth and differentiation factor neuregulin-1 (NRG-1), into the spinal cord. Three examples are shown. (1) Neuronal NRG-1 acts on microglial erbB2, leading to IL-1β release. (2) Microglial cathepsin S (catS) cleaves neuronal CX3CL1, which binds CX3CR1 and stimulates phosphorylation of p38 MAPK (mitogen-activated protein kinase) in microglia. This pathway may be inhibited by protein-coupled receptor kinase 2 (GRK2). (3) ATP binds P2X4 and induces BDNF release from microglia, which upon binding TrkB receptor induces a shift in the chloride anion gradient and $GABA_A$ receptor-mediated depolarization in dorsal horn neurons. (b) Astrocyte-neuron interactions. (1) Astrocytes release glutamate and D-serine, which bind extrasynaptic and synaptic NMDA receptors on neurons, respectively. (2) Injury-induced down-regulation of astrocytic GLT-1 alters glutamate homeostasis in the synaptic cleft. (3) TNF-α activates the JNK1 (c-Jun N-terminal kinase 1) pathway, which leads to release of CCL2 and alterations in NMDAR and AMPAR activity. (c) Cross-talk between nerve terminals, astrocytes and glia. (1) TLR priming and purinergic signaling increase IL-1β release by glia, which modulates NMDAR activity on postsynaptic neurons. TIMPs (tissue inhibitor of metalloproteinases) in astrocytes inhibit MMP (matrix metalloproteinase)-mediated cleavage of pro–IL-1β. (2) Microglial IL-18 binds IL18R on astrocytes and induces NF-κB activity and up-regulation of inflammatory cytokines. Dashed lines represent multiple intermediate signaling events (From Ren and Dubner, 2010).

early development, whereas astrocytes function to sustain neuropathic pain (Raghavendra et al., 2004; Raghavendra et al., 2003; Colburn et al., 1999). Microglial activation leads to the release of signaling proteins, such as IL-1β, into the cell interstitium and to some extent the cerebrospinal fluid. These signaling proteins bind to specific sites on the astrocyte membrane initiating cell activation (Sama et al., 2008; Schubert et al., 2000). Upon activation, a positive feedback cycle occurs whereby astrocytes release inflammatory mediators, e.g., TNF-α, which in turn can activate other glial cells (Sama et al., 2008). Astrocyte activation is accompanied by a decrease in microglial activity over time (Tanga et al., 2004). In an animal model, intrathecal administration of activated microglial cells decreased pain threshold while a similar application of activated astrocytes did not (Narita et al., 2006), further demonstrating that activated astrocytes are not predominantly involved in the development of a pathological pain state, but rather its potentiation.

1.5.4 Chemokines and neuropathic pain

The chemotactic cytokines represent a large subfamily of cytokines generally referred to as chemokines. Characteristics common among all chemokines include both structural and functional features: the conservation of a cysteine motif in the N-terminal region of the protein and the induction of their effects via various 7-transmembrane G-protein-coupled receptors. Chemokines play a dual role in the immune system: they act as chemoattractants during inflammation and as traffickers of hematopoietic stem cells during development and differentiation (Charo et al., 2006; Lapidot et al., 2005). Chemokine binding to receptors triggers downstream signaling cascades that ultimately result in Ca^{2+} influx (Gillard et al., 2002; Oh et al., 2001; Murphy, 1994). Chemokines can be synthesized throughout the body from a wide variety of cells with expression being variable depending on the immunological state. Some chemokines are homeostatic, such as those that guide lymphocytes to lymphoid tissues, while others are only expressed to facilitate the localization of the immune response around the site of injury or infection (Fernandez and Lolis, 2002). Numerous rodent neuropathic pain models have demonstrated an up-regulation of the chemokine receptors CX3CR1 or CCR2 as well as MCP-1 (recently termed CCL2) in neural tissues after an injury such as partial ligation of the sciatic nerve (Lindia et al., 2005; Tanaka et al., 2004; Abbadie et al., 2003), chronic constriction injury of the sciatic nerve (Sun et al., 2007; Kleinschnitz et al., 2005; Holguin et al., 2004), chronic compression of the L4-L5 DRG (Sun et al., 2006; Kim et al., 2005), bone cancer pain (Vit et al., 2006), and zymosan-induced inflammatory pain (Xie et al., 2006; Holguin et al., 2004; Milligan et al., 2004). The importance of the chemokine

receptor CCR2 in neuropathic pain may be very significant as revealed in genetically engineered mice lacking the *CCR2* gene. These mice failed to display a detectable change in acute pain behavior for mechanical hyperalgesia after partial ligation of the sciatic nerve (Abbadie et al., 2003). Chemokines' ability to alter nociception can occur via induction by pro-inflammatory agents. For example, chemokines were expressed by endothelial cells after administration of LPS, IL-1β, or TNF-α (Beck et al., 1999). Furthermore, chemokines and their receptors have been shown to facilitate pain via injection of stromal cell-derived factor-1a (CXCL12), Regulation upon Activation Normal T cell Expressed and Secreted (CCL5), or macrophage inflammatory protein-1a (CCL3) into the non-inflamed rat hindpaw inducing dose-dependent tactile allodynia (Oh et al., 2001). Chemokines such as stromal cell-derived factor-1a/CXCL12 acting on neurons and/or astrocytes are believed to affect the release of glutamate, potentially affecting neuronal excitation (Bezzi et al., 2001; Limatola et al., 2000). Application of Regulation upon Activation Normal T cell Expressed and Secreted /CCL5 or MCP-1/CCL2 to DRG cultures results in the release of substance P (Oh et al., 2001) and CGRP (Qin et al., 2005), respectively. Substance P and CGRP are potent peptides and neurotransmitters with established roles in pain transmission. MCP-1/CCL2 produces membrane threshold depolarization and action potentials in neuronal cell bodies (Sun et al., 2006, 2005; Kim et al., 2005). These excitatory effects on sensory neurons are believed to facilitate the release of CGRP (Qin et al., 2005). Furthermore, the increase in electrical activity after peripheral injury may stimulate the release of MCP-1/CCL2 into the dorsal horn of the spinal cord, further activating CCR2-bearing glial cells and/or neurons (Sun et al., 2007; Abbadie et al., 2003; De Leo et al., 2002).

1.5.5 Neurotrophic factors and neuropathic pain

Neurotrophic factors are protein molecules that promote the survival, growth, and maintenance of neurons. Upon tissue injury neurotrophic factors act to prevent damaged neurons from initiating programmed cell death. The term neurotrophic factor describes three major families including neurotrophins, the GDNF family, and neuropoietic cytokines. In mammals there are only 4 members of the neurotrophin family: NGF, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, Boid et al., 2003). Under normal physiological conditions, neurotrophins are secreted by peripheral targets (such as skin, muscle, and viscera) and transported retrogradely to the neuron cell body (Zhou et al., 1996; Helgren et al., 1997). Both BDNF and NT-3 also undergo anterograde transport to neurons and target cells, thereby

potentially acting as a neuromodulator and trophic factor (Altar et al., 1998). Furthermore, activated brain astrocytes are a source of NGF, BDNF, and NT-3 around the site of an injury (Appel et al., 1997). NT-4 is synthesized by most DRG neurons and dorsal and ventral horn neurons (Heppenstall et al., 2001).

NGF is the most extensively studied neurotrophin. Aside from its role in developing nervous tissues, NGF is most commonly known for its role as a major regulator of inflammatory and homeostatic pain states (Theodosiou et al., 1999; Lewin and Mendell, 1993; Ramer 1998; Herzberg et al., 1997; Woolf 1996; Verge et al., 1995; Woolf et al., 1994). Elevated NGF levels enhance expression of the neuropeptides substance P and CGRP (Jongsma Wallin et al., 2001; Fernyhough et al., 1998; Schuligoi and Amann, 1998; Amann et al., 1996; Verge et al., 1995; Zhang et al., 1995; Dickinson et al., 1999). Thermal hyperalgesia and mechanical allodynia, in both animal and human studies, have been linked to elevated NGF (Theodosiou et al., 1999; Sommer et al., 1998 Dyck et al., 1997; Lewin et al., 1994). Expression of NGF is often biphasic, with the secondary increase correlating temporally with IL-1β expression (Heumann et al., 1987). Further evidence supporting the secondary increase linked to IL-1β expression, likely released by macrophages, was characterized in three ways: (i) by a temporal relationship between macrophage invasion and the secondary increase in NGF; (ii) it was mimicked *in vitro* using activated macrophages or recombinant IL-1β; (iii) inhibition of the secondary expression of NGF was seen when using IL-1 β antibodies (Heumann et al., 1987; Lindholm et al., 1987). Unlike NGF, BDNF is expressed at basal levels in sensory neurons. BDNF synthesized in neurons is transported anterogradely to the spinal cord in secretory vesicles (Mannion et al., 1999; Tonra, 1999; Zhou and Rush, 1996) in addition to target-derived BDNF that is retrogradely transported to the cell body (Tonra, 1999). Under inflammatory conditions, BDNF is regulated in an NGF-dependent fashion (Cho et al., 1997a, b; Apfel et al., 1996) and appears as an important mediator of centrally sensitized inflammatory pain via inhibition of chloride ion excretion resulting in further depolarization of the neuron (De Koninck, 2007; Obata et al., 2006; Mannion et al., 1999; Kerr et al., 1999). TNF-α up-regulates expression of BDNF in primary astrocytes, which are activated earliest during inflammation (Saha et al., 2006). BDNF may signal through the DRG to the dorsal horn of the spinal cord (Obata et al., 2006a, b). A direct role for BDNF in the generation of neuropathic pain was demonstrated by administering BDNF which subsequently induced both thermal hyperalgesia and mechanical allodynia (Miki et al., 2000; Zhou et al., 2000b; Shu et al., 1999).

NT-3 exerts an anti-inflammatory role in neuropathic pain by modulating injury-associated increases in BDNF (Karchewski et al., 2002), substance P (Gratto et al., 1995, 1994), and IL-6 (Verge et al., 2000). NT-3 also antagonizes the NGF pro-inflammatory pathway, inhibiting the production of pro-inflammatory mediators such as NO, TNF-α, and IL-1β (Wilson-Gerwing and Verge, 2006; Tzeng et al., 2005; Gratto and Verge, 2003; Tzeng and Huang, 2003). Nerve injury-induced phenotypic changes in DRG neurons can be reversed by exogenous NT-3 as reflected by decreased BDNF expression (Karchewski et al., 2002).

Little is known about the specific role of NT-4 regarding glial responses and the immune system. One study showed that like BDNF, NT-4 can sensitize sensory afferent neurons to thermal stimulation (Shu and Mendell, 1999). However, NT-4 antibodies failed to abolish thermal hyperalgesia in a chronic pain model (Yajima et al. 2002), indicating that NT-4 plays a supporting role. The specific role of NT-4 in inflammatory pain has yet to be fully elucidated.

The GDNF family of neurotrophic factors is composed of secreted proteins that are structurally related, namely GDNF, neurturin, artemin, and persephin. In regards to neuropathic pain, the GDNF family has not been as intensely studied as the neurotrophins. Most research has centered on GDNF, while artemin, neurturin, and persephin are still considered fairly new. GDNF is expressed in almost all tissue types such as spinal cord, cartilage, stomach, intestine, and kidneys, and virtually every brain region (Lin, 1996). Artemin, neurturin, and persephin are expressed in many tissues, although typically at very low levels (Lagercranz et al. 2002; Fernyhough et al., 1999). GDNF primary role seems to be in the repair or neuroprotection of nerves after injury. In one study, GDNF was released by activated microglia, restorating locomotor function after LPS-induced inflammation (Lin, 1996). A separate study showed that administration of IL-1β, IFNγ, TNF-α, or LPS to cultured astrocytes increases GDNF expression, indicating that GDNF in astrocytes is regulated by inflammatory stimuli (Appel et al., 19997). Partial sciatic nerve ligation and spinal nerve ligation, both established pain models, induce inflammation, resulting in mechanical allodynia and thermal hyperalgesia. In both injury paradigms, intrathecal GDNF treatment attenuated hyperalgesia by significantly increasing the withdrawal threshold for mechanical and thermal stimulation (Boucher and McMahon, 2001). Similarly, artemin injections produced a time- and dose-related reversal of tactile and thermal hypersensitivity, which was maintained with sustained artemin administration (Gardell et al., 2003). However, conflicting data have since shown that intrathecal artemin injections fail to inhibit the development of hyperalgesia after nerve ligation (Bolon et al., 2004). Interestingly, expression

of GDNF, artemin, and neurturin is enhanced after nerve injury (Sarabi et al., 2001; Zhou et al., 2000a; Tang et al., 1998) or chemically-induced inflammation (Malin et al., 2006; Hashimoto et al., 2005; Amaya et al., 2004). There is considerable debate around the involvement of GDNF, artemin, and neurturin in thermal hyperalgesia and expression of transient receptor potential vanilloid 1 (TRPV1) (Malin et al., 2006; Vellani et al., 2006; Amaya et al., 2004), a protein known to facilitate thermal hyperalgesia. Both NGF, a pro-inflammatory neurotrophic factor, and GDNF, an anti-inflammatory neurotrophic factor, have been shown to increase TRPV1 expression. However, studies suggest that NGF and GDNF act on distinctive neuronal cell populations to induce TRPV1 expression (Amaya et al., 2004). Very little is known regarding persephin and its role in neuropathic pain. The entire GDNF family, with the sole exception of persephin, showed increased gene expression following administration of capsaicin, a known activator of TRPV1 (Schmutzler et al., 2009). More research is needed to determine any role, if any, that persephin plays in neuropathic pain.

The neuropoeitic cytokine family consists of IL-6, IL-11, leukeaemia inhibitory factor, oncostatin-M, ciliary neurotrophic factor, cardiotrophin-1, cardiotrophin-like cytokine, and neuropoietin. The neuropoietic cytokine family has two general unifying characteristics. First, neuropoietic cytokines all have a degree of homology to IL-6, and second, they share a common signal-transducing receptor, glycoprotein 130 (gp130). Given that all neuropoietic cytokines rely on gp130 to induce their cellular response, it is important to note that gp130 is up-regulated in peripheral nerves, DRG, and spinal cord in a variety of pain models (De Jongh et al., 2003). Neuropoietic cytokines have both pro- and anti-inflammatory characteristics and are major players in hematopoiesis, acute-phase responses, and immune responses (Heinrich et al., 2003). Most of the neuropoietic cytokines play a minor role in pain or inflammation. However, cardiotrophin-1 was recently shown to induce IL-6 mRNA and protein expression in a time- and dose-dependent manner (Fritzenwanger et al., 2007). As with almost all neuropoietic cytokines, IL-6 is important for differentiation, survival, and nerve regeneration. However, it plays a significant role in chronic pain. IL-6 represents a typical defence hormone involved in the activation of the immune and acute-phase responses (Heinrich et al., 1998). IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to signals such as IL-1β, TNF-α, and prostaglandins (Zumwalt et al., 1999; Murphy et al., 1999; DeLeo et al., 1996; Ringheim et al., 1995). Centrally, IL-6 is produced by neurons as well as astrocytes and microglia (Zumwalt et al., 1999; Frei et al., 1989; Le JM and Vilcek, 1989). Following peripheral axotomy, the presence of IL-6 mRNA is one of the earliest changes observed in DRG and brain (Murphy et al., 1995; Keifer et al.,

1993). IL-6 is produced both locally, at the site of peripheral nerve injury, and centrally, in response to nerve damage (De Leo et al., 1996). Following an injury, IL-6 mRNA and protein were primarily found in neurons; however, microglia and astrocytes are also known sources of IL-6 production (Arruda et al., 1998; De Leo et al., 1996). Intraplantar injection of IL-6 in rats induced dose-dependent mechanical hyperalgesia (Cuhna et al., 1992). Within 3 hours after a sciatic nerve crush injury, IL-6 was produced both distally and proximally at the site of injury. Interestingly, a similar case was seen when the nerve was transected, indicating a source other than the neuronal cell body, such as macrophages or Schwann cells at or near the injury site as responsible for producing inflammatory cytokines (Kurek et al., 1996; Bolin et al., 1995). Mechanical allodynia and up-regulation of IL-6 were observed in the sciatic nerve after 14 days following chronic constriction injury, crush injury, and axotomy (Cui et al., 2000). After sciatic nerve injury, IL-6 was found in both the ipsilateral and contralateral dorsal and ventral horns with the increase in IL-6 paralleling pain behaviors over time (Winkelstein et al., 2001; Arruda et al., 1998; De Leo et al., 1996). IL-6 is considered a pro-inflammatory cytokine; however, it has some anti-inflammatory characteristics as well, for instance IL-6, as well as leukeaemia inhibitory factor and ciliary neurotrophic factor, have been shown to inhibit TNF-α expression (Waring et al., 1995; Benigni et al., 1995; Ulich et al., 1994; Alexander et al., 1992 Aderka et al., 1989).

1.6 TOLL-LIKE RECEPTORS

1.6.1 Overview of TLRs

Innate immunity represents the first line of defence against invading microbes. The innate immune system relies on germline-encoded receptors that are directed against broadly defined molecular motifs. These molecular determinants, termed pathogen-associated molecular patterns (PAMPs), are conserved among large classes of micro-organisms and, as such, are unlikely to undergo mutation as they are essential for pathogen survival (Medzhitov et al., 2000; Takeuchi and Akira, 2010; Kaisho et al., 2004; Qureshi et al., 2003) . These PAMPs are sensed by pattern recognition receptors, which comprise multiple receptor families located in both the extracellular and intracellular milieu. One such group of pattern recognition receptors is TLRs, which are not only critical for eliciting innate immune responses to invading pathogens, but are also important in initiating adaptive immunity (Hoebe et al., 2004; Pasare et al., 2003). Specifically, TLR engagement on antigen-presenting cells induces cytokine release and co-stimulatory molecule expression, priming these cells for subsequent activation and expansion of antigen-specific T-cells (Hoebe et al., 2004; Pasare et al., 2003; Hertz et al., 2001; Boonstra et al., 2003). In addition to detecting molecular patterns associated with many micro-organisms, TLRs have also been implicated in recognizing an array of endogenous molecules, the danger-associated molecular patterns (DAMPs) (Kaisho et al., 2004; Kirschning et al., 2002; Piccinini et al., 2010; Karikó et al., 2004). In general, most DAMPs are sequestered from the immune response; however, during CNS infection and disease, self antigens and/or danger signals may be liberated as a consequence of cell death, necrosis or tissue remodelling. The expression of TLRs and related signaling proteins has been demonstrated in all major glial cell types, including microglia, astrocytes and oligodendrocytes, as well as a more limited repertoire in neurons (Kielian, 2006; Lafon et al., 2006; Cameron et al., 2007; Farina et al., 2005). Thus TLR engagement may be elicited by a combination of PAMPs and/or DAMPs during inflammatory CNS disorders.

The TLR family members, 13 described in mice and 11 in humans (**Table 1.2**), can be separated into two broad categories based on their subcellular localization patterns. To date, TLR2 and TLR4 are the best characterized and recognize the microbial motifs PGN (peptidoglycan)/lipoproteins/dectin and LPS, respectively. These PAMPs are expressed within the pathogen cell wall and are accessible for TLR recognition once they come into contact with the cell surface (Takeuchi and Akira, 2010).

Following infection, it is likely that these intracellular TLRs serve to amplify responses initially triggered by extracellular TLRs to ensure effective pathogen clearance. In the case of neurodegenerative diseases without evidence of an infectious aetiology, pathological engagement of TLRs by DAMPs may contribute to exacerbated immune responses and enhanced neuropathology. Alternatively, some studies suggest a neuroprotective role for TLR signaling, indicating that the context and intensity of TLR engagement may dictate whether TLRs exert beneficial compared with detrimental properties during CNS disorders. Depending on the particular TLR, receptor engagement can culminate in the induction of NF-κB, mitogen-activated protein kinases (MAPKs) and/or interferon-regulatory factor signaling pathways, which regulate the expression of a wide array of genes involved in inflammatory responses. The majority of TLRs utilize the central adaptor molecule MyD88 (myeloid differentiation factor 88), with the exception of TLR3, to bridge the receptor to downstream signaling intermediates (Takeuchi and Akira, 2010; Coll et al., 2010). TLR activation results in MyD88 recruitment, which is associated with the serine/threonine kinase IRAK⁄IL-1R [IL-1 receptor]-associated kinase. Subsequently, IRAK interacts with the tumour necrosis factor-receptor-associated factor (TRAF) adaptor protein TRAF6 and provides a link to NF-kB-inducing kinase), resulting in phosphorylation of inhibitory kB kinase and subsequent IkB phosphorylation. IkB phosphorylation targets the protein for ubiquitination and proteasome-mediated degradation, resulting in the release and nuclear translocation of NF-kB, whereupon it can influence the expression of numerous immune response genes. In addition to MyD88, alternative adaptor molecules have been identified that initiate responses emanating from TLR3 and TLR4 (Takeuchi and Akira, 2010). These MyD88-independent adaptors include TRIF (TIR (Toll/IL-1R) -domain containing adaptor protein inducing IFN-β) and TRIF-related adaptor molecule, which are pivotal for the expression of IFN-inducible genes following TLR4 activation (Takeuchi and Akira, 2010; Yamamoto et al., 2004; Yamamoto et al., 2003). TRIF is also required for TLR3-mediated signaling in response to dsRNA and is responsible for the induction of type I IFNs (i.e. IFN-α and -β), which are a hallmark of the host innate immune response to viral infection. Following receptor engagement, TLRs initiate signaling cascades that lead to the production of a wide array of pro-inflammatory mediators, including reactive oxygen/nitrogen intermediates, cytokines and chemokines (Aloisi et al., 2001; Tambuyzer et al., 2009; Häusler et al., 2002). In general, the net effect of TLR signaling during CNS infection is to facilitate innate immune activation, as well as to recruit and activate various immune cell populations. However, it is important to acknowledge that the outcomes of TLR signaling in response to endogenous ‘danger’ signals may elicit a

different secretory response tailored to the type of insult. The following discussion is limited to TLRs in CNS health and disease, focusing on the mechanisms and therapeutic potential of TLRs in the brain.

Tab. 1.2 Human TLRs and their principal agonists

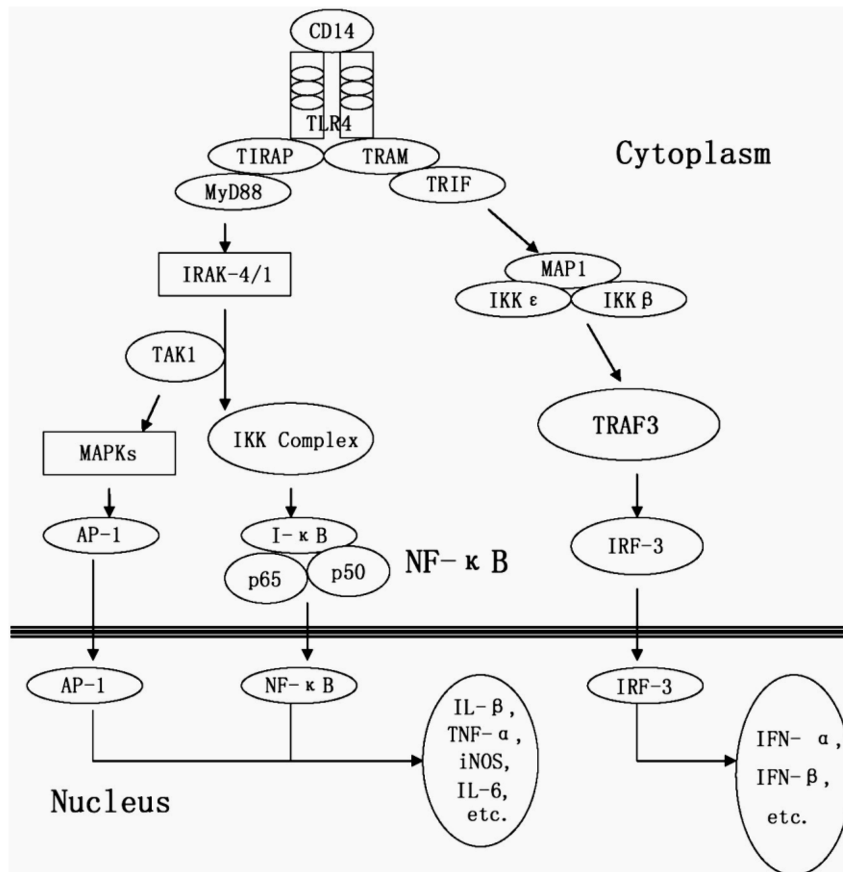
TLR	Agonists	References
1/2	Triacylated lipoproteins and peptidoglycan	Lauw et al., 2005 Yarovinsky et al., 2005 Zhang et al., 2004
2	Lipoproteins and peptidoglycan	
3	dsRNA	Kaisho et al., 2004 Alexopoulou et al., 2001 Takeda et al., 2003
4	LPS	Takeuchi and Akira, 2010
5	Flagellin	Lauw et al., 2005 Yarovinsky et al., 2005 Zhang et al., 2004
6/2	Diacylated lipoproteins and zymosan	
7	ssRNA	Kaisho et al., 2004 Alexopoulou et al., 2001 Takeda et al., 2003
8	ssRNA	
9	Non-methylated CpG-containing ligonucleotide DNA	
11	*Toxoplasma gondii* profiling-like protein and E. coli	Lauw et al., 2005 Yarovinsky et al., 2005 Zhang et al., 2004

1.6.2 TLR4

TLR4 is one of the most actively investigated members of the TLR family. In humans, the gene encoding TLR4 is located on chromosome 9q32-q33, contains 4 exons, and is highly expressed in lymphocytes, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, and splenocytes (Opal et al., 2003; Aderem and Ulevitch, 2000). TLR4 expression is observed in many other types of cells, including epithelial (Yu et al., 2010) and endothelial cells (Lagos et al., 2008). TLR4 is expressed in certain tumor cells, such as human lung cancer cells (He et al., 2007), ovarian cancer cells (Kelly et al., 2006), melanoma cells (Molteni et al., 2006), and head-and-neck squamous cell carcinoma (Szczepanski et al., 2009).

Interestingly, TLR4 expression is reduced in leukocytes during leukemia (Webb et al, 2009) and in cervical cells during cervical intraepithelial neoplasia (Yu et al., 2010).

Fig. 1.6. The TLR4 transduction pathway



Surface TLR4 recognizes LPS from bacterial cell walls and transmits the signal downstream via MyD88-dependent or MyD88-independent pathways, resulting in phosphorylation, dimerization and nuclear translocation of IRF5 and IRF3, and activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways. MyD88, upon binds to TLR4 C-terminal domain by the Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP), and activates the interleukin-1 receptor-associated kinase 1/4 (IRAK1, 4), a serine/threonine kinase that phophorylates the IKK complex to activate NF-kB. IRAK4/1 can activate also TGF-β-activated kinase 1 (TAK1), a MAP3K activated by mitogen and inflammatory stimuli, that can phosphorylate and activate the MAPKs and activator protein 1 (AP-1) transcriptional pathway.
The independent pathways, via TRAM (TRIF-related adaptor molecule) and TRIF, activate the microtubule associated protein 1 (MAP1) and then TNF-receptor-associated factor 3 (TRAF) which, in turn can activate the interferon regulatory factor 3 (IRF3) to induce the transcription of IFN genes.

After binding of microbial ligands, such as LPS (Chow et al., 1999), respiratory syncytial virus fusion protein (Kurt-Jones et al., 2000), or glucuronoxylomannan (Roeder et al., 2004), TLR4 activates downstream transcription factors by various adapter proteins and stimulates production of cytokines and other innate immune response components (Armant et al., 2002; Liew et al., 2005). Besides recognizing microbe-derived ligands, TLR4 binds various endogenous ligands, including heat shock proteins HSP60 (Ohashi et al., 2000), HSP70

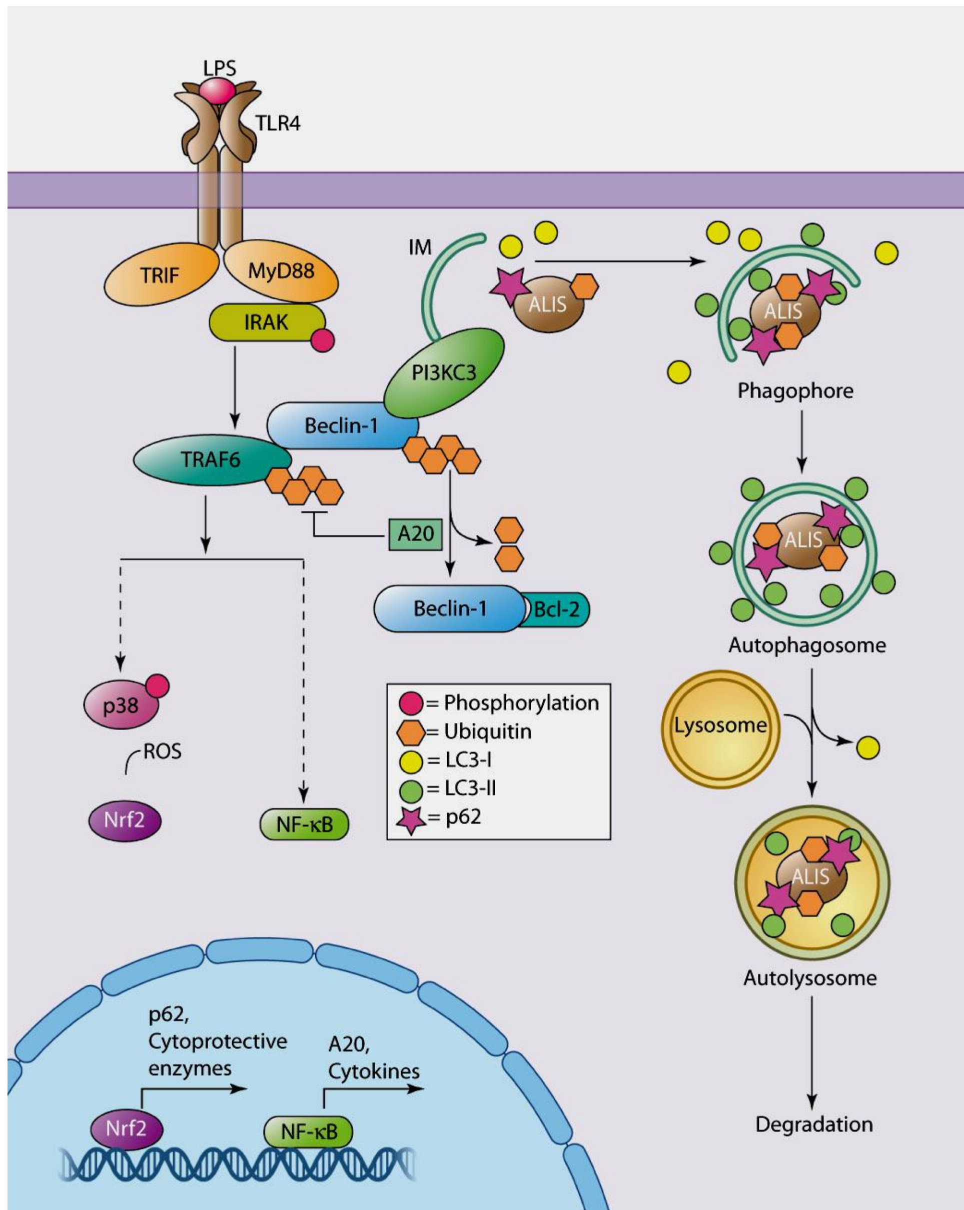
(Vabulas et al., 2002) and gp96 (Warger et al., 2006), β-defensin (Lapteva et al., 2009), Tamm-Horsfall protein (SÅemann et al., 2005), biglycan (Schaefer et al., 2005), fibrinogen (Smiley et al., 2001), surfactant protein (Guillot et al., 2002), low-molecular-weight oligosaccharide fragments of hyaluronan (Termeer et al., 2002), fibronectin (Okamura et al., 2001), and heparin sulfate (Johnson et al., 2002). Hence, the TLR-mediated immune response can be activated in the absence of foreign microbes (Tsan et al., 2002)**.**

The interaction between LPS and TLR4 is a multistep process. LPS is first bound by circulating LPS-binding protein which works as an opsonin for CD14 (Schumann et al., 1990). CD14 catalyzes the binding of LPS to myeloid differentiation protein-2 (MD-2) (Wright et al., 1990). After LPS is transferred to MD-2, this complex interacts directly with TLR4, and formation of a new complex LPS/MD-2/TLR4 occurs (Kim et al., 2007). This, in turn results in dimerization of the TLR4 cytoplasmic domain (Akira and Takeda, 2004), thereby initiating signal transduction (Akira and Takeda, 2004).

Two pathways of downstream signal transduction have been identified (**Fig. 1.6**). The first involves the adapter molecule MyD88, and the second pathway is mediated by the TIR-containing protein and by TRIF domains, as for others TLR receptors. Initiation of the latter pathway leads to activation of IFN regulatory factor 3 (IRF3), and to expression of IFN-inducible genes and IFN-β (Akira and Takeda, 2004). The interaction between TLR4 and antigen-presenting cells results in up-regulation of co-stimulatory molecules such as CD40, CD80, and CD86 on antigen-presenting cells (Pesare et al., 2005), attraction of neutrophils and activated macrophages (or microglia in the CNS) to the inflammation site, T-cell activation, inhibition of regulatory T-cell activity, and activation and maturation of B cells (Rakoff-Nahoum et al., 2009; El-Omar et al., 2008).

TLR4 signaling also regulates autophagy in macrophages, and perhaps in microglia (**Fig. 1.6**, Shi et al., 2010; Xu et al., 2007) by recruiting Beclin-1 to MyD88 as part of the TLR4 signaling complex. Beclin-1 is a component of the PI3KC3 complex and acts as a scaffolding protein to localize other proteins that are necessary for phagophore formation. TRAF6 can be catalyzed the Lys63-linked ubiquitination of the Lys117 residue of Beclin-1, and regulatesTLR4-mediated autophagy in macrophages. Interestingly, Beclin-1 and TRAF6 are both substrates for A20, a ubiquitin-editing NF-kB inhibitor protein, implying that reduced ubiquitination of Beclin-1 occurs through both direct and indirect mechanisms (**Fig. 1.6**) (Shi et al., 2010).

Fig. 1.7. Ub-mediated, selective autophagy in TLR4 signaling–induced macrophages



Activation of TLR4 with LPS enhances the E3 activity of TRAF6, which undergoes auto-ubiquitination and ubiquitinates Beclin-1. Lys[63]-linked polyubiquitination of Beclin-1 in the PI3KC3 complex triggers the nucleation of an isolation membrane (IM), which elongates to form a phagophore. TLR4 signaling also up-regulates p62 through the ROS-p38-Nrf2 pathway and A20 through the NF-kB pathway. A20 modulates TLR4-induced autophagy by de-ubiquitinating Beclin-1 and TRAF6. The interaction of Beclin-1 and Bcl-2 inhibits autophagy, and ubiquitination of Beclin-1 blocks the Bcl-2 binding site. Induction of p62 by nuclear factor erythroid 2-related factor-2 (Nrf2) leads to the formation of aggresome-like induced structures (ALISes) and recruitment of LC3-I. ALISes are engulfed into elongating phagophores by converting cytoplasmic LC3-I to membrane-bound LC3-II. Phagophores matures into autophagosomes, which fuse with lysosomes to form autolysosomes, ultimately degrading the sequestered components (Shi et al., 2010; Fujita et al., 2011). From Sriram et al., 2011.

1.6.3 The major signal adaptor proteins downstream TLR4

1.6.3.1 MyD88 and protein kinase C epsilon (PKCε)

MyD88 is the universal adaptor for TLRs, as well as members of the IL1 receptor subfamily, which share similarities in signaling via the TIR domain (Medzhitov et al., 1998). It was postulated that MyD88 is involved in PKCε activation, as mice lacking PKCε are hypersensitive to both gram negative and gram positive bacterial infection (Castrillo et al., 2001). A key target for PKCε is TRAM, which is required for TLR4 to signal IRF3 activation via TRIF. TRAM undergoes phosphorylation by PKCε on ser16 and this is required for TLR4 to signal (McGettrick et al., 2006). A recent study has revealed a mechanism for the involvement of PKCε in TLR signaling which involves MyD88 (Faisal et al., 2008). Serine 350 of PKCε is first phosphorylated by the c/d isoforms of p38 (Durgan et al., 2008), followed by phosphorylation on serine 346 by glycogen synthase kinase (GSK) and serine 368 by PKC. PKCε is phosphorylated on both sites in response to all TLR ligands (with the exception of TLR3) suggesting the link to MyD88. Knock-down of MyD88 using siRNA in 293-TLR4 cells resulted in a decrease in phosphorylation and prevented PKCε binding to TLR4. Therefore, MyD88 is required for PKCε activation, 14-3-3 binding and for the ability of PKCε to interact with TLR4. Since the TRAM pathway can function in the absence of MyD88 (Oshiumi et al., 2003), it is possible that additional mechanisms exist for PKCε activation in TLR signaling.

1.6.3.2 Mal

Mal acts a bridging adaptor for MyD88, only in the context of TLR2 and TLR4 stimulation (Yamamoto et al., 2002). MyD88, TLR2 and TLR4 are largely electropositive on their surfaces: hence MyD88 is unable to bind these TLRs. Mal on the other hand is mainly electronegative on its surface, allowing it to bind TLR2 and -4 and bring MyD88 into the signaling complex (Dunne et al., 2003). A region in the C-terminus of Mal contains a caspase-1 cleavage site (Miggin et al., 2007). Bioinformatics hinted at a putative caspase-1 cleavage site in Mal at amino acid 198 (an aspartic acid). Treatment of Mal with caspase-1 led to the cleavage of Mal at this position which could be blocked with a caspase-1 inhibitor. The cleavage of Mal is required for its activation in the signaling pathways of TLR2 and -4, pointing to an important synergy between TLRs and nucleotide-binding oligomerization domain-like receptors that activate caspase-1. Activation of nucleotide-binding oligomerization domain-like receptors such as Nalp3 will therefore potentiate signaling by TLR2 and TLR4 via cleavage of Mal (Miggin et al., 2007). Mal becomes phosphorylated on

tyrosines 86 and 106 by Brutons tyrosine kinase. The phosphorylation of Mal on tyrosines 86, 106 and 159 is induced by LPS. Mutations in Mal at these positions result in decreased ability to activate p38 phosphorylation, IkBα degradation and NF-kB activation (Gray et al., 2006; Piao et al., 2008).

1.6.3.3 TRIF

In TLR4 signaling TRIF couples to TLR4 through the bridging adaptor TRAM (Yamamoto et al., 2003). Activation of the MyD88- and TRIF-dependent pathways in TLR4 signaling is thought to be related to the induction of the early (MyD88) and late (TRIF) signaling pathways (Nunez et al., 2007). The relative importance of early and late activation of NF-kB for the ultimate outcome in terms of gene expression changes remains unclear. Curiously, few additional studies have been carried out on TRIF, other than the mapping of domains involved in IRF3 and NF-kB activation (O'Neill et al., 2007).

1.6.3.4 TRAM

TRAM acts as a bridging adaptor allowing activation of the TRIF-dependent pathway in response to LPS (Oshiumi et al., 2003). The downstream effect of TRAM is the activation of IRF3 and late activation of NF-kB. It is regulated by myristoylation (Rowe et al., 2006), which is required to localise it to the plasma membrane, and it is also regulated by PKCε. TLR4 signaling therefore involves all four adaptor proteins with MyD88/Mal believed to be involved in the early LPS responses, with the TRIF/TRAM pathway being initiated at a later stage (Covert et al, 2005). It is yet to be clarified if Mal and TRAM bind to TLR4 in a mutually exclusive manner (Nunez et al., 2007). Two recent studies into TRAM localisation have clarified this to some degree. One study stated that stimulation with LPS led to TLR4 and TRAM co-localisation at the plasma membrane and also recruited TRIF (Tanimura et al., 2008). LPS stimulation then caused co-translocation of TRAM and TLR4 to endosomes where TRAF3 was recruited to TRIF allowing the activation of IFN-β, and it was shown that endocytosis of TLR4 was controlled by dynamin. The ability of TRIF/TRAM to phosphorylate IRF3, with subsequent induction of IFN-β and RANTES was blocked in the presence of dynasore, a dynamin ihibitor, indicating TLR4 must be endocytosed to activate the TRIF-dependent pathway (Kagan et al., 2008). However, dynasore did not affect the localisation of TRAM to the membrane and early endosomes, suggesting that TLR4 and TRAM traffic to endosomes via different routes. TRAM shows a bipartite localisation motif: an N-terminus myristoylation site followed by a polybasic domain. Other proteins that contain

this bipartite motif are involved in signaling at both the plasma membrane and in endosomes. Mutation studies revealed that both regions of this motif are required for plasma membrane localisation, while the myristoylation site is solely needed for endosomal localisation. TRAM mutants lacking the myristoylation site were unable to produce IL6 or RANTES in response to LPS, whereas mutations in the polybasic region did not affect these responses (Kagan et al., 2008).

1.6.3.5 SARM

SARM is the fifth TIR domain-containing adaptor that also contains sterile a-(SAM) and HEAT/armadillo motifs. There are conflicting reports as to its function. SARM is unable to induce NF-kB activation when over-expressed (Liberati et al., 2004), but it can down-regulate the TRIF (but not MyD88) -dependent pathway (Carty et al., 2006). SARM protein mRNA levels were greatly up-regulated after LPS stimulation. Examination of components of the TRIF pathway showed that SARM acted directly on TRIF. Truncated forms of SARM were used to identify the domains in SARM necessary to cause this inhibitory effect. Both the TIR and the SAM domains were proven to be vital. Deletion of the N-terminus of SARM prevented its up-regulation after LPS stimulation, hinting at a domain present in that region involved in the post-transcriptional regulation of SARM. RNA interference of TRAM expression led to enhanced *ifnb* promoter, TNF-α and RANTES expression upon polyI:C and LPS stimulation (Carty et al., 2006).

In the mouse, SARM is mainly localised in brain. Mice expressing GFP-SARM show neuronal localisation and was associated with mitochondria and microtubules (Kim et al., 2007). Truncated forms of SARM revealed that the HEAT/armadillo motif was required for association with mitochondria. SARM interacts with c-Jun N-terminal kinase 3, which is involved in neuronal cell death during stress; this interaction became more pronounced after induction of stress through UV light. As mitochondria and c-Jun N-terminal kinase 3 are involved in apoptosis upon stress in neurons SARM-deficient mice were generated to test its role in this cell death. Neurons from SARM-deficient mice were more resistant to apoptosis induced by oxygen and glucose deprivation than neurons from wild-type mice, demonstrating the role of SARM in inducing stress-related cell death in neurons. Macrophages from these mice responded normally to TLR2, 3, 4 and 9 ligands, indicating no role for SARM in these TLR pathways (Kim et al., 2007).

The role of SARM that is revealed from these studies is not fully clear. On the one hand, SARM is inhibitory for TLR4 signaling, due to its ability to block TRIF activity. On the other

hand, SARM-deficient mice reveal no apparent role for SARM in TLR4 signaling (and its expression appears to be limited to neurons). Further studies are needed to elucidate the exact function of SARM in the TLR pathway.

AIM OF THE THESIS

In the CNS, glial cells not only serve supportive and nutritive roles for neurons, but also respond to protracted stress and insults by up-regulating inflammatory processes. Reactive astrocytes and microglia have been detected in animal models of neuronal injury such as ischemia, axotomy, and neurotoxic insult, and in the human brain in neurodegenerative diseases. Reactive glial cells produce a wide array of pro-inflammatory molecules, including NO, cytokines and chemokines. The complexity of studying glial activation in vivo has led to the widespread adoption of in vitro approaches, for example the use of the bacterial toxin LPS, a ligand for TLR4 as an experimental model of glial activation. In the latter, however, the contribution of microglia, if any, to the response by astrocytes remains an open question, as such astrocyte cultures frequently contain minor numbers of microglia (≤5%) (Saura, 2007).

Primary rodent purified microglia and astrocyte-enriched cultures, prepared mainly from cortical tissue, have been used extensively to study glial cell biology *in vitro*, but their relative influence on each other in response to inflammatory stimuli has not been fully addressed.

To gain an improved understanding of the exact role of the glia in the CNS, we set up an *in vitro* cellular model to analyse selected molecular and cellular parameters involved in astrocyte-microglia interactions in the genesis and maintenance of inflammation and pain. Purified microglia and astrocyte-enriched cultures will be treated with LPS, and will be analysed for the expression of mRNAs of particular significance to pain and inflammation (e.g. iNOS, IL-1β, IL-6, and TNF-α), and for the release of their co-respective mediators. Moreover, the effect of different compounds (peptides involved in pain or anti-depressants) will be studied, in order to evaluate possible pharmacological down-regulation of glia in response to LPS.

In another series of experiments enriched astrocyte cultures will be purified by treatment with L-leucine methylester (L-LME). Although it relatively easy to obtain purified microglial cultures, only recently has it been possible to purify astrocyte cultures *in vitro* and to begin to observe the exact role in different conditions, including inflammation. Two groups purified astrocytes in two different ways, with not obvious results. Hamby and colleagues showed that a unpurified mixed glia culture from CNS tissues, treated in a first time with cytosine-β-d-arabinoside (ara-C), and, in a second time, with L-LME, can lead to a purified astrocyte population (Uliasz et al., 2012; Hamby et al., 2006). Another way is to differentiate, *in vitro*, astrocytes from neural precursor cells grown in fetal calf serum (FCS)-containing cell culture

medium (Welser-Alves et al., 2011). While all cells expressed the astrocytic marker GFAP, they were unresponsive to LPS stimulation.

Using purified microglia and astrocyte-purified cultures with or without the addition of microglia, we will evaluate the interactions between the two types of glia, in terms of expression of a pro-inflammatory phenotype.

The astrocyte/microglia co-culture paradigm described here may provide a useful starting point to elucidate the molecular mechanisms underlying astrocyte- and microglia-specific responses pertaining to, although not limited to, CNS inflammation, especially where TLR activation plays a role.

2. MATERIALS AND METHODS

2.1 Primary culture reagents

Tissue culture media, antibiotics, and FCS were purchased from Life Technologies (U.S.A.); ATP, LPS (E. coli 026:B6), Griess reagent, poly-L-lysine hydrobromide (mol wt 70,000-150,000), rat tail type I collagen solution, papain, DNase I (bovine pancreas), trypsin inhibitor, L-LME and all other biochemicals were purchased from Sigma-Aldrich (U.S.A.) unless noted otherwise; Falcon tissue culture plasticware was purchased from BD Biosciences (U.S.A.). Sterilin petri plastic dishes (10 cm Ø) were from Sarstedt GmbH (Germany). Glass tips and pipettes were sterilized by autoclaving (30 minutes at 121°C under 101.325 kPascal (Pa). Substance P (SP), calcitonin gene related peptide (CGRP) and vasoactive intestinal peptide (VIP) were purchased from Sigma-Aldrich.

2.2 Primary culture of microglia and astrocytes

Rat microglia were isolated from mixed glial cell cultures as previously described (Rosin et al., 2004). Animals were sacrificed in accordance with institutional guidelines. Briefly, cells dissociated from P1-P2 rat pups (strain: CD) cerebral cortices or spinal cord were plated in 75-cm^2 poly-L-lysine-coated tissue culture flasks at a density of 1.5 brains (or 5 spinal cords) per flask and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamine, 50 units/ml penicillin/50 μg/ml streptomycin, 50 μg/ml gentamycin and 10% FCS (glia growth medium). Culture medium was changed after 24 h and then twice per week. Microglia were isolated between days 7-10 by shaking the flasks on an orbital shaker at 200 rpm for 1 h (37°C). The attached cell monolayers were highly enriched in astrocytes (<5% microglia). The culture supernatant (containing mainly microglia) was transferred to plastic Petri dishes (Sterilin) and incubated for 45 min at 37°C (5% CO_2, 95% air) to allow differential adhesion of microglia. The adherent microglial cells (99% pure) were detached by mechanically scraping into glia growth medium and replated in this same medium, on poly-L-lysine-coated microwell culture plates or dishes. In the case of re-addition to microglia-depleted astrocyte cultures, microglia on Sterilin dishes were maintained in growth medium for a further 2 days until harvest.

To eliminate residual microglia, astrocyte monolayers were incubated 1 h with 50 mM L-LME, a lysosomotropic agent (Hamby et al., 2006) dissolved in growth medium. Culture medium was exchanged for fresh medium, and allowed to recover for 1 day in growth medium prior to experimentation. Cultures were visually inspected to ensure microglial lysis. Care must be taken, as longer exposure times can lead to astrocytic toxicity.

2.3 Immunofluorescence

Cortical astrocytes were seeded on poly-L-lysine-coated 12-mm diameter cover-glasses (Menzel-Gläser, Menzel GmbH, Germany) placed in the wells of a 24 well multiwall plate, at a density of 15,000 cells per well in glial cell growth medium and allowed to adhere for 4 days. Later cells were treated with 50 mM L-LME for 1 h, and allowed to recover for 1 day in growth medium. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS), pH 7.4, for 30 min at 4ºC, and washed 4x5 min with PBS/0.05% Triton X-100 (PBS-T, Sigma-Aldrich), 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: p65 (1:250, Santa Cruz Biotechnology, USA) or GFAP (1:000, Sigma-Aldrich, Milan, Italy). Cells were then washed 5x5 min with PBS, and incubated for 1 h at room temperature with anti-mouse-AlexaFluor555 or anti-rat-AlexaFluor488 secondary antibody (1:500, Life Technologies). 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).

2.4 Total RNA extraction

TRIzol® Reagent (Life Technologies) was used for total RNA isolation from cells and tissue, according to the manufacturer's instructions. The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, which maintains RNA integrity during sample homogenization while disrupting cells and dissolving cell components. Cells were lysed in TRIzol for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, and than collected in 1.5 ml tubes. Then chloroform (≥ 99.8% ACS, Sigma-Aldrich) was added to TRIzol in a 1:5 ratio, and the samples shaken manually for 15 seconds, and re-incubated in ice

for 15 min. Samples were then centrifuged at 11,000 g for 15 min at 4°C to separate the mixture into 3 phases: a lower red, phenol-chloroform organic phase containing proteins, an interphase containing DNA, and a colorless upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh vial and RNA precipitated by mixing with an equal volume of isopropyl alcohol (J.T.Baker, U.S.A.). Samples were incubated at 4°C for 15 min and centrifuged at 12,000 g for 15 min at 4°C. RNA appeared as a gel-like pellet, and the supernatant was removed. RNA was washed with 75% ethanol (Carlo Erba Reagenti, Italy) in distilled and autoclaved, RNAse-free water with (0.01% (v/v) diethylpyrocarbonate,(Sigma-Aldrich), and centrifuged at 11,000 g for 15 min at 4°C. Afterwards, RNA was briefly air-dried, then dissolved in RNAse-free water and stored at -80°C until use.

2.5 Agarose gel electrophoresis of RNA for quality determination

Agarose (Ultra pure, electrophoresis grade, Life Technologies) was dissolved by boiling in 1X TAE buffer (0.8 mM Trizma base (Sigma-Aldrich), 20 mM glacial acetic acid (Carlo Erba Reagenti), 0.02 mM EDTA (pH 8.0) (Sigma-Aldrich)). The gel was cast on a gel bed with a suitable comb using a horizontal gel apparatus, and then placed in an electrophoresis tank containing 1X TAE buffer to a level just above the gel surface. For gel electrophoresis, to each RNA sample was added 0.62 mM Ficoll 400 (Sigma Aldrich), a highly branched sucrose and epichlorohydrin copolymer that acts as an electrolyte stabilizer, and 4 µg/ml Orange G sodium salt (Sigma-Aldrich), as tracking dye for nucleic acids. During electrophoresis an electrical potential difference of 6 V/cm was applied to the gel. The gel was then placed on a UV transilluminator for viewing. High molecular weight RNA is visible as a double band, referred to rRNA 18S and 25/28S, without any fluorescent smear directed to the anode (indicative of degradation).

2.6 RNA spectrophotometric quantification

Total RNA yield was determined by measuring spectrophotometric absorption at 260 nm. The absorbance of RNA samples at 260 nm and 280 nm, diluted in sterile distilled water, was used to evaluate protein contamination (A_{260}/A_{280} ratio). A_{260} should be higher than 0.15, with an absorbance of 1 unit at 260 nm corresponding to 40 µg RNA per ml. This relation is valid only for measurements at neutral pH. The A_{260}/A_{280} ratio was determined and used to assess sample purity.

The concentration of purified RNA was calculated using the following equation:

$$[RNA] \text{ in } \mu g/\mu l = (A_{260} \times D)/1000$$

Where 1 optical density unit is equivalent to 40 μg/ml single-stranded RNA and D is the dilution factor.

2.7 Preparation of RNA sample prior to RT-PCR

Duplicate tubes were prepared for the positive and negative reverse transcriptase (RNA) samples used in the amplification reaction. To avoid DNA contamination samples were treated at room temperature for 15 min with deoxyribonuclease I, amplification grade (Life Technologies). To a RNase-free, 0.5-ml microcentrifuge tube were added 8.5 μl RNA sample 1 μl 10X DNase I Reaction Buffer (200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl) 0.5 μl DNase I, amplification grade. DNase I was inactivated by the addition of 1 μl of 25 mM EDTA solution (pH 8.0) (Life Technologies) to the reaction mixture. DNase I was completely denatured by heating at 70°C for 15 min.

2.8 First-Strand cDNA Synthesis

Retrotranscription (RT) reaction mixture was prepared in a final volume of 10 μl. In a nuclease-free microcentrifuge tube were added 250 ng of random primers, 5 mM dNTP Mix (Life Technologies), and 5 μg of total RNA. The mixture was heated to 65°C for 6 min and incubated on ice for at least 1 min for primer annealing. Then the reaction was performed in 1X First-Strand Buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2), 1 mM dithiothreitol, 10 units of RNase OUT Recombinant Ribonuclease Inhibitor, and 50 units of SuperScript™ III Reverse Transcriptase (Life Technologies). SuperScript™ III Reverse Transcriptase is an engineered version of M-MLV (Moloney Murine Leukemia Virus) RT, with RNase H activity and an increased thermal stability. RT reaction was performed at 50°C for 70 min and inactivated by heating at 75°C for 15 min. cDNA was stored at -20°C until use.

2.9 Amplificability of cDNA samples

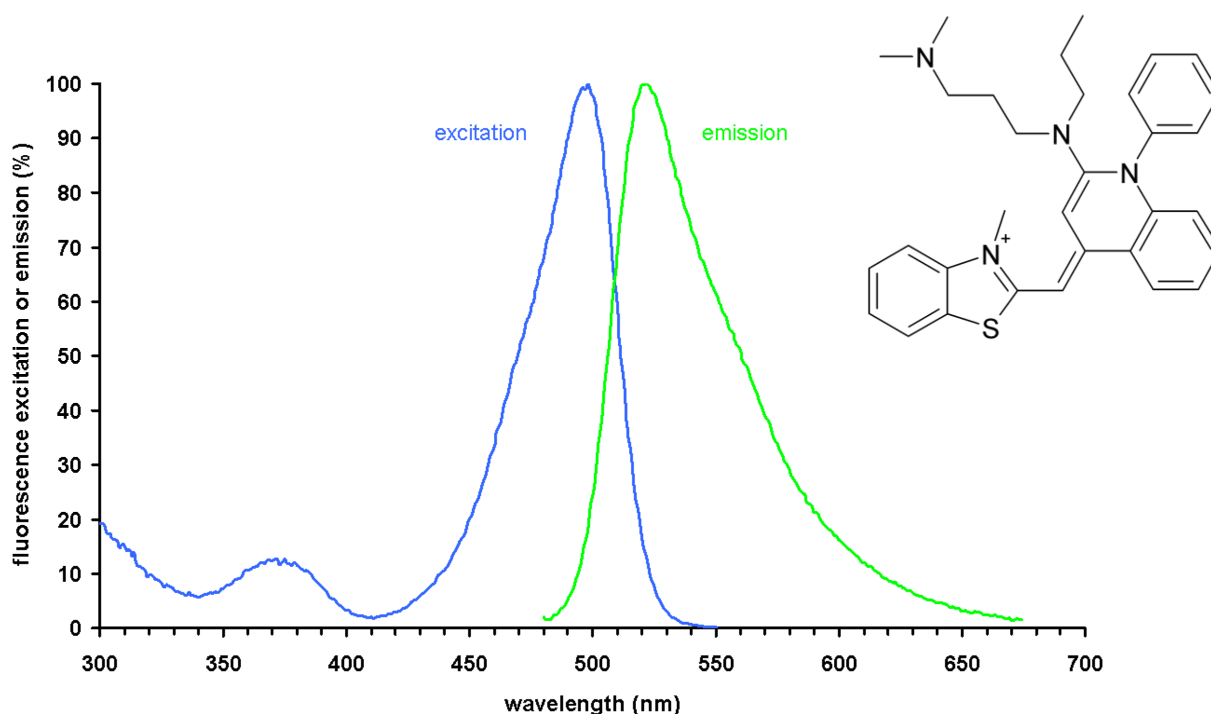
Many substances are reported as PCR inhibitors, including hemoglobin, urea, humic acid, citric acid, polysaccharides, bilirubin, heparin, residual reagents used during DNA or RNA extraction, chelating agents (EDTA) and detergents (Triton X-100, sodium dodecyl phosphate), among others (Wilson, 1997). In order to verify the amplificability of all RNA samples, a prior PCR amplification of a fragment of the complement DNA (cDNA) of the messenger of GAPDH gene were made. Non-amplified samples were re-extracted or further

purified in TRIzol, and the amplification of the GAPDH housekeeping gene was repeated. Primers are shown in **Table 2.1**. PCR conditions included 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 72°C for 30 s. At the end, the mix is maintained at 72°C for 7 min, and then cooled to 25°C until reaction stop. Amplification was performed using 0.5 µl of cDNA sample in a reaction mix containing 500 nM of each primer (Sigma-Aldrich), Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7, 800 mM deoxynucleotide triphosphates (Life Technologies), and 0.5 unit of HotStarTaq Polymerase (Qiagen, U.S.A.) in a final volume of 10 µl. The final PCR products were detected by agarose gel electrophoresis and stained with 1.6 % GelRed (Biotium Inc., U.S.A.).

2.10 qPCR: methods and principles

The qPCR technology allows one to quantitfy a DNA or RNA template copy number with high reproducibility. This technology uses a traditional primer pair or a fluorescence-marked oligonucleotide or a fluorescence dye that stains double-stranded DNA, and a Taq DNA polymerase that retains 5’→3’ exonuclease activity (Heid et al., 1996). The Sybr Green I fluorescence probe (**Fig. 1**) was used here, which is an asymmetrical cyanine dye (Zipper et al., 2004) less mutagenic than ethidium bromide (Ames test). However, anything capable of binding DNA with high affinity is a possible carcinogen, including SYBR. Moreover, Sybr Green I readily penetrates living cells, whereas ethidium bromide is efficiently excluded (Singer VL et al., 1999). The resulting DNA-Sybr Green complex absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm – **Fig. 2.1**). The stain preferentially binds to double-stranded DNA, but will stain single-stranded DNA with lower performance. SYBR green can also stain RNA with a lower performance than DNA. Taq DNA polymerase, used for the amplification reactions, is an engineered enzyme, relatively stable and inactive at room temperature, and fully activated only after the denaturation temperature is reached (Hot Start PCR). This change is not fundamental for PCR, but minimizes the risk of nonspecific amplification.

Fig. 2.1 Spectrogram and molecular structure of SYBR Green I



2.11 Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

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 (Life Technologies), 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 (Life Technologies). The qRT-PCR is a real time PCR reaction, 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 (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each standard dNTP, stabilizers, 0.05 u/μl Taq DNA Polymerase, JumpStart Taq antibody, and SYBR Green I, Sigma-Aldrich). At room temperature, JumpStart Taq is inactivated by an antibody; heating at 70°C or more, in the first denaturation step of the cycling process, dissociates the complex and the DNA polymerase becomes fully active. In order to normalize mix distribution due to errors in pipetting, the mix contains the internal

Table 2.1 Selected Primers for this study

Primer	Direction	Sequence
GAPDH	F	5'-CAAGGTCATCCATGACAACTTTG-3'
	R	5'-GGGCCATCCACAGTCTTCTG-3'
β-ACT	F	5'-CCCCATTGAACACGGCATTGTCA-3'
	R	5'-ACCCTCATAGATGGGCACAGTGT-3'
IL-1β	F	5'-TGTGGCAGCTACCTATGTCT-3'
	R	5'-GGGAACATCACACACTAGCA-3'
iNOS	F	5'-CACACAGCGCTACAACATCC-3'
	R	5'-CCATGATGGTCACATTCTGC-3' R
TNF-α	F	5'-CATCTTCTCAAAACTCGAGTGACAA-3'
	R	5'-TGGGAGTAGATAAGGTACAGCCC-3'
IL-6	F	5'-TCACAGAAGGAGTGGCTAAGG-3'
	R	5'-GCTTAGGCATAGCACACTAGG-3'
IL-10	F	5'-ACCATGGCCCAGAAATCAAG-3'
	R	5'-GGGGAGAAATCGATGACAGC-3'
Iba1	F	5'-AACTGGAGGCCTTCAAGACG-3'
	R	5'-AACCCCAAGTTTCTCCAGCA-3'
galC	F	5'-AGCCCTTCCCAACCAGCTAT-3'
	R	5'-TCTGAACGCATGCTCAAGGT-3'
TLR2	F	5'-TCCATGTCCTGGTTGACTGG-3'
	R	5'-AGGAGAAGGGCACAGCAGAC-3'
TLR4	F	5'-GATTGCTCAGACATGGCAGTTTC-3'
	R	5'-CACTCGAGGTAGGTGTTTCTGCTAA-3'
CD14	F	5'-TATGCTCGGCTTGTTGCTGT-3'
	R	5'-GTTGCAGTAGCAGCGGACAC-3'
GFAP	F	5'-TGGAGGTGGAGAGGGACAAT-3'
	R	5'-AGATCCACACGAGCCAAGGT-3'
β-III tub	F	5'-AGCAGTACCAGGACGCCACT-3'
	R	5'-CCAGCTGCAAGCAACTTCAC-3'
vWF	F	5'-GAGTCCCAATGACCCTCAGC-3'
	R	5'-TCAACATATGGGGTGGTGGA-3'
Mcam	F	5'-CACCCTCACACCTGACTCCA-3'
	R	5'-ATCACAGCCACGATGACCAC-3'

GAPDH, gliceraldeide 3-phosphate dehidrogenase; β-ACT, β-actin; IL-1β, intereukin-1β; iNOS, inducible nitric synthase; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; Iba1,_ionized calcium binding adaptor molecule 1; galC, galactosylceramidase; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; CD14 and MD2 (), TLR4 co-receptor protein; GFAP, glial fibrillary acid protein; β-III tub, β-III tubulin; vWF, von Willebrand factor; Mcam, melanoma cell adhesion molecule. F, forward; R, reverse.

reference dye 6-carboxy-X-rhodamine. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm.

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 55°C for GAPDH primers and 60°C for all other primers, for 30 s, and extension at 72°C for 1 min, followed by a dissociation thermal profile of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Primer pairs used are shown in **Table 2.1**: 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.

2.12 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.

2.13 NO Assay

NO has a relatively short half-life. 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. Equal volumes of cell culture medium and Griess reagent (Sigma-Aldrich) were incubated for 15 min, and the amount of nitrite quantified using a standard curve of sodium nitrite at O.D. 540 nm.

2.14 Cytokine enzyme-linked immunosorbent assay (ELISA) Assays

Cells were stimulated to release pro-inflammatory mediators in medium containing the indicated concentration of LPS. Cell supernatants were harvested after 24 h and cytokine release was assayed by ELISA according to the manufacturer's instructions (IL-1β and TNF-α: Korma Biotech, Korea) (IL-6: Bio Scientific, Texas). The IL-1β ELISA assay kit does not distinguish between the inactive 33-kDa precursor (pro-IL-1β) and the bioactive 17-kDa mature form (as is the case for all commercially available kits), but the specificity for the

mature form versus the pro-IL-1β is between 15:1 and 20:1, respectively (personal communication by Rolan Stall, Lundbeak, U.S.A.).

2.15 Statistics

Data are given as mean ± s.e.m. (standard error of the mean). 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 test for comparisons involving more than two data groups.

3. RESULTS

3.1 Optimization of "real time" PCR technique

In order to analyze the transcriptional course of cortical and spinal cord microglia and astrocyte cultures stimulated with LPS or other compounds, we optimized the real time PCR conditions. The tested genes included inflammatory gene transcripts (IL-1β, IL-6, IL-10, TNF-α, iNOS), genes used to characterize microglia and astrocytes (GFAP, Iba1, integrin alpha M or ITGAM, galactosylcerebroside, β-III tubulin, melanoma cell adhesion molecule, von Willebrand factor, TLR4, CD14), and genes for normalization of data (GAPDH and β-actin). Reaction efficiency was evaluated for each qPCR reaction. This parameter assesses the ability of Taq DNA polymerase to identify the DNA template, and to amplify it completely. It varies from 0 to 1. Maximum efficiency is reached when the reaction has the highest speed and the greatest amount of amplification. To calculate the efficiency of PCR it is necessary to prepare a set of progressive standard DNA dilutions and, for each dilution, to evaluate at which PCR cycle a detection of fluorescence is achieved. Most notably, the PCR curves are sigmoids: after a starting latent period, the reactions reach the exponential phase, in which the PCR proceeds at a high and constant speed. After a plateau is reached the reaction slows, until coming to a complete stop. The best point for quantification is when PCR is in the exponential phase, because the PCR product is proportional to the initial template DNA, according to the formula: $N_{(t)} = N_{(0)}(1+E)^t$, were $N_{(t)}$ is the number of amplified molecules at time t, $N_{(0)}$ the number of molecules at time 0, t the time (PCR cycle), and E the reaction efficiency. At the end of PCR, for each reaction, a threshold cycle (Ct) is chosen. A two-dimensional plot of Ct defines a logarithmic curve, according to the formula: $Ct = m(Log_{10}N_{(t)}) + n$, in which m and n are constants. The relationship can be linearized by placing $X_{(t)} = Log_{10}N_{(t)}$ to get: $Ct = mX_{(t)} + n$.

Where m is the slope of the line, and n is the intercept with the Ct axis. The slope is directly correlated to the following formula: $E = 10^{-1/m} - 1$. The maximum efficiency is achieved when the primer pairs range between 100 and 200 nM per reaction, as described in Materials and Methods.

In order to obtain a more robust normalizing gene transcript system, we prepared purified microglia and enriched astrocyte cell cultures from cortex and spinal cord from 2-day-old rat pups. Cultures were stimulated with different LPS concentrations (0.001-10 ng/ml) for 6 h. Samples were prepared for qPCR, as described in Materials and Methods, and then quantified

by normalizing data related to the expression of GAPDH or β-actin mRNAs. Results for IL-1β in cortical glial cells are shown in **Table 3.1**.

There are no significant differences in the relative quantitation of IL-1β, and similar results where obtained for the other inflammatory gene transcripts (iNOS, TNF-α). Hence, for all following PCR quantitations we decided to use the expression of GAPDH mRNA.

Table 3.1 Comparison between GAPDH and β-actin normalization

		GAPDH		β-actin	
		Microglia	Astrocytes	Microglia	Astrocytes
CTR		100 ± 10	100 ± 18	100 ± 8	100 ± 11
LPS	0.001	109 ± 7	89 ± 8	119 ± 9	124 ± 16
	0.01	111 ± 16	67 ± 26	55 ± 19	123 ± 30
	0.1	407 ± 59	97 ± 19	247 ± 85	173 ± 80
	1	3719 ± 1157	3060 ± 671	5019 ± 1298	5704 ± 738
	10	2090 ± 463	27318 ± 2861	2016 ± 539	29463 ± 5897

Purified microglia and enriched astrocyte 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 GAPDH or β-actin levels, and are representative of 3 experiments. Qualitatively similar results were obtained with spinal cord microglia and astrocytes.

3.2 Characterization of Microglia and Astrocyte Cultures

Purified cortical microglia expressed high levels of mRNA for the macrophage/microglia protein Iba1 relative to astrocyte-enriched cultures (purified microglia 14245 ± 1260 % vs enriched astrocytes 242 ± 109; **Fig. 3.1a**), when expressed as a ratio to the astrocyte-specific gene GFAP. In addition, both cultures express the TLR4 gene with or without LPS treatment (**Fig 3.1b**). At the higher concentration of LPS (100 ng/ml) there is a reduction of the TLR4 transcription in microglia, which could reflect either down-regulation and/or reduced cell vitality due to the very strong stimulus. Astrocytes express lower levels of TLR4, which does not appear to be affected by LPS treatment. Similar results were obtained for spinal cord microglia and astrocytes (data not shown).

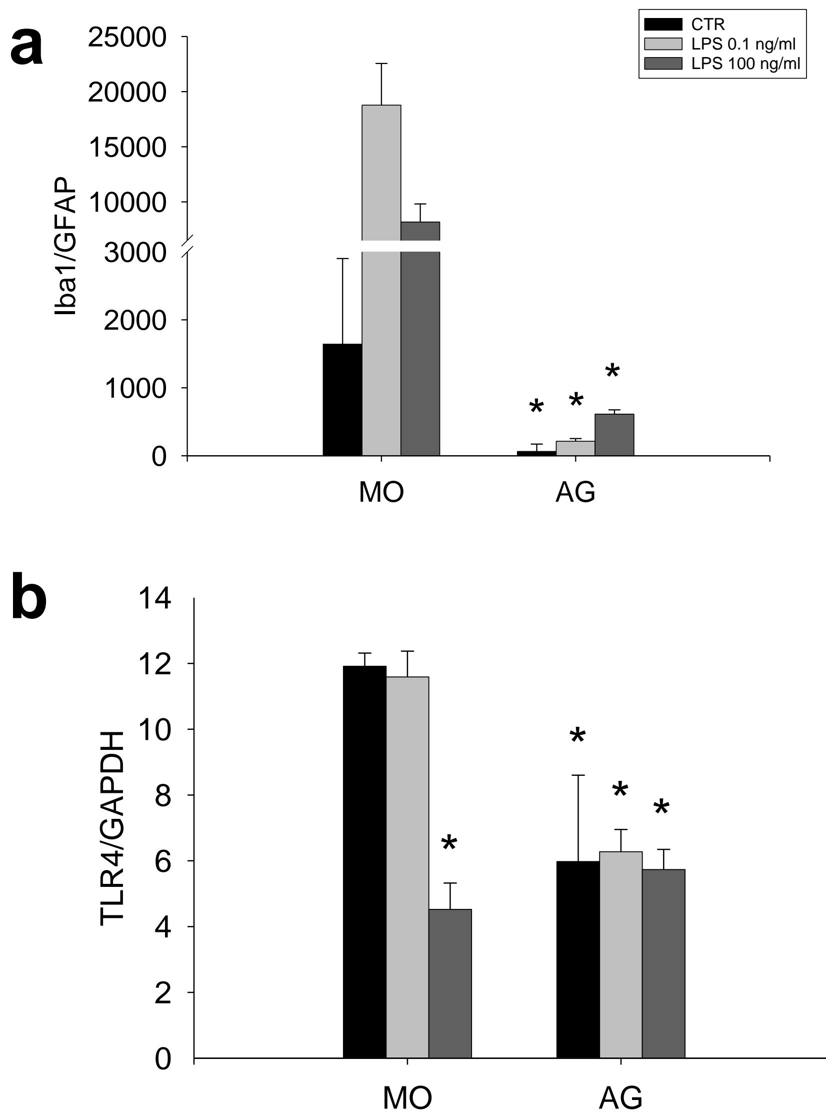
In enriched astrocyte cultures, remaining microglia express Iba1 and TLR4, instead astrocytes express GFAP and TLR4 protein (**Fig. 3.2**). Astocyte expression of TLR4 appears to be less robust than for microglia.

3.3 iNOS, IL-1β, and TNF-α mRNAs expression in cortical mixed astrocyte and microglia cell cultures

In order to evaluate induction of the transcription of pro-inflammatory genes (iNOS, IL-1β, TNF-α), we prepared mixed glial cell cultures from cortex and spinal cord of 2-day-old rats. Results for mixed cortical glial culture are shown in **Fig. 3.3a**. Similar results are obtained with cultures from spinal cord (data not shown). Cultures were stimulated with 100 ng/ml of LPS with or without 10 ng/ml of IFNγ for 4, 6, and 24 h. iNOS, IL-1β and TNF-α transcripts are up-regulated after treatment versus control. IFNγ is used to enhance the response to LPS. mRNAs have a peak of induction at 4 h of treatment (1359702 ± 255000 % vs CTR ± 5 % for iNOS; 21088 ± 2555 % vs CTR ± 7 % for IL-1β; 11262 ± 5568 % vs CTR ± 3 %), while at longer times their values decrease. At 24 h all three mRNAs are still up-regulated (332481 ± 50000 % vs CTR ± 5 % for iNOS; 13059 ± 6541 % vs CTR ± 7 % for IL-1β; 363 ± 89 % vs CTR ± 3 %). In these culture conditions, it seems that LPS is responsible for almost full transcriptional activation of these three genes in astrocytes and microglia. Adding 10 ng/ml of IFNγ appears not to produce an additive effect, as reported in the literature (Sheng et al., 2011; Kao et al., 2010; Kitamura et al., 1998).

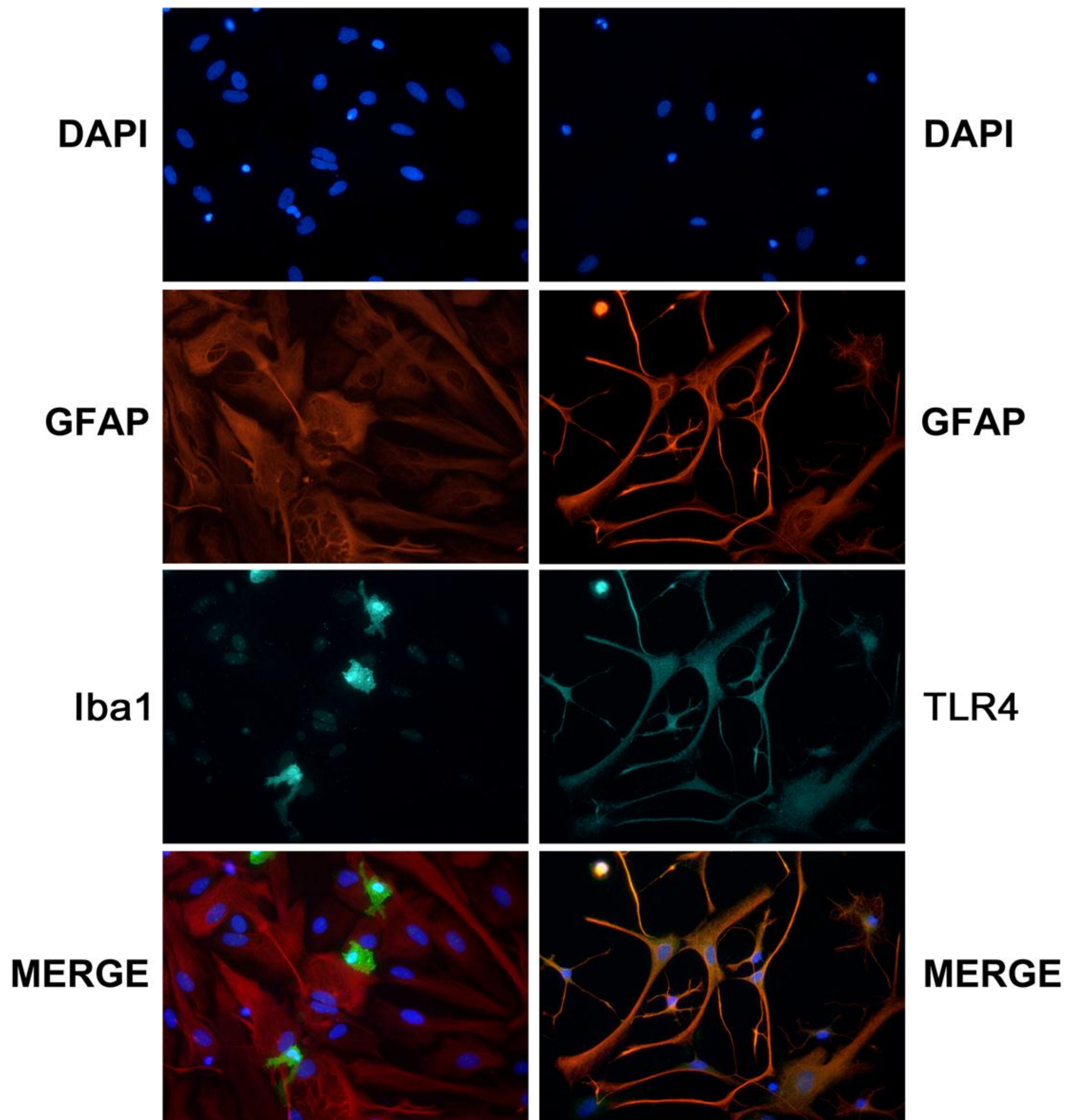
Next, mixed cortical and spinal cord cultures were treated for 6 h with different concentrations of LPS (0.1, 1, 10, 30, 100 ng/ml) and transcription levels determined. **Figure 3.3b** reports the three most effective concentrations (spinal cord cultures have similar results). iNOS, IL-1β and TNF-α transcripts are up-regulated at higher levels with 10 ng/ml (1357489 ± 255000 % vs CTR ± 6 % for iNOS; 19275 ± 2546 % vs CTR ± 4 % for IL-1β; 10281 ± 5420 % vs CTR ± 4 %), and did not change significantly with the remaining two higher concentrations. In these conditions iNOS mRNA appears to be the major up-regulated transcript after LPS or LPS/IFNγ treatments. It should be noted that iNOS mRNA expression without LPS or LPS/IFNγ treatment was very low or undetectable, while IL-1β and TNF-α transcription levels were low but still measurable.

Fig. 3.1 Iba1 and TLR4 mRNA expression in purified microglia and enriched astrocytes



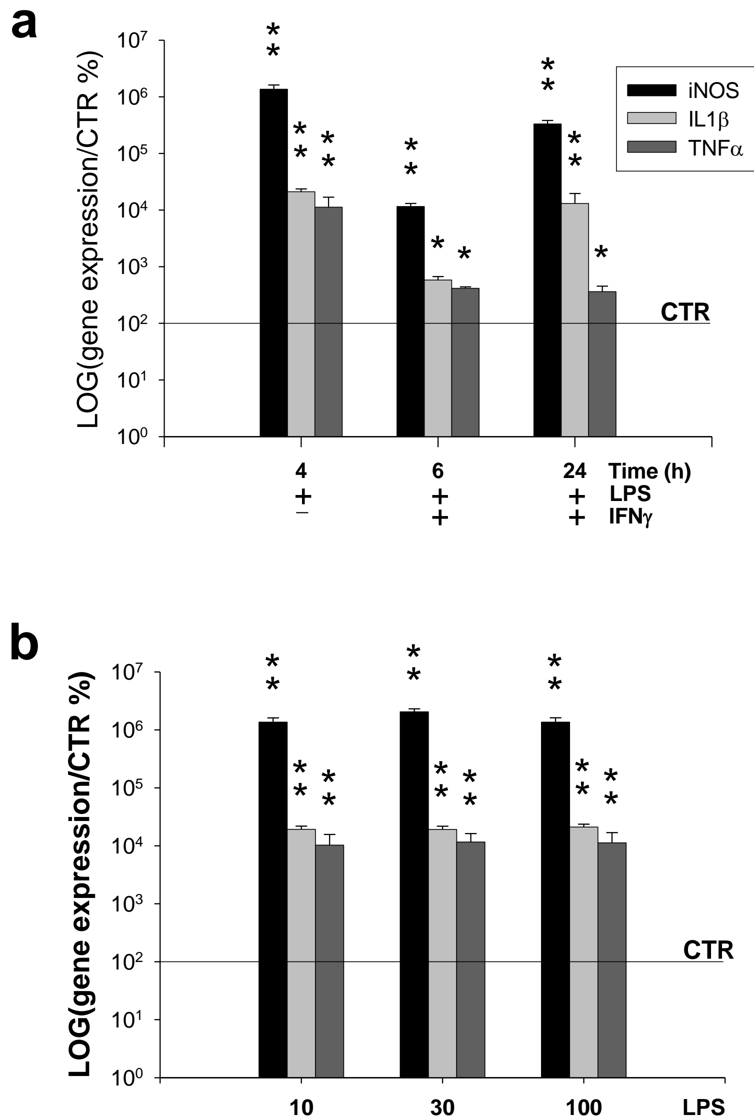
Cortical microglia express high levels of Iba1 mRNA relative to astrocytes. Purified microglia (MO) and enriched astrocyte (AG) cell populations prepared from neonatal rat cortex were incubated with the indicated concentrations of LPS for 6 h, and then processed for RT-PCR analysis. Data, given as means ± 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. *$p<0.05$ vs CTR (Anova and Bonferroni exact test).

Fig. 3.2 Iba1 and TLR4 protein expression in enriched astrocytes



Cortical enriched astrocyte cell populations prepared from neonatal rat cortex were marked for DAPI (nuclei), GFAP (astrocytes), Iba1 (microglia), and TLR4. Qualitatively similar results were obtained with spinal cord astrocytes.

Fig. 3.3 Up-regulation of iNOS, IL-1β and TNF-α mRNAs in mixed cultures of astrocytes and microglia after LPS/IFNγ treatment



LPS and LPS/IFNγ induce pro-inflammatory gene expression in microglia and astrocyte mixed cultures prepared from neonatal rat cortex. a) Cultures were incubated with 100 ng/ml LPS or 100 ng/ml LPS and 10 ng/ml IFNγ for 4, 6, 24 h, and then processed for RT-PCR analysis of iNOS, IL-1β, and TNF-α mRNA. b) Cultures were incubated with 10, 30, and 100 ng/ml LPS for 6 h, and then processed for RT-PCR analysis of iNOS, IL-1β, and TNF-α mRNA. Data are means ± sem (duplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. *$p<0.05$ and **$p<0.001$ vs CTR (Anova and Bonferroni exact test).

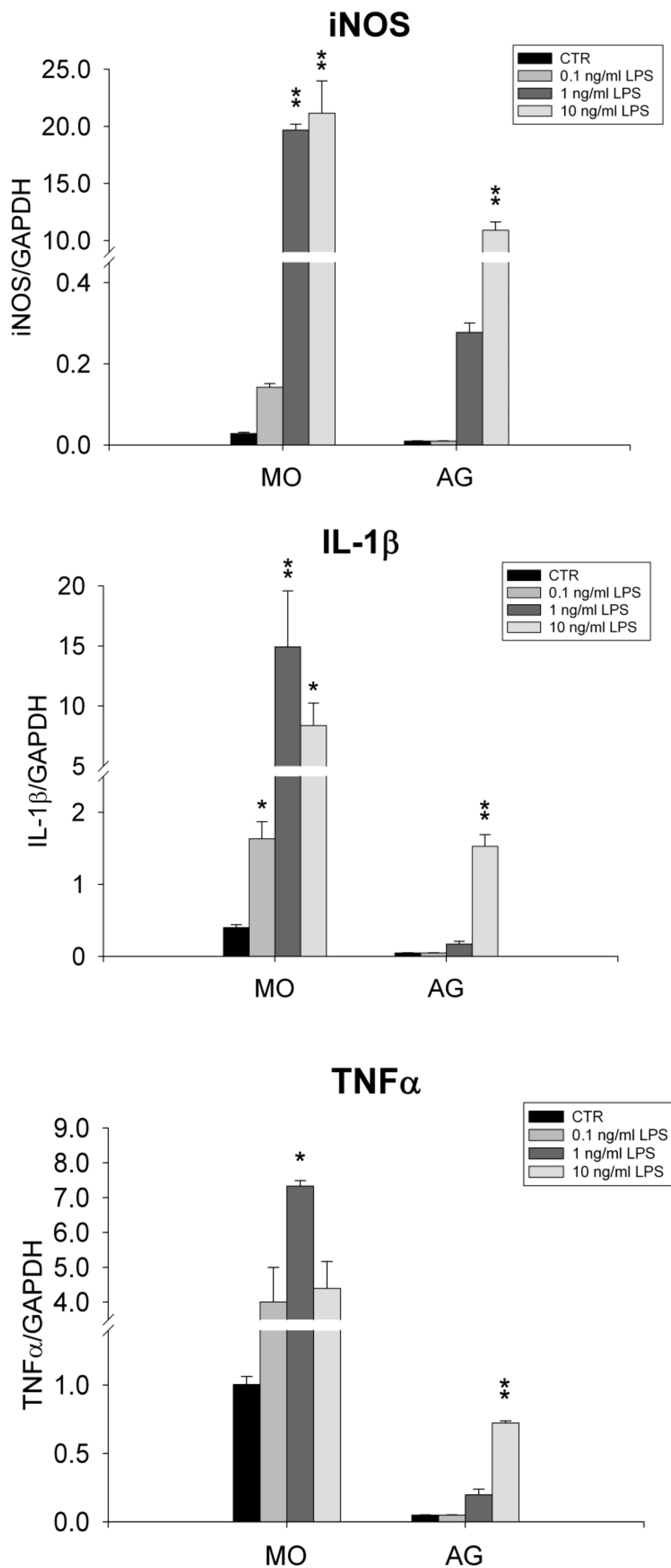
3.4 iNOS, IL1-β, and TNF-α mRNAs expression in cortical purified microglia and enriched astrocyte cell cultures

In order to evaluate the induction of pro-inflammatory gene transcripts (iNOS, IL-1β, and TNF-α), we prepared purified microglia and enriched astrocyte cell cultures from cortex and spinal cord of 2-day-old rat pups. Results for cortical glial cultures are shown in **Fig. 3.4**. Cultures were stimulated with 0.1, 1, and 10 ng/ml of LPS 6 h. Addition of IFNγ was omitted, because we did not observe additional effects with LPS. iNOS, IL-1β and TNF-α transcripts are up-regulated after treatment versus control. In microglia cultures, mRNAs have a peak of induction at 1 ng/ml of LPS (19.68 ± 0.04 vs CTR 0.030 ± 0.003 for iNOS; 14.93 ± 4.65 vs CTR 0.40 ± 0.04 for IL-1β; ± vs CTR ±; 7.33 ± 0.16 vs CTR 1.04 ± 0.06 for TNF-α), while astrocytes required a 10-fold greater concentration (10.92 ± 0.71 vs CTR 0.010 ± 0.001 for iNOS; 1.53 ± 0.16 vs CTR 0.050 ± 0.001 for IL-1β; 0.72 ± 0.01 vs CTR 0.050 ± 0.001 for TNF-α). Similar results are obtained with cultures from spinal cord (data not shown). LPS also stimulated, in a concentration-dependent manner the output of NO and IL-1β from both cortical and spinal cord microglia and astrocytes, with microglia again being more responsive (for cortical cultures, see **Fig. 3.5**). The colorimetric cell vitality assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Manthorpe et al., 1986, Mosmann, 1983) did not show differences in cell viability at 24 h of treatment, versus control at the same LPS concentrations (data not shown).

3.5 SP, VIP and CGRP treatments of cortical purified microglia and enriched astrocyte cell cultures

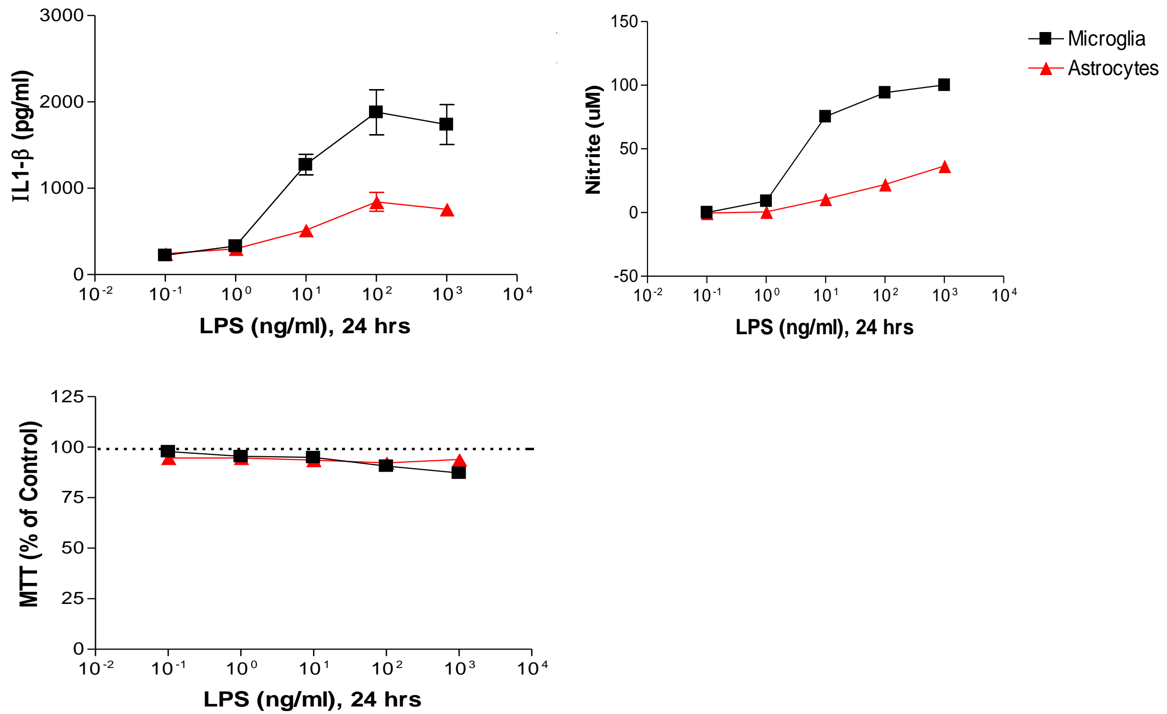
In order to evaluate the induction of pro-inflammatory gene transcripts (iNOS, IL-1β, and TNF-α) after treatment with three peptides involved in pain transmission (SP, VIP, and CGRP), we prepared purified microglia and enriched astrocyte cell cultures from cortex and spinal cord of 2-day-old rat pups. Results for cortical glial cultures are shown in **Fig. 3.6**. Cultures were stimulated with 10-500 ng/ml of LPS for 6 h as positive control, and/or 10 nM SP, or 1 μM VIP, or 10 and 100 nM CGRP. Peptide and LPS concentrations were chosen based upon previously published data, which reported that SP and CGRP increase transcription and release of inflammatory factors (Priller et al., 1995; Martin et al., 1993; Lubed-Narod et al., 1994; Zhou et al., 2009), and VIP promotes release of astroglia-derived factors, such as IL-1, IL-6, chemokines, neurotrophin-3, nexin-1, RANTES, activity-dependent neurotrophic factor

Fig. 3.4 iNOS, IL-1β, and TNF-α expression in purified microglia and enriched astrocyte cultures



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, IL-1β, and TNF-α mRNAs. Data are means ± sem (duplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. *$p<0.05$ and **$p<0.01$ vs CTR (Anova and Bonferroni exact test).

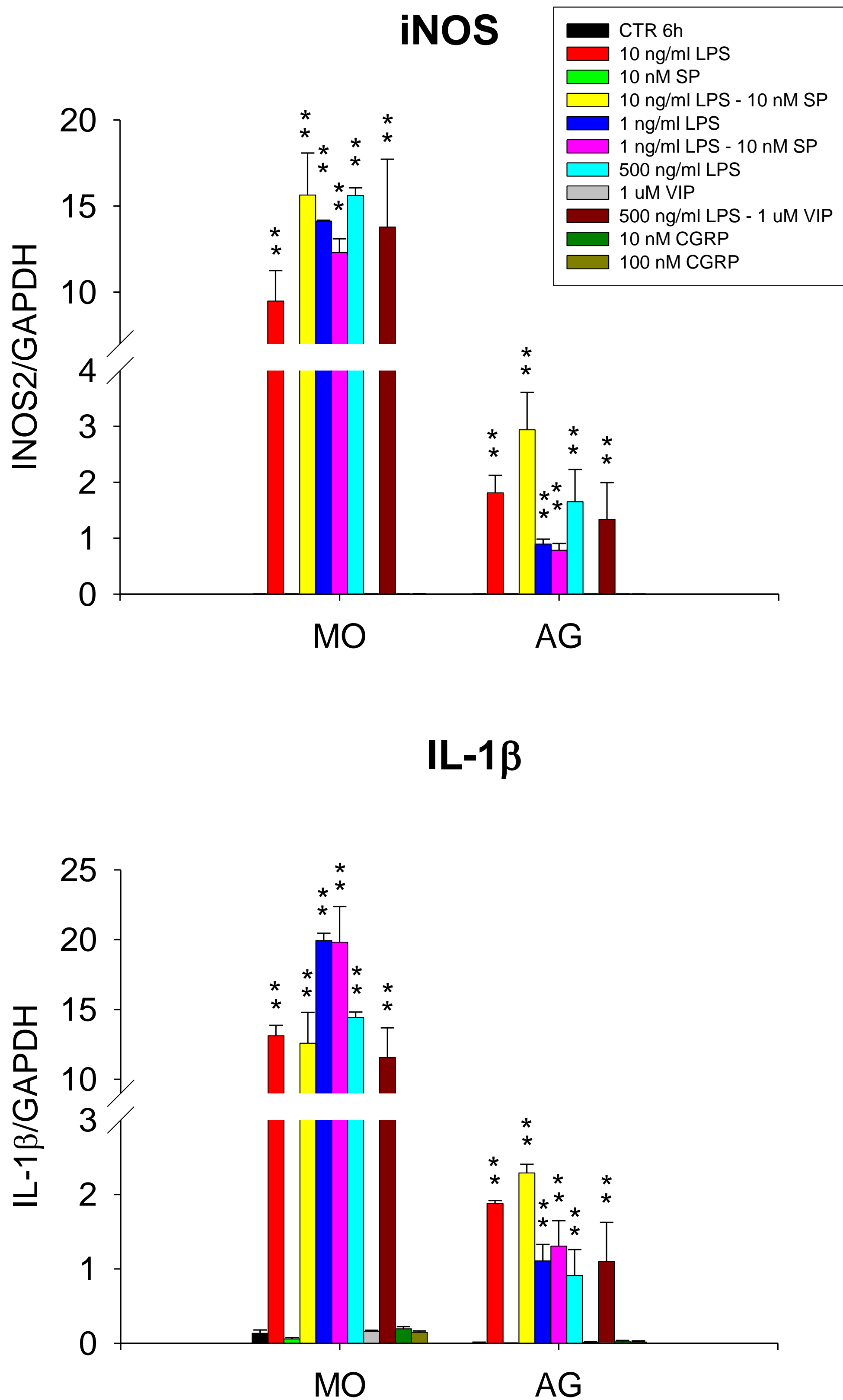
Fig. 3.5 Effect of LPS concentration on mediator release and MTT in microglia and enriched astrocyte cultures

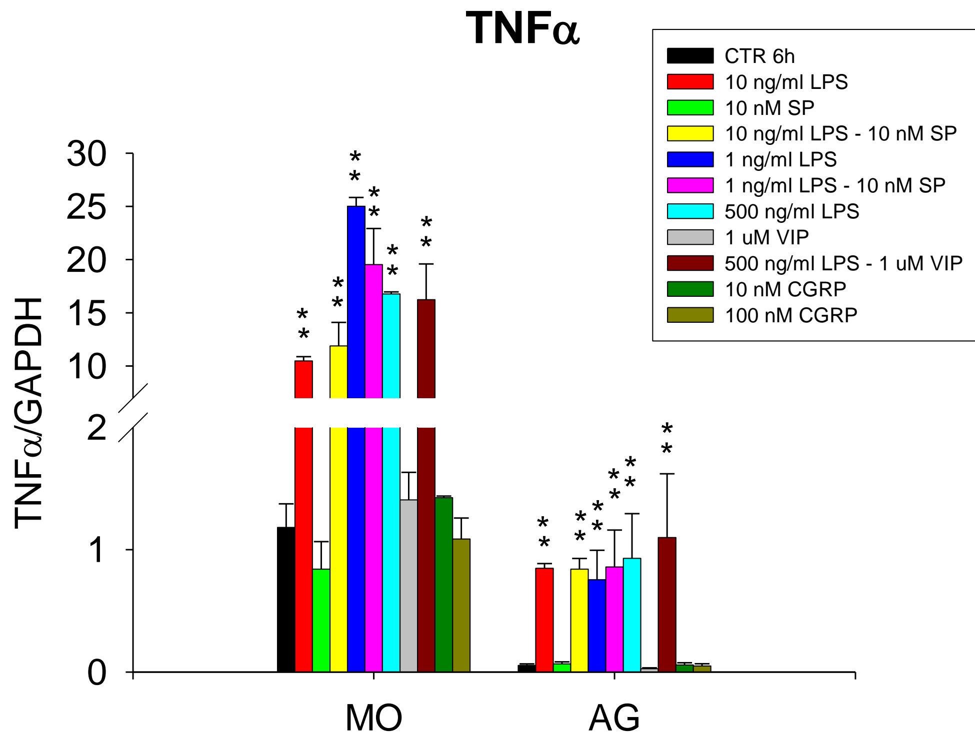


LPS induces release of NO and IL-1β from cortical microglia and astrocytes. Purified microglia and enriched astrocyte cell populations prepared from neonatal rat cortex were incubated with the indicated concentrations of LPS for 24 h, and analysis of NO and IL-1β was performed by ELISA. Data are means ± sem (triplicate culture wells), and are representative of 3 experiments.

and activity-dependent neuroprotector homeobox (Brenneman et al., 1998), or blocks microglia activation and production of TNF-α, IL-1β, and NO (Delgado & Ganea, 2003). Unlike these data, in our culture conditions microglia and enriched astrocytes where not affected by peptide treatment with or without LPS addition. Similar results are obtained with cultures from spinal cord (data not shown). Peptides do not modify the output of NO and IL-1β at 24 h, from either cortical or spinal cord microglia and astrocytes. The MTT test did not show differences in cell viability at 24 h of treatment, versus control at the same concentrations of LPS (data not shown).

Fig. 3.6 Effects of SP, VIP and CGRP treatment on iNOS, IL-1β and TNF-α mRNA expression in purified microglia and enriched astrocyte cultures





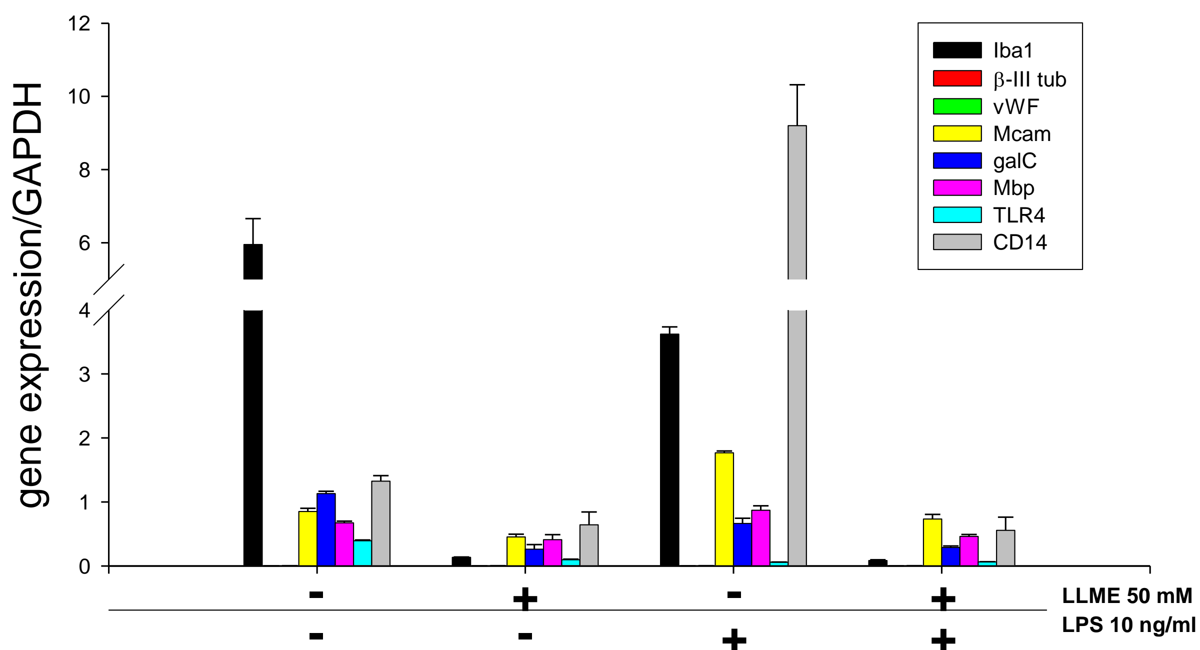
LPS, but not SP, VIP, or CGRP induces pro-inflammatory gene expression in cortical microglia and astrocytes. Purified microglia (MO) and enriched astrocyte (AG) cell populations prepared from neonatal rat cortex were incubated with the indicated concentrations of LPS or/and peptides for 6 h, and then processed for RT-PCR analysis of iNOS, IL-1β, and TNF-α mRNAs. Data are means ± sem (duplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. **$p<0.01$ vs CTR (Anova and Bonferroni exact test).

3.6 Characterization of purified Astrocyte Cultures

A large number of studies have been published using astroglial-enriched cultures to describe the expression of a variety of molecules, e.g. cytokines, chemokines, and adhesion molecules. Many such reports assume that the astrocyte is the cell type causing the observed effect (Saura, 2007). This assumption may sometimes be incorrect, as shown for iNOS (Solà et al., 2002) and apolipoprotein E (Saura et al., 2003) expression. To more fully characterize the role of microglia in our cultures, a specific microglia toxin was used. L-LME, a lysosomotropic agent originally used to selectively destroy macrophages (Thiele et al., 1983), has also been employed to eradicate microglia from neural cell cultures including astrocytes (Giulian et al., 1993; Guillemin et al., 1997; Hamby et al., 2006) and oligodendrocytes (Hewett et al., 1999). Confluent enriched astrocyte monolayers were treated by brief (60 min) exposure to a high concentration (50 mM) of L-LME (cf. Hamby et al., 2006) followed 24 h later by a 6-h challenge with LPS. Both enriched and purified astrocyte cultures were then examined for the

presence of transcript markers of: microglia (Iba1), astrocytes (GFAP), neurons (β-III tubulin), oligodendrocytes (galactosylcerebroside), schwann cells (myelin basic protein), endothelial cells (von Willebrand factor, and melanoma cell adhesion molecule), as well as for the presence of TLR4 and CD14, a protein involved in LPS-TLR4 binding. The cultures were stimulated with 10 ng/ml LPS for 1 h after L-LME treatment. Astrocyte cultures show a negligible expression of endothelium and neuron transcripts while presenting detectable levels of oligodendrocyte and Schwann cell markers, as described by others (Albuquerque et al., 2009). However, the absolute numbers of oligodendrocytes and schwann cells are very low, as demonstrated by immunostaining for cell-type specific markers (data not shown). L-LME treatment abolishes Iba1 gene expression (after L-LME 0.13 ± 0.01 vs CTR 5.95 ± 0.71) in cortical and spinal cord astrocytes (**Fig. 3.7**). LPS up-regulates CD14 expression, which is abolished after L-LME treatment. CD14 expression may be due to the few remaining microglia in the enriched astrocyte cultures. Similar results are obtained with cultures derived from spinal cord (data not shown).

Fig. 3.7 mRNA expression characterizing cell types in enriched and purified astrocytes



Cortical enriched astrocytes express high levels of Iba1 mRNA relative to purified astrocytes. Enriched astrocyte cell populations prepared from neonatal rat cortex were incubated with 50 mM L-LME for 1h followed 24 later by a 6-h challenge with LPS, and then processed for RT-PCR analysis. Data, given as means ± sem (duplicate culture wells) are normalized to GAPDH levels, and are representative of 3 experiments. Qualitatively similar results were obtained with spinal cord microglia and astrocytes. β-III tub, β-III tubulin; vWF, von Willembrand factor; Mcam, melanoma cell adhesion molecule; galC, galactosylcerebroside; Mbp, myelin basic protein.

3.6 Iba1, ITGAM, iNOS, IL-1β, IL-6 and TNF-α mRNAs expression in cortical purified astrocyte cell cultures

In order to evaluate the induction of Iba1 and pro-inflammatory gene transcripts (iNOS, IL-1β, IL-6 and TNF-α), we prepared purified astrocyte cultures by treating with 50 mM of L-LME cortex and spinal cord enriched astrocyte cultures. Twnty-four hours later cultures were challenged with LPS for 6 h and then processed. Results are shown in **Fig. 3.8**. Similar results are obtained with cultures from spinal cord (data not shown).

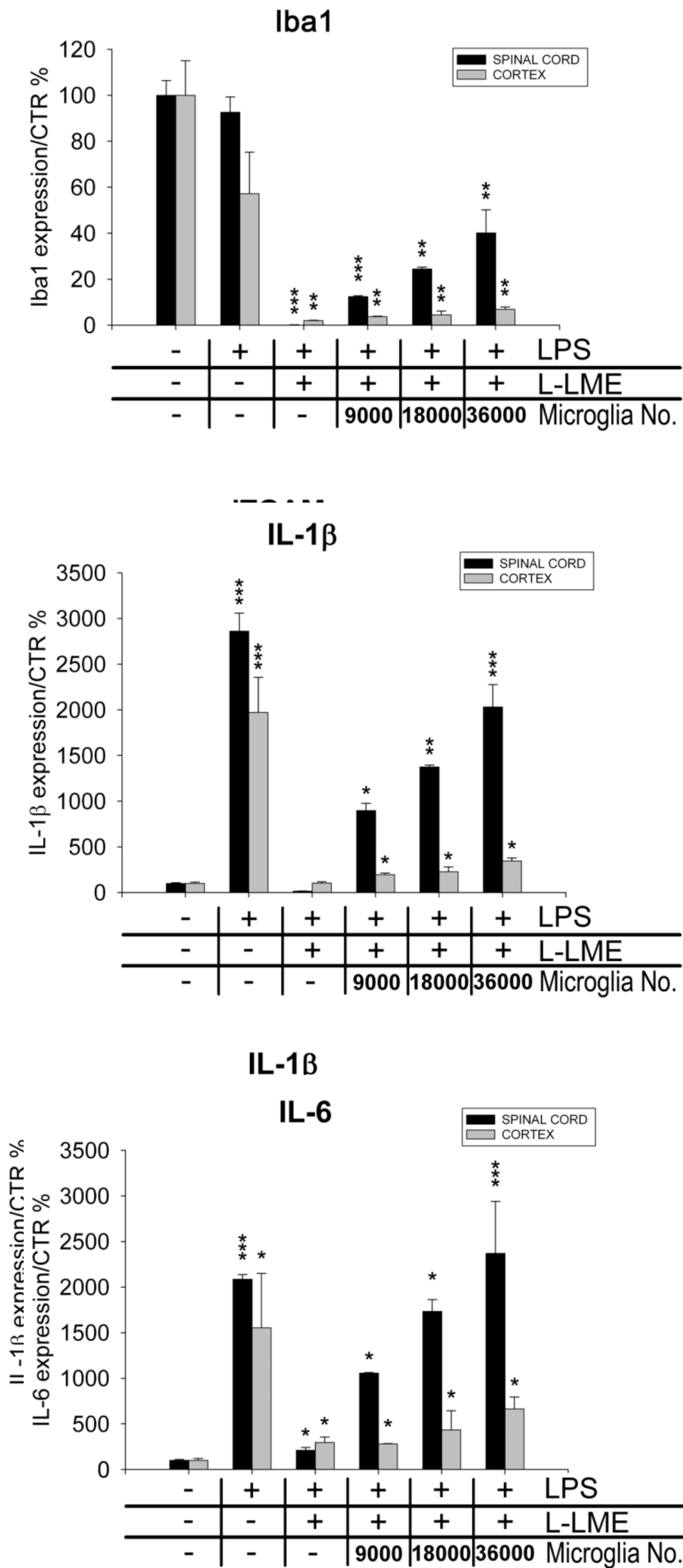
Under these conditions L-LME (first three sets of columns from the left in each figure): abolished Iba1 and ITGAM gene expression in both cortical and spinal cord astrocytes; eliminated the LPS induction of IL-1β mRNA in cortical and spinal cord astrocytes; reduced, but did not fully abolish the LPS-induced rise in IL-6 and TNF-α mRNA; abolished in spinal cord, but did not cortical astrocytes the LPS-induced rise in iNOS mRNA expression. 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) and *in vivo* (Murphy, 2003). Reducing the L-LME concentration to 5 mM proved ineffective, while incubating purified microglia with 50 mM (but not 5 mM) L-LME for 60 min resulted in complete destruction of the cells 24 h later, assessed with MTT test. Astrocytes treated with 50 mM L-LME did not evidence any overt morphological changes or loss of MTT reactivity.

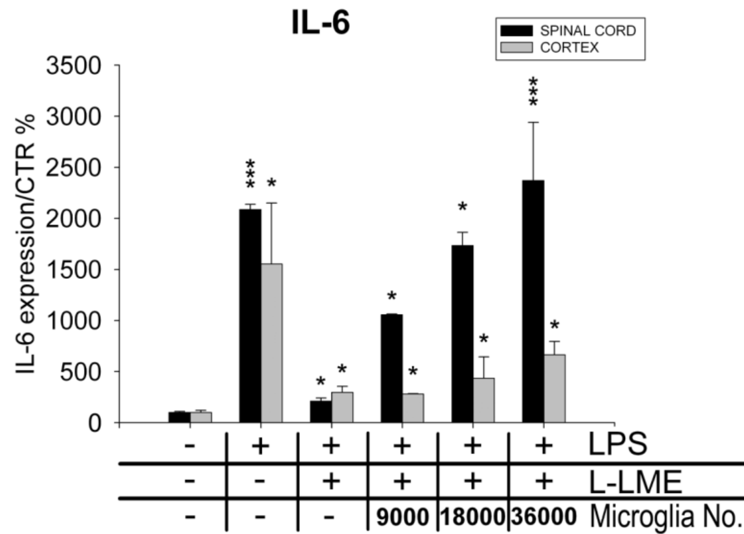
Nuclear factor-kB (NF-kB) is an important transcription factor that controls the expression of inflammatory mediators, such as iNOS, cyclooxygenase-2, TNF-α, and IL-1β. Inflammatory stimuli induce IkBα phosphorylation, releasing NF-kB and allowing it to activate expression of inflammatory mediators (Ghosh and Hayden, 2008; Surh et al., 2001). Astrocytes treated with 50 mM L-LME retained their responsiveness to LPS, as evidenced by the appearance of p65 staining within the nucleus (**Fig. 3.9**).

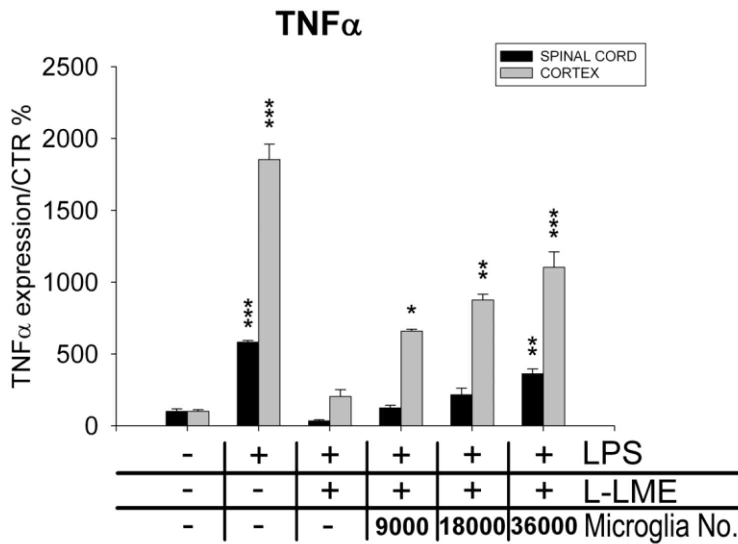
3.7 Microglia Addition to L-LME-Treated Astrocytes Restores LPS Responsiveness

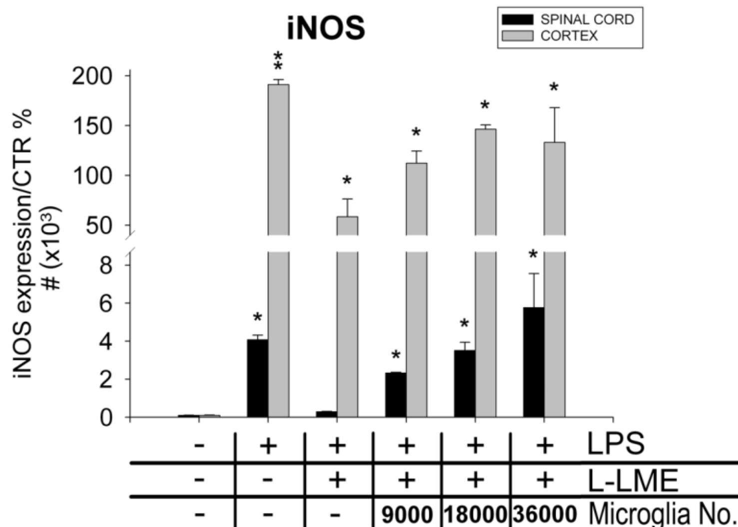
The above results suggest that microglia-astrocyte interaction may be a necessary condition to elicit responsiveness to LPS. To test this possibility, fixed numbers of purified microglia were added to L-LME-treated astrocytes. Upon so doing, the Iba1 signal became detectable, being more evident in the spinal cord co-cultures (**Fig. 3.8**, second three sets of columns).

Fig. 3.8 Iba1, ITGAM and Inflammatory mRNA expression in enriched and purified astrocytes





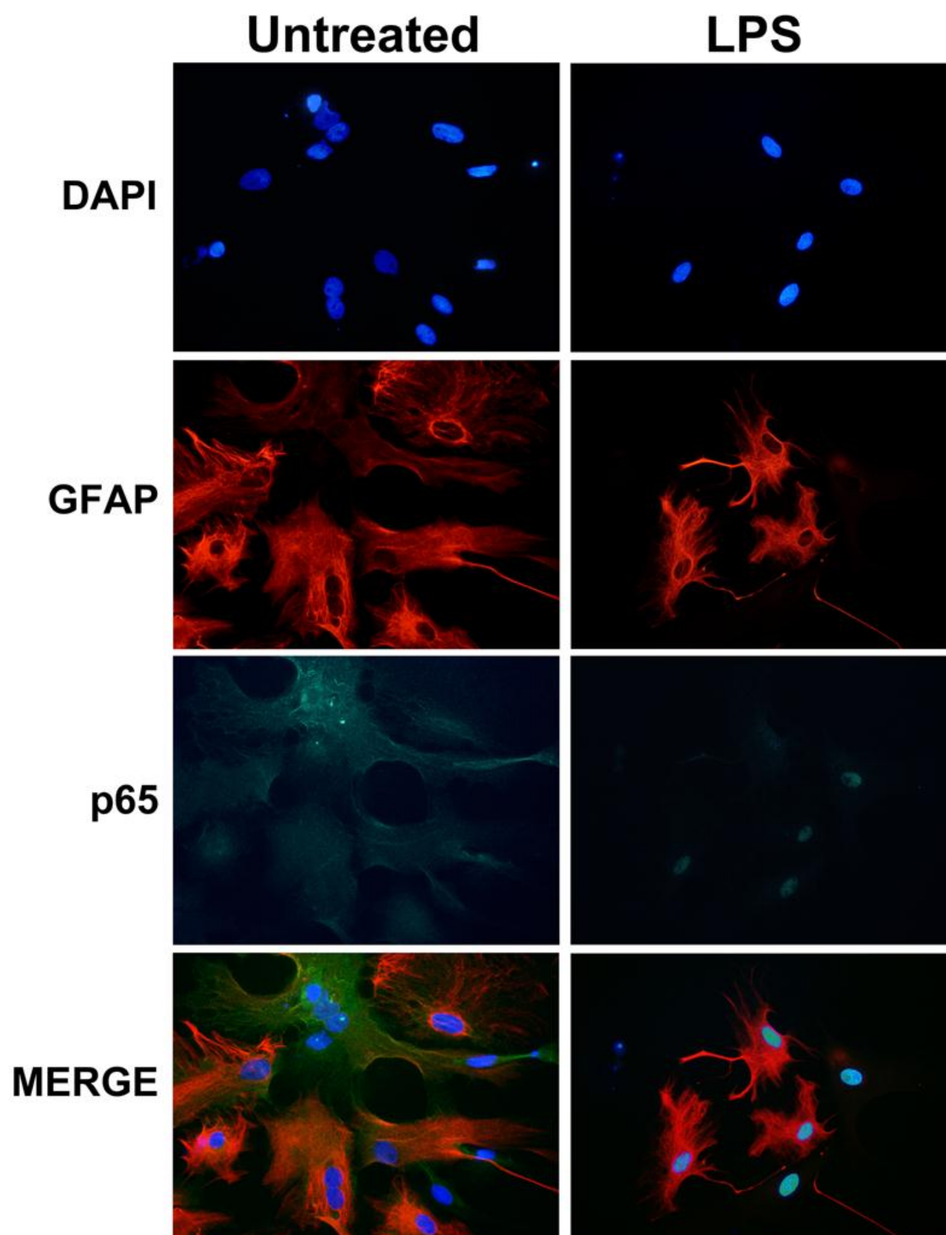




L-LME treatment of cortical and spinal cord astrocytes eliminates Iba1 and ITGAM gene expression, and limits pro-inflammatory gene induction by LPS: effect of microglia addition. Enriched astrocyte cell populations prepared from either tissue were treated for 60 min with 50 mM L-LME, and returned to 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, ITGAM, IL-1β, IL-6, TNF-α, iNOS 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. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs CTR (Anova and Bonferroni exact test).

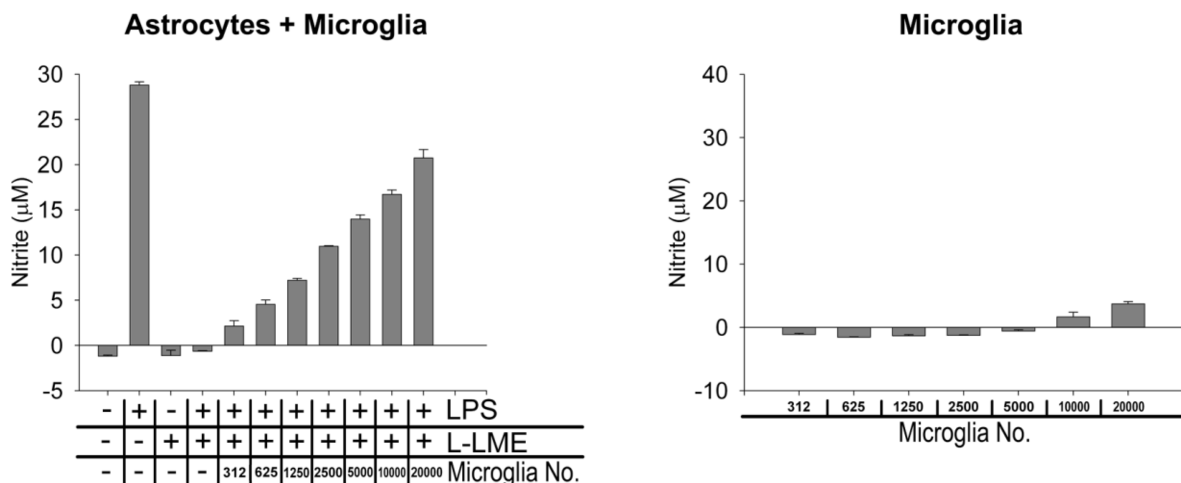
Interestingly, reintroduction of microglia (second three sets of columns in each panel) restored LPS responsiveness in terms of IL-1β and IL-6 release, and the effect was more pronounced in spinal cord glia co-cultures. In the latter, expression levels clearly exceeded those for L-LME-treated astrocytes 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. 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. 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 expressed in terms of mediator output. For example, addition of microglia to L-LME-treated astrocytes resulted in NO release in proportion to the number of microglia added, becoming already detectable with less than 1000 cells per culture well. 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 (**Fig. 3.10**).

Fig. 3.9 p65 protein expression in purified astrocytes



LPS induces NF-κB nuclear translocation in L-LME-treated cortical astrocytes. Purified cortical astrocytes were treated for 60 min with 50 mM L-LME, and returned to fresh culture medium for 24 h. After this time cells were incubated with 10 ng/ml of LPS for 3 h, and then processed for p65 immunostaining, DAPI (nuclei), and GFAP (astrocytes). Qualitatively similar results were obtained with spinal cord microglia and astrocytes.

Fig. 3.10 NO release in purified astrocyte cultures with addition of microglia



Addition of microglia to L-LME treated 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 min, 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 release. (Right panel) The same numbers of microglia were cultures in a parallel plate, treated with LPS as above and medium analyzed for NO content. All data are means ± sem (n=3).

4. DISCUSSION

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. Furthermore, we are now coming to more fully appreciate the importance of interactions between the immune and nervous systems in neuropathology, including chronic pain. Microglia are the primary immune effector cells in the brain (Streit et al., 2004) and play a pivotal role in the neuroinflammatory processes associated with a variety of neurological disorders (Bjorkqvist et al., 2008; Lopes et al., 2008). Astrocytes, the predominant CNS cell type also become reactive following injury and have been implicated in the pathogenesis of CNS inflammation in several instances (Dong and Benveniste, 2001; John et al., 2005; Murphy, 2000). In the present study we used the bacterial toxin LPS as an experimental model of glial activation in vitro, and show that inflammatory gene and mediator up-regulation induced by LPS depends on an interplay between microglia and astrocytes. While purified microglia and enriched astrocytes from rat cortex and spinal cord were responsive to LPS in terms of pro-inflammatory mediator induction, astrocytes depleted of microglia after treatment with the lysosomotropic agent L-LME became unresponsive. However, co-culture of the microglia-depleted astrocytes with increasing numbers of microglia restored sensitivity to LPS. Surprisingly, the same numbers of microglia, cultured alone were largely insensitive to activation by LPS.

The complexity of glial activation studies in vivo has led to the widespread utilization of ex vivo glial cell systems to investigate, under defined conditions, the mechanisms underlying inflammatory processes. While microglia can be obtained relatively pure (>99%), many in vitro studies with astrocytes utilize 'enriched' preparations, i.e. they contain a minor population (up to 5%) of microglia which may confound interpretation of the results (Giulian and Baker, 1986; Ciccarelli et al., 2000; Solà et al., 2002; Saura, 2003; Hamby et al., 2006). While the availability of highly-enriched astrocyte cultures (>99%) may often be desirable, the presence of microglia can allow one to study astroglial-microglial cross talk that is extremely important in glial cell activation. To address this issue, we applied a specific microglia toxin to remove residual microglia from our enriched astrocyte cultures, followed by reintroduction of fixed numbers of microglia (see, for example, Hamby et al., 2006). Not unexpectedly, LPS up-regulated mRNA for iNOS, IL-1β and TNF-α (as well as NO and IL-

1β release) in both purified microglia and enriched astrocytes cultured from cortex and spinal cord, with the responses being more robust in microglia. The ability of L-LME to eradicate microglia from these astrocyte populations was shown by the disappearance of Iba1 and integrin alpha M gene expression (Fig. 3.8). L-LME treatment abolished LPS induction of IL-1β mRNA in cortical and spinal cord astrocytes; reduced, but did not fully eliminate LPS-induced rise in IL-6 and TNF-α mRNA; abolished in spinal cord, but did not cortical astrocytes the LPS-induced rise in iNOS mRNA. Astrocyte elaboration of the respective gene products was also essentially abolished following L-LME treatment. Introducing fixed numbers of purified microglia to L-LME-treated astrocytes restored LPS responsiveness in terms of IL-1β and IL-6 gene induction, the effect being more robust in spinal cord glia co-cultures. In the case of TNF-α, astrocyte/microglia co-cultures derived from cortex appeared to be more sensitive to the presence of microglia in comparison to spinal cord. Addition of spinal cord microglia to L-LME-treated astrocytes restored iNOS gene induction by LPS, although the effect was less evident for cortical astrocytes, as iNOS mRNA expression remained relatively high even after L-LME treatment.

The LPS responsiveness of microglia/astrocyte co-cultures was also evident at the level of mediator output as for example, with NO release in proportion to the number of microglia added. In contrast, the same numbers of microglia alone failed to produce NO when incubated with LPS, except at the highest number.

The reason for loss of astrocyte sensibility to activation by LPS after microglial depletion is not known. Expression of TLR4 in astrocytes in vitro, for which LPS is a ligand, appears controversial (Kielian, 2006; Crack and Bray, 2007). Some studies were unable to detect TLR4 expression (Farina et al., 2005; Kielian, 2006), whereas others have shown a constitutive expression of TLR4 in astrocytes and an up-regulation following activation (Bsibsi et al., 2002; Bowman et al., 2003; Carpentier et al., 2005) or in CNS pathology (Casula et al., 2011). Astrocytes used in the present study show co-localization of immunoreactivity for GFAP and TLR4. These discrepancies may reflect species differences, as well as differences in culture conditions (Kielian, 2006). Moreover, glial TLR4 expression may be influenced by pro-inflammatory cytokines (e.g. IL-1β) or molecules released by injured tissue (Bianchi and Manfredi, 2009). Although we observed nuclear translocation of NF-kB in LPS-treated, microglia-depleted astrocytes, a role for microglia-astrocyte interaction in mediator up-regulation cannot be ruled out.

The molecular basis for the observed astrocyte-microglia cross talk remains to be determined. IL-1β can activate astrocytes (Guo et al., 2004; Wu et al., 2008), including increased

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 (our unpublished observations). It is worth pointing out that while introduction of microglia to cultures of L-LME-treated astrocytes restored LPS responsiveness across a range of parameters (pro-inflammatory mediator genes and products), the response levels reached were generally below those seen for the same number of enriched (untreated) astrocytes. It is possible that astrocyte-microglia coupling is less efficient when isolated microglia are added to toxin-treated, microglia-depleted astrocytes as compared to when the two cell populations mature together ('enriched' astrocytes). Given that microglia are a minor CNS cell population compared to astrocytes, the astrocyte/microglia co-culture behavior seen here may well underestimate what occurs in vivo. Soluble factors released by astrocytes have been suggested to not be directly responsible for such an effect (Solà et al., 2002), although insoluble factors present in the cell membrane of astrocytes could play a role. We are currently exploring this idea, which may shed light on each cell type's contribution to LPS action. Astrocytes can produce microglial mitogens (Shafit-Zagardo et al., 1993; Ringheim, 1995); 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 more robust responses.

Neuroinflammation in CNS disorders was viewed at one time as an epiphenomenon following neuron damage. Emerging evidence now challenges this earlier "neuron-chauvinistic" 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 – and may even contribute to schizophrenia, depression, and other psychiatric disorders. 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. Prior work suggests that TLR4 recognizes not only LPS, but also ligands called damage associated molecular patterns, released by the injured tissue (Bianchi and Manfredi, 2009), including fibronectin A (Okamura et al., 2001), heat shock protein 60 (Ohashi et al., 2000), heat shock

protein 70 (Asea et al., 2002), and tenascin C (Midwood et al., 2009). The astrocyte/microglia co-culture paradigm described here may provide a useful starting point to elucidate the molecular mechanisms underlying astrocyte- and microglia-specific responses pertaining to, although not limited to, CNS inflammation.

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