Targeting Cytokine Systems to Achieve Neuroprotection: Experimental Models of Seizures and Neurodegeneration

Thesis

The Open University, UK

Advanced School of Pharmacology
Dean, Enrico Garattini M D

Mario Negri Institute for
Pharmacological Research

Targeting cytokine systems to achieve neuroprotection: experimental models of seizures and neurodegeneration

by

Silvia Balosso, degree in Chemistry and Pharmaceutical Technology

Mario Negri Institute for Pharmacological Research, Milan, Italy

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L. Bernardino, S. Balosso, T. Ravizza, N. Marchi, G.Ku, JC Randle, JO Malva and A. Vezzani "Transient inflammatory events in hippocampal slice cultures prime neuronal susceptibility to excitotoxic injury: a crucial role of P2X7 receptor-mediated IL-1beta release" J Neurochem 2008; 106(1): 271-80

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COLLABORATIONS

1 - HPLC detection of GABA and Glutamate into dialysate samples was performed by Dr Eleonora Calcagno, Mario Negri Institute for Pharmacological Research

2 - ELISA measurements were performed by Dr George Ku, Vertex Pharmaceuticals, Inc

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LIST OF ABBREVIATIONS

AED	Antiepileptic drug
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein-1
APC	Antigen presenting cell
A-SMase	Acid sphingomyelinase
ATP	Adenosine 5'-triphosphate
BBB	Blood-brain barrier
BBG	Brillant blue G
BSA	Bovine serum albumin
BzATP	2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium
Ca^{2+}	calcium
CA	Ammon's horn
CA1	Ammon's horn hippocampal subfield 1
CA2	Ammon's horn hippocampal subfield 2
CA3	Ammon's horn hippocampal subfield 3
cAMP	Cyclic-AMP
CNS	Central nervous system
COX-2	Cyclooxigenase-2
CREB	cAMP responsive element binding
CVO	Circumventricular organ
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DD	Death domain
DG	Dentate gyrus
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay

ERK1/2	Extracellular signal-regulated kinases 1 and 2
FADD	Fas-associated death domain
FAN	Factor associated with neutral sphingomyelinase activation
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF-2	Fibroblast growth factor-2
GABA	γ-amino butirric acid
GBSS	Gey's balanced salt solution
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HPA	Hypothalamic-pituitary-adrenal
ICAM	Intercellular adhesion molecule-1
ICE	Interleukin-1 converting enzyme
icv	Intracerebroventricular
IFN-γ	Interferon-γ
IκB	Inhibitor proteins of NFκB
IKK	IκB kinase complex
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RI or II	Interleukin-1 receptor I or II
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
KA	Kainic acid
LPS	Lipopolysaccharide
MAP-2	Microtubule-associated protein-2
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase

mRNA	Messenger ribonucleic acid
MTLE	Mesial temporal lobe epilepsy
MyD88	Myeloid differentiation factor 88
Neun	Neuronal nuclear protein
NFκB	Nuclear factor κB
NMDA	N-methyl D-aspartic acid
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-1
NSAIDs	Non-steroidal anti-inflammatory drugs
NSD	Neutral sphingomyelinase activation domain
N-SMase	Neutral sphingomyelinase
oATP	Oxidized ATP
OX-42	Complement receptor type 3
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PC-PLC	Phosphatidylocholine-specific phospholipase C
PCR	Polymerase chain reaction
PECAM	Platelet/endothelilal cell adhesion molecule-1
PI	3,8-diamino-5-(3-(diethylmethyilamino)propyl)-6-phenyl phenanthridinium diiodide; propidium ioide
PGE_2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLAD	Pre-ligand-binding assembly domain
PLC	Phospholipase C
PN	Postnatal
RIG-1	Retinoic acid inducible gene 1
RIP	Receptor interacting protein
ROS	Reactive oxigen species
RT	Room temperature

SE	Status epilpeticus
SRF	Serum response factor
TACE	TNF-α converting enzyme
TBS	Tris-buffered-saline
TIM	Traf interactive motif
TIR	Toll/interleukin-1 receptor
TLE	Temporal lobe epilepsy
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TNFR 1 and 2	Tumor necrosis factor receptor 1 and 2
Tollip	Toll-interacting protein
tPA	Tissue plasminogen activator
TRADD	TNF receptor-associated death domain
TRAF 2 and 6	TNF receptor-associated factor 2 and 6
VCAM-1	Vascular cell adhesion molecule-1
WT	wild-type
zVAD-fmk	z-Val-Ala-DL-Asp (Ome)-fluoromethylketone

ABSTRACT

This study investigated whether IL-1β and TNF-α have a role in the pathophysiology of seizures by altering neuronal excitability and cell survival and also probed new targets to achieve an effective anticonvulsive action.

The first set of experiments evaluated if acute and spontaneous seizures can be effectively inhibited by blocking the brain production of IL-1β using a selective inhibitor of caspase-1, the key enzyme specifically involved in the production of the releasable and biologically active form of IL-1β. Caspase-1 inhibition significantly reduced the number and the total time spent in seizures, indicating that this treatment represents an effective and novel anticonvulsive strategy.

The second set of experiments investigated the intracellular pathway involved in the IL-1β proconvulsant actions which appear to occur via a novel non-transcriptional pathway: namely, the IL-1β-mediated activation of neutral-sphingomyelinase, the production of ceramide and the subsequent phosphorylation of Src-family of tyrosine kinases and the target receptor protein NR2B subunit of the NMDA receptor, resulting in the potentiation of NMDA function. This fast post-translational effect of IL-1β represents a novel and non-conventional pathway by which inflammatory molecules produced in epileptic tissue can affect neurotransmission.

Then, this work demonstrated that a brief pro-inflammatory stimulus, characterized by the activation of microglia and subsequent lasting release of critical concentrations of IL-1β, primes neuronal vulnerability to a subsequent excitotoxic insult, highlighting one

mechanism by which a pre-existent pro-inflammatory state may increase hippocampal neuronal susceptibility to the excitotoxic damage associated with seizures.

Thus, pharmacological approaches specifically targeted to block the overproduction of IL-1β and its functions in diseased conditions may represent new, nonconventional strategies for the treatment of seizure disorders, which are refractory to classical anticonvulsant treatments.

The second part of this thesis demonstrated that TNF-α significantly decreases epileptic activity by specifically acting on neuronal p75 receptors. The actions of TNF-α on neuronal excitability strictly depend on whether p55 or p75 receptors are preferentially involved and appear to be mediated also by changes in the assembly of glutamate receptor subunits.

These novel functional glia-neuronal interactions add important insights into the mechanism of ictogenesis and seizure-associated neuronal cell death, highlighting innovative pharmacological strategies to block the activation of cytokine-mediated signalling in diseased conditions.

1.1 Epilepsy: definition and classification

Epilepsy is one of the most common neurological disorders that occurs in about 1% of the population worldwide, with an incidence of 50 per 100.000 people per year in developed countries. It can occur at all ages, from neonates to elderly people and has different etiologies.

In the past it was known as the "Sacred disease" because it was often associated with religious experiences and even considered as a manifestation of demonic possession because of its peculiar behaviour caused by epileptic seizures. Hippocrates was the first to dissociate epilepsy from religion and magic, defining it as a disease of the brain that must be treated by diet and drugs.

The International League against Epilepsy (ILAE) has recently defined epilepsy as "a chronic condition of the brain characterized by an enduring propensity to generate epileptic seizures, and by neurobiological, cognitive, psychological and social consequences of this condition. The definition of epilepsy requires the occurrence of more than one unprovoked seizures" (Fisher et al., 2005). This definition indicates that the diagnosis of epilepsy requires not only the occurrence of at least one seizure, but also the presence of an intrinsic chronic epileptogenic tissue that generates seizures. The process that turns a normal neuronal network into a permanent state of hyperexcitabilty and hypersynchrony (i.e., an epileptic tissue), is called *epileptogenesis.* The mechanisms that lead to an epileptogenic network are complex and not yet fully elucidated (Pitkanen and Sutula, 2002).

The term "epilepsy" indicates clusters of disorders that are characterized by different symptoms but all capable of generating recurrent seizures which are the hallmark of

epilepsy. Seizures result from a "transient alteration of the EEG and/or behavior due to abnormal excessive neuronal activity in the brain" (Fisher et al., 2005). Therefore, to generate seizures, two concurrent events are required:

1) hyperexcitabilty of neurons that is necessary to exceed a certain level of excitability (seizure threshold); 2) synchronization of neural networks; i.e. a population of neurons firing at the same time at a similar rate (Stafstrom, 2006). At the level of single neurons, there is a sustained neuronal depolarization resulting in a burst of action potentials, followed by a rapid repolarization and then hyperpolarization. This sequence is called *the paroxysmal depolarizing shift (PDS).* When a large population of neurons is recruited and fires PDSs in a synchronous manner, then a seizure is triggered. The clinical manifestations of seizures depend on which brain area is involved. In 1981 ILAE developed an international classification of epileptic seizures dividing seizures into two major classes: partial (focal) and generalized.

Partial seizures arise in one hemisphere and are divided in simple partial seizures when the consciousness is unaffected or complex partial seizure when there is loss of consciousness. A partial seizure may spread within the brain and generate a *secondarily generalized seizure*, which often begins with an aura (a simple partial seizure) that evolves into complex and then into generalized tonic-clonic seizure.

Generalized seizures arise in both hemispheres and include absence (petit mal), myoclonic, and tonic-clonic seizures (grand mal).

Status epilepticus consists of a very prolonged seizure or seizures occurring so frequently that they do not allow for full recovery of normal brain function. From a clinical perspective (Engel, 1996), epilepsy is divided into: *Idiopathic epilepsies* which are not associated with

identifiable brain pathologies and include all genetically-determined forms; C*ryptogenic epilepsies* when their etiology can not be determined; *Symptomatic epilepsies,* disorders due to a specific epileptogenic lesion which may be genetic (e.g. tuberous sclerosis or malformations of cortical development) or acquired (e.g head trauma, status epilepticus, stroke, infection, febrile seizures). These initial insults may trigger molecular, cellular and network changes which possibly lead to the occurrence of spontaneous seizures; this process called epileptogenesis is schematically depicted in Fig. 1.1. Symptomatic epilepsies are divided into generalized (generalized seizures) and localization-related disorders (partial or secondarily generalized seizures). Of particular relevance among symptomatic localization-related epilepsies are those of temporal lobe origin, called *Temporal Lobe Epilepsy* (TLE) which represents the most common form of human epilepsy. The most frequent epileptic syndrome of temporal-lobe origin is *Mesial Temporal-Lobe Epilepsy* (MTLE), characterized by hippocampal sclerosis.

The new ILAE definition of epilepsy also considered that the chronic and repeated seizures can lead to psychological, social and intellectual impairments. Indeed, patients with epilepsy often exhibit memory impairments, live in fear waiting for the next seizure and may be unable to drive or work. So it is important to achieve seizure remission to permit good quality of life; this task can be obtained in about 70% of patients using the current antiepileptic drugs (AEDs), which reduce or abolish the occurrence of seizures. However, approximately one-third of patients still has uncontrolled seizures, and others suffer from severe side effects of AEDs. When three anticonvulsant drugs used at maximal therapeutic doses do not control seizures, the patient is defined pharmacoresistant (Duncan et al., 2006; Perucca et al., 2007). In about 10% of instances, pharmacoresistant patients are eligible for

surgery consisting in the resection of the epileptogenic zone. The current AEDs are symptomatic drugs, thus they can suppress the symptoms (seizures) but do not arrest, or interfere with, the epileptogenic process (Perucca et al., 2007; Loscher and Schmidt, 2004).

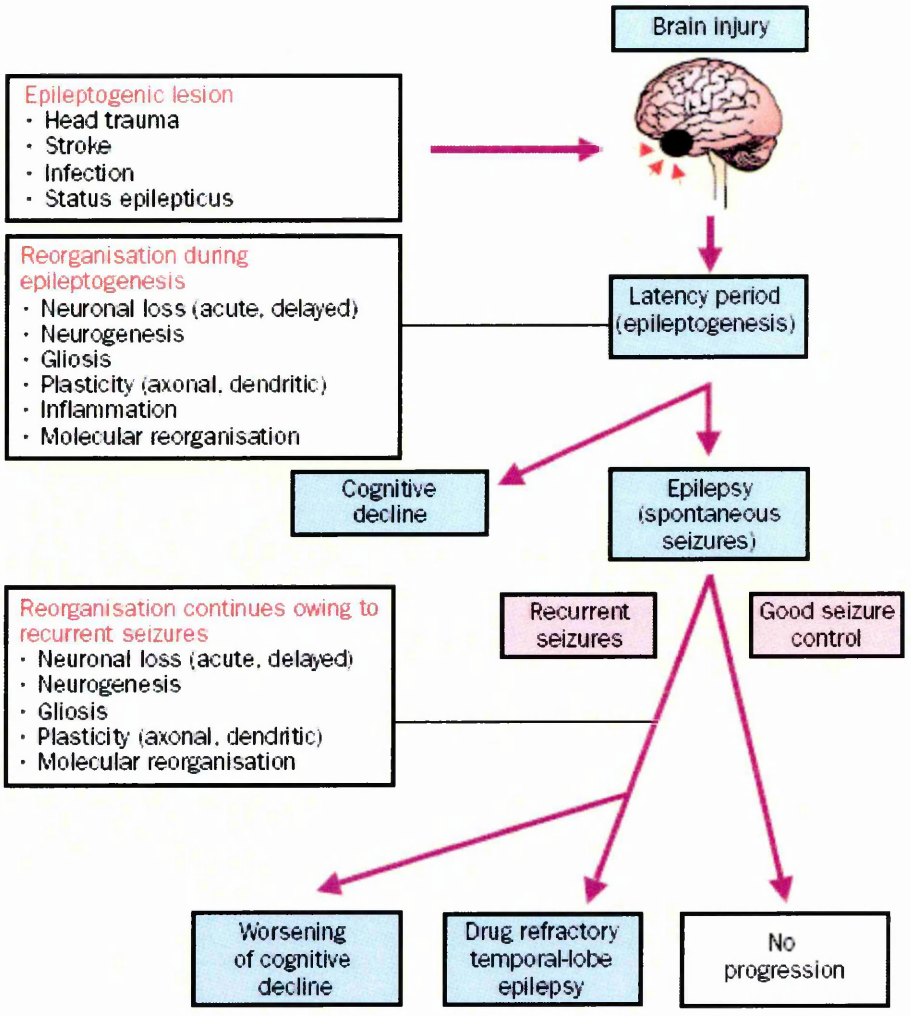


Figure 1.1. Epileptic process in symptomatic temporal-lobe epilepsy
An epileptogenic lesion can occur after a primary brain injury. This lesion leads to epileptogenesis that is characterized by cellular and molecular alterations including cell death, gliosis, axonal and dendritic plasticity, inflammation and angiogenesis. This period can last from weeks to years and induces a permanent hyperexcitability leading to the occurrence of spontaneous seizures (epilepsy). 70% of patients treated with AEDs have a good seizure control; however, ~30% of patients continue to have recurrent seizures that may contribute to the progression of the disorder, inducing a worsening of cognitive decline and contributing to drug refractoriess (Pitkanen and Sutula, 2002).

1.2 Inflammation in the Central Nervous System

The CNS has been commonly considered as an immune-privileged organ because of the blood-brain barrier (BBB) that reduces the exposure of the brain to immune/inflammatory events and the lack of lymphatic drainage (Glezer et al., 2007). However, various studies published during the past ten years have shown that an innate immune response and the subsequent recruitment of adaptive immunity cells from the blood (Nguyen et al., 2002) can occur in the CNS following various injuries suggesting that the brain is indeed an immunologically specialized site.

The innate immune system provides immediate defense against infections by recognizing and responding to pathogens in a non-specific manner; the innate immune cells can kill the invaders directly or activate phagocytic cells to ingest and remove them (Rivest, 2003). A key component of the innate immune response is the induction of inflammation which is characterized by the presence of inflammatory mediators such as cytokines and their receptors, chemokines, proteins of the complement system and prostaglandins (Glezer et al., 2007; Gosselin and Rivest, 2007; Rivest, 2003). These molecules are produced by brain parenchymal cells such as resident microglia (Garden and Moller, 2006; Hanisch and Kettenmann, 2007), astrocytes (Farina et al., 2007; Seifert et al., 2006) neurons (Friedman, 2001; Sairanen et al., 2001) and also by cell components of the BBB (Webb and Muir, 2000). Cytokines produced during the innate immune response have an important role in shaping the subsequent development of the adaptive immunity and can determine whether the response is predominantly T-cell mediated or humoral. Indeed, the adaptive immune system is triggered when a pathogen evades the innate immune system and is considered a

specific response that can recognize and remember specific pathogens. The cells of the adaptive immune systems are the lymphocytes, manly B and T cells. The first play a large role in the humoral immune response inducing the production of the antibodies; the second are intimately involved in cell-mediated immune responses and comprise the cytotoxic T-cells and helper T-cells.

A rapid and robust inflammatory reaction in the brain in response to systemic infection can be mimicked in rodents by the administration of lypopolisaccharide (LPS), the major component of the outer membranes of Gram-negative bacteria (Laflamme and Rivest, 2001; Nguyen et al., 2002; Rivest, 2003). LPS is an element of pathogen-associated molecular patterns that are recognized by phagocitic cells such as microglia and macrophages throught the Toll-like receptors (TLRs), RIG-1 and NOD-like receptors (Rietdijk et al., 2008). These belong to a group of pattern recognition receptors that detect the presence of infectious agents by recognizing specific and highly conserved structures produced by these pathogens which are not expressed by eukaryotic organisms (Glezer et al., 2007; Rivest, 2003). Increasing evidence indicates an important role of TLRs in several inflammatory CNS pathologies such as Alzheimer's disease, Streptococcus pneumoniae meningitis and Herpex simplex virus encephalitis (Farina et al., 2007).

LPS induces downstream signaling events by interacting with TLR-4 (Akira et al., 2001). Recent data show that TLR-4 receptors are expressed by microglia and macrophages, in the circumventricular organs (CVOs), choroid plexus, the microvasculature and in the ependymal cells of the ventricules (Chakravarty and Herkenham, 2005). Thus, circulating LPS targets its TLR-4 expressed on macrophages and microglia in CVOs leading to the activation of mitogen-activated protein kinase (MAPK) pathways and nuclear factor kB

(NFkB), which can induce various proinflammatory genes (Glezer et al., 2007; Nguyen et al., 2002). Initially, these events involve the CVOs and thereafter the brain parenchyma (Laflamme and Rivest., 2001; Nguyen et al., 2002; Rivest, 2003). An increase in CD14, tumor necrosis factor (TNF)-α and TLR-2 mRNA was found in the brain in response to systemic administration of LPS (Nguyen et al., 2002; Rivest, 2003). It is likely that TNF-α produced in the CVOs by LPS can mediate the spread of LPS-induced inflammation in the brain by activating receptors on parenchymal microglial cells both in autocrine and paracrine manners (Bette et al., 2003; Nguyen et al., 2002). These data indicate that the activation of microglia by endotoxemia appears to be a crucial step in the CNS inflammatory response to systemic infection.

The innate immune response and related inflammatory reactions can occur not only during infection (Glezer et al., 2007) but also after neurotrauma, ischemia (Allan and Rothwell, 2001; Lucas et al., 2006), autoimmune and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Hungtington's disease, multiple sclerosis, amyotrophic lateral sclerosis and also epilepsy (Aarli, 2000; Bauer et al., 2001; Billiau et al., 2005; Nguyen et al., 2002). Indeed, endogenous ligands such as signals from damaged cells (e.g. heat shock proteins, components of degradated extracellular matrix, mRNA released from necrotic cells) or molecules entering into the brain because of BBB damage can trigger an immune response in the CNS by stimulating the TLRs expressed both on microglia and astrocytes (Akira et al., 2001; Farina et al., 2007) and inducing the activation of these resident glial cells.

1.2.1 Microglia function and activation

Microglia form 10-20% of the total glia population and are considered the resident immune cells of the CNS. Microglia are always vigilant for changes in the microenvironment and prepared to respond to brain injuries (Lucas et al., 2006; Schwartz et al., 2006). *In vivo* two-photon microscopic studies in transgenic mice expressing enhanced green fluorescent protein in microglia demonstrate that microglial processes are highly mobile, continually rebuilt and appear to monitor the neuronal microenviroment (Nimmerjahn et al., 2005; Davalos et al., 2005). The presence of non-physiological factors such as proteins that occur in abnormal format, damaged cells and debris, transform the ramified and resting microglia into activated cells characterized by round-shape morphology with thickened and short processes (Kreutzberg, 1996). Thus, numerous signals that compromise the structural and functional integrity of the CNS can activate microglia, rapidly changing their functional phenotype into a reactive profile (Hanisch and Kettenmann, 2007; Lucas et al., 2006). In particular ATP which is released by damaged neurons following brain insults, is considered the main mediator of these early microglial morphological changes through actions on its P2 receptors (Garden and Moller, 2006). Neurotransmitters such as glutamate and GABA also can modulate the microglial response to CNS injury (Hanisch and Kettenmann, 2007) since microglial cells express various neurotransmitter receptors (Pocock and Kettenmann, 2007). Microglia can detect various microenvironmental changes because they express membrane receptors that play a critical role in initiating and regulating the immune response. These receptors recognize cytokines, chemokines, bacterial products, complement, misfolded proteins and immunglobulins (Garden and Moller, 2006). Activated microglia immediately migrate to the inflammatory

site moving along a gradient of soluble factors such as chemokines or ATP (Davalos et al., 2005). At the injury site microglia can release inflammatory molecules such as cytokines (Bianco et al., 2005) and prostaglandins. These released mediators have multiple paracrine and autocrine effects. They can recruit more microglia to the site of activation and activate neighboring astrocytes, which amplify the inflammatory response resulting in the accumulation of neurotoxic factors (Garden and Moller, 2006). Microglia can acquire varying phenotypes resulting in different functional properties. This versatility strictly depends on the stimulus and its intensity (Lai and Todd, 2008). In this respect, in response to LPS, β-amyloid aggregates or high doses of IFN-γ, microglia acquire a phagocyte and cytotoxic phenotype and release proinflammatory mediators. On the other hand, after IL-4 or low doses of IFN-γ, microglia can support neurogenesis and offer neuroprotection by releasing anti-inflammatory factors or removing extracellular glutamate (Hanisch and Kettenmann, 2007; Schwartz et al., 2006). When the activating stimuli fade, activated microglia can down-regulate the immune response secreting anti-inflammatory molecules such as IL-13 and IL-4 that induce the apoptosis of the same microglia (Garden and Moller, 2006; Yang et al., 2002).

The available evidence therefore suggests that the transient and focal activation of microglia could be beneficial by limiting and repairing the damaged tissue. However, excessive response of microglia may lead to sustained inflammatory reactions that progress toward a pathological process (Hanisch and Kettenmann, 2007; Schwartz et al., 2006).

1.2.2 Astrocytic function and activation

The inflammatory response also rapidly activates astrocytes, which are the most abundant glial cells of the CNS (Raivich et al., 1999; Chen and Swanson, 2003). In mouse hippocampus, at least two astrocytic populations have been identified: Glutamate transporter (GluT) expressing astrocytes are enriched in glial fibrillary acidic protein (GFAP, a cytoskeletal protein) with irregular cell bodies and branched processes, prominent glutamate uptake and gap-junctional coupling; glutamate receptor expressing (GluR) astrocytes are characterized by low GFAP expression, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), low glutamate uptake and not coupled with gap-junction (Matthias et al., 2003).

Astrocytes give a physical and metabolic support to neurons, meninges and vasculature. They are implicated in the maintenance of K^+ and H_2O homeostasis through K^+ and water channels that are co-localized on astrocytic processes near synapses and capillaries (Jabs et al., 2008). Astrocytes are interconnected via gap junctions to form a large intercellular network. This connection allows dissipating ion and metabolites that could be detrimental if accumulated in the extracellular space (Pekny and Nilsson, 2005). It has also been reported that neuronal stimulation induces Ca^{2+} increases in astrocytes which can spread to neighbouring astrocytes indicating the presence of a functional astrocyte network (Bezzi et al., 2001b; Jabs et al., 2008; Volterra and Meldolesi, 2005). Other important functions of astrocytes are the removal of neurotransmitters such as glutamate, which is considered the main mediator of neuronal excitability and excitotoxicity, and the protection of neurons from oxidative stress via a glutathione dependent mechanism (Pekny and Nilsson, 2005; Dringen et al., 2000). Astrocytes can release also gliotransmitters that activate neuronal

receptors and modulate the strength of inhibitory or excitatory synaptic transmission suggesting that glial cells are integral and active components of the functional brain network (Bezzi et al., 2001b; Volterra and Meldolesi, 2005; Haydon, 2001). Three dimensional reconstruction of astrocyte-neuron structural relations in hippocampus (Ventura and Harris, 1999) or electronic microscopy studies (Volterra and Meldolesi, 2005) have demonstrated that astrocytic processes are intimately apposed to synapses and are rich in docked vesicles with high probability of neurotransmitter release (Bezzi et al., 2004). Following injury, astrocytes become activated and proliferate with hypertrophy of cellular processes (astrogliosis) and up-regulatation of GFAP protein (Chen and Swanson, 2003; John et al., 2005). Astrogliosis can create a physical barrier, termed the glial scar, which isolates the damaged tissue from healthy cells (Raivich et al., 1999). This scar can be beneficial favouring the repair and neuronal survival as shown in mice in which reactive astrocytes were ablated. In these mice, astrocyte loss was associated with a significant neuronal cell death and a failure of BBB repair following brain injury. However, astroglial scar functions as an impediment to axonal regeneration, restricting the neuronal outgrowth at the brain injury site (Bush et al., 1999; John et al., 2005; Pekny and Nilsson, 2005). Astrocytic neuroprotective effects can be related to their ability to release neurotrophic factors and anti-inflammatory cytokines such as ciliary neurotrophic factor, insulin-like growth factor-1, nerve growth factor, IL-10 and IL-1 receptor antagonist (IL-1ra) promoting the CNS repair (Allan and Rothwell, 2001). On the other hand, during sustained inflammation the potentially protective functions of astrocytes such as glutamate uptake, K^+ buffering, eliminations of free radicals are reduced and the astrocytes can release molecules

contributing to neuronal cell damage and dysfunction (John et al., 2005; Pekny and Nilsson, 2005).

Microglial and astrocytic response is versatile and can vary with the nature of the stimulus and its intensity and duration, and the microenvironment in which these cells are activated (Hanisch and Kettenmann, 2007; Pekny and Nilsson, 2005).

1.2.3 Adaptive immunity mechanisms

The innate immune response may be not sufficient to eliminate the pathogens and cell debris from the damage tissue. Thus, inflammatory cytokines, chemokines, adhesion molecules released by glial cells and neurons following TLRs/PAMPs interaction stimulate the traffic of adaptive immune cells such as T and B lymphocytes to the sites of lesion in the brain. For this reason, TLRs as well as inflammatory mediators are considered the critical proteins linking innate and acquired immunity (Akira et al., 2001) that are not separate entities, but sequential, complementary and functionally interconnected by cell-to-cell contact and by soluble inflammatory mediators (Abreu and Arditi, 2004; Olson and Miller, 2004; Medzhitov and Janeway, 1997).

In healthy brain, only few activated T lymphocytes can pass the BBB (Engelhardt, 2006; Ransohoff et al., 2003). However, in pathological conditions characterized by inflammatory reactions, T cells, primed in peripheral lymphoid organs, infiltrate brain parenchyma and accumulate at injury sites (Engelhardt, 2006; Man et al., 2006). The release of cytokines facilitates leukocyte migration across the BBB inducing the catabolism of arachidonic acid and subsequent production of prostaglandins and the upregulation of selectins and

adhesions molecules (ICAM-1, VCAM-1, PECAM-1, E-selectin) in the brain microvasculature (Webb and Muir, 2000).

Trafficking of leukocytes into the CNS may occur through at least three routes (Ransohoff et al., 2003): 1-leukocytes pass through the fenestrated endothelium in the choriod plexus stroma, cross the epithelium of the choroid plexus and reach the cerebral spinal fluid; 2-leukocytes can extravasate across postcapillary venules at the pial surface of the brain into subarachnoid space or across the Virchow-Robin perivascular space which is in direct communication with the cerebral spinal fluid; 3-leukocytes can enter the brain parenchyma by extravasation across the BBB non-fenestrated endothelium and basal lamina.

In the CNS leukocytes can be restimulated by antigen presenting cells (APCs), a specific group of highly specialized immune cells that include microglia, macrophages, dendritic cells, perivascular cells and epithelial cells of the choroid plexus (Aloisi et al., 2000; Williams et al., 2001). During inflammation, APCs express the major histocompatibility complex (MHC) and co-stimulatory molecules that promote the activation of T cells. Restimulated T cells can exibit either a Th1 phenotype characterized by the secretion of IL-2, IFN-γ and TNF-β and are implicated in the clearance of virus and in the regulation of the cellular immunity exacerbating CNS inflammation or a Th2 phenotype that produces IL-4, IL-10 and IL-13 and promotes the humoral immunity decreasing CNS inflammation (Aloisi et al., 2000). The regulation of T cell phenotype is affected by immunoregulatory mediators such as chemokines, cytokines, colony-stimulating factors, prostaglandins-E2 that are released by APCs during inflammation.

The activation of immune cells and the release of immune molecules also support neurogenesis from neural stem and progenitor cells and this non-classical immune activity

takes place constantly under normal physiological conditions in the adult brain (Ziv and Schwartz, 2008). Indeed, neurogenesis in neurogenic niches such as the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus is significantly decreased in the T-cell-deficient mice, suggesting that T cells facilitate neurogenesis affecting in particular the proliferative capacity of neural precursor cells (Ziv et al., 2006). In particular, T cell-induced modulation of the activity of the local microglia and macrophages seems to have a role in the adult neurogenic niches. The activation of microglia by Th1 cytokine IFN-γ or Th2 cytokine IL-4 induces neuronal and oligodendroglial differentiation from adult neural precursor cells (Butovsky et al., 2006). Thus, the activation of the immune system can constitute a beneficial endogenous response as occurring during the classic immune response to infection, or consolidate inflammation in the brain inducing an excessive and long-lasting release of inflammatory mediators, that promote neuronal degeneration and dysfunctions. An important role in the regulation of immune response is covered by the BBB, which strictly regulates the entry of molecules and immune cells into the brain and responds rapidly to circulating endotoxins, bacteria or viruses.

1.2.4 Blood brain barrier

The BBB is formed by nonfenestrated endothelial cells connected by tight junctions and surrounded by end-feet processes of astrocytes (Abbott et al., 2006; Ballabh et al., 2004; Pachter et al., 2003). It is a physical and metabolic barrier which serves to regulate and protect the microenvironment of the brain limiting the entry of molecules and cells. However, a variety of CNS injuries including infections, ischemia, trauma and seizures can

change the functionality and the integrity of BBB (Oby and Janigro, 2006; Ballabh et al., 2004). This could be caused by increased local blood pressure, free radical formation, inflammatory responses including the release of cytokines and leukocyte recruitment. Inflammatory mediators can induce the activation of metalloproteinases that damage the basal lamina of vessels, enhance the intracellular Ca^{2+} concentration that mediates the loss of the tight junction molecules, events that lead to an increase of endothelial permeability (Ballabh et al., 2004; Oby and Janigro, 2006; Webb and Muir, 2006). One of the consequences of increased BBB permeability is the accumulation of serum protein into the brain, which may contribute to increased excitability. Recent data have shown that BBB opening in the rat results in serum albumin extravasation into the brain and this is sufficient to induce epileptiform activity. These data suggest that neurons tend to fire abnormally when exposed to molecules that extravasate through a leaky BBB and this may contribute to the pathogenesis of focal epilepsies (Seiffert et al., 2004). Moreover, BBB permeability positively correlates with seizure frequency in chronic epileptic rats indicating that BBB leakage could contribute both to the increased excitability and to the maintenance of epileptic activity (van Vliet et al., 2007). The mechanisms that alter neuronal excitability after BBB opening are still unknown; the activation of astrocytes, the development of Ca^{2+} waves in astrocytes and the decrease of K^+ buffering appear to be involved (Nadal et al., 1997; Seiffert et al., 2004). An impairment of BBB integrity and an inflammatory state are common features of several neurological conditions associated with the late onset of epilepsy (Vezzani and Granata, 2005).

1.3 IL-1 and TNF-α: brief overview on related molecules, receptors and signal transduction pathways

This study focused on two pro-inflammatory cytokines, namely IL-1β and TNF-α. For a quicker and better comprehension of the incoming dissertations, a brief overview on IL-1 and TNF-α as well as their related molecules, receptors and signal transduction pathways is provided.

1.3.1 IL-1 system

IL-1 is considered the prototypic inflammatory cytokine (Dinarello, 1996); the IL-1 gene family comprises three members: IL-1α and IL-1β, which are agonists, and IL-1 receptor antagonist (IL-1ra) that is the natural occurring competitive receptor antagonist of IL-1 type 1 receptor (IL-1R1). Both IL-1α and IL-1β are synthesized as 31-kDa precursor proteins and are cleaved by specific cellular proteases to constitute the 17 kDa mature forms. Pro-IL-1α is biologically active and remains mostly intracellular; it has been shown that intracellular proIL-1α exerts its effects after binding to an intracellular pool of IL-1R1 and subsequent formation of a ligand-receptor complex which translocates to the nucleus where it binds DNA (Weitzmann and Savage, 1992). Pro-IL-1α can also be released in the extracellular space by dying cells where specific Ca^{2+}-activated cytoplasmic proteases (calpain) cleave pro-IL-1α to generate the mature protein.

Pro-IL-1β is biologically inactive and it is localized in the cytoplasm until its cleavage by *IL-1β converting enzyme* (ICE/Caspase-1) that produces the 17 kDa mature and active protein which is secreted in the extracellular space (Dinarello, 2005; Fantuzzi and

Dinarello, 1999). It has been observed that cleavage of proIL-1β can be also accomplished by other enzymes in proximity of residues asp (116)-ala (117) where the ICE/Caspase-1 usually acts. Thus, trypsin (Kobayashi et al., 1991), elastase (Fantuzzi and Dinarello, 1999), chymotrypsin (Mizutani et al., 1991a), a mast cell chymase (Mizutani et al., 1991b) and a variety of proteases (Hazuda et al., 1991), metalloprotease 9 (Schonbeck et al., 1998) commonly found in inflammatory fluids have been shown to cleave proIL-1β to biologically active IL-1β species.

Both IL-1α and IL-1β exert their biological effects by binding the membrane 80 kDa IL-1 receptor type-1 (IL-1R1), which requires the recruitment of IL-1R accessory protein (IL-1R-AcP) to induce signal transduction (Rothwell and Luheshi, 2000). IL-1R-AcP does not bind IL-1β but appears to increase the affinity of IL-1R1 for IL-1β. The IL-1R signal transduction system is quite efficient since it is known that fewer than 10 ligand-bound receptors are enough to trigger a sustained response (Dinarello, 1996), whereas for the majority of receptor systems 10 to 100 fold higher receptor occupancy is usually required. This feature could be explained considering that IL-1β can activate a complex cascade resulting in signal amplification (O'Neill and Dinarello, 2000). There is also a type 2 IL-1 receptor (IL-1R2). The extracellular domains of the two receptors, responsible for ligand binding, are made of three immunoglobulin G-like domains and share a significant homology with each others (Greenfeder et al., 1995; Sims et al., 1988).

IL-1R1 possesses a single transmembrane segment with a long cytoplasmic domain, while IL-1R2 has a short amino acid sequence (29 residues) linked to its transmembrane domain. The lack of this transducing cytoplasmic signal domain makes the IL-1R2 unable to induce an intracellular signal and thus acts as a competitive *decoy* receptor to reduce the IL-1 activity

(Colotta et al., 1993; Sims et al., 1993). IL-1R1, IL-1R2 and IL-1R-AcP exist also in soluble forms that can function as inhibitors of IL-1β mediated signal transduction (Allan et al., 2005).

IL-1Ra is produced as pro-IL-1Ra and there are at least three intracellular isoforms and one secreted isoform. The last can bind the IL-1R1 with the same affinity as IL-1β, but without inducing the signal transduction. IL-Ra prevents the formation of the complex between IL-1R1 and IL-1R-AcP that is essential for a fully functional intracellular signal (Rothwell and Luheshi, 2000).

In rat and mouse brain, in situ hybridization and autoradiographic studies have shown high density of IL-1 receptors in the granule cell layer of dentate gyrus of the hippocampus and a moderate signal was obtained in the pyramidal cell layer of the hylus and CA3 subfield (Ban, 1994; Takao et al., 1990). Immunohistochemistry studies have shown receptor protein expression on glial cells (astrocytes and microglia), neurons and cerebral blood vessels following a CNS injury while barely detectable staining is observed in physiological conditions. The same holds true for IL-1β and IL-1Ra.

The presence of multiple regulator mechanisms such as IL-1R2, IL-1Ra, the soluble forms of IL-1R1, IL-1R2 and IL-1R-AcP, suggest that the biological activity of IL-1β requires a tight control mechanism.

1.3.1.1 Interleukin-1β converting enzyme (ICE/Caspase-1)

ICE is the enzyme responsible for cleavage of proIL-1β to mature IL-1β specifically. This enzyme is synthesized as inactive 45-kDa precursor (Cerretti et al., 1992; Thornberry et al., 1992) that requires two internal cleavages in order to be enzymatically active. Its

active tetramer conformation is made of two chains of 10- and 20-kDa respectively, with the cysteine-active site located on the 20-kDa chain. ICE itself can contribute to processing of the ICE precursor (Wilson et al., 1994).

ICE/caspase-1 is a cytoplasmatic cysteine protease that cleaves the precursor of IL-1β at the aspartic acid residue located at position 116 and alanine residue at position 117 genereting the active form of this proinflammatory cytokine (Lindberg et al., 2004). Thus ICE is called also caspase-1 for this enzimatic activity and it is not directly involved in apoptotic processes.

ICE/caspase-1 can also cleave proIL-18 (after the aspartic acid 19) thus yielding the mature IL-18 protein (Gu et al., 1997).

In health tissue, ICE/caspase-1 mostly exists as inactive precursor indicating that the levels of active caspase-1 are usually low, but they can be induced in neurons and glia in response to various brain injury such as ischemia, endotoxin, excitotoxic damage (Lindberg et al., 2004).

1.3.1.2 IL-1β synthesis and secretion

The synthesis of the inactive IL-1β precursor is induced by various proinflammatory stimuli including several brain injuries and infections (Allan et al., 2005). These events induce the production of transcriptional factors including nuclear factor-kB, activator protein-1 or the activation of mitogen activated protein kinase (MAPK) pathway that are involved in the transcriptional activation of IL-1β gene (Simi et al., 2007). Other proinflammatory signals, such as complement components and PGE2, can increase the rate of the transcription of IL-1β gene (Allan et al., 2005).

Most of synthesized proIL-1β remains in the cytosol while a fraction moves into secretory lysosomes where pro-IL1β colocalizes with the procaspase-1. The next step is the processing of pro-IL-1β in the mature active form that depends on cleavage by caspase-1 which is present in resting cells as procaspase-1 and binds to a large inhibitor molecule preventing its activation (Dinarello, 2005). The conversion of the inactive pro-caspase-1 to the active one depends on the recruitment of adaptor molecules complex termed inflammasome (Martinon et al, 2002; Ogura et al., 2006). The IL-1β processing and release are closely linked, although cellular release of this cytokine is still poorly understood and several mechanisms have been proposed. At least two stimuli seem to be required to induce the processing and secretion of IL-1β. For example, microglial cells synthesize proIL-1β in response to proinflammatory stimuli, subsequent activation of $P2X_7$ receptors by ATP triggers the efflux of K^+ and influx of Ca^{2+} which activates both phospholipase A2 (required for caspase-1 processing in lysosome) and phospholipase C which favors the lysosomal excitotosis and IL-1β release (Andrei et al., 2004).

Other possible mechanism involved in the IL-1β release is the shedding of microvescicles containing IL-1β from the plasma membrane (Bianco et al., 2005). There is also the possibility the IL-1β is secreted directly across the plasma membrane even if a transport protein has not been identified (Brough and Rothwell, 2007) or that proIL-1β could be cleaved in the extracellular milieu at sites of tissue injury (Simi et al., 2007).

Figure 1.2. represents the processing and secretion of IL-1β.

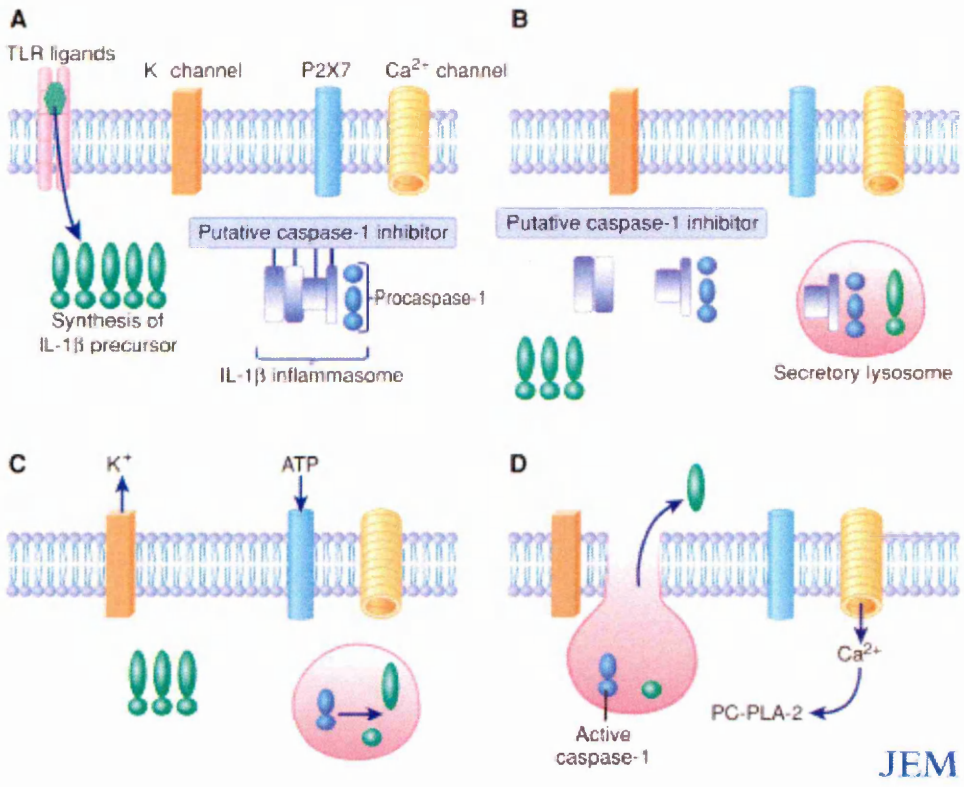


Figure 1.2. Schematic representation of processing and secretion of IL-1β
Various stimuli including TLR ligands and seizures trigger gene expression and synthesis of the IL-1β precursor, which remains in the cytosol. In the same cell, inactive procaspase-1 is bound to components of the IL-1β inflammasome, that is kept in an inactive state by binding to a large molecular weight putative inhibitor (A). After brain stimuli, most of synthesized proIL-1 β remains in the cytosol, while a fraction moves into secretory lysosomes where pro-IL1β colocalizes with the procaspase-1 (B). Activation of the $P2X_7$ receptor by ATP initiates the efflux of potassium from the cell via a potassium channel. The efflux of potassium activates the autocatalytic processing of procaspase-1. Active caspase-1 cleaves the IL-1β precursor in an active cytokine (C). The efflux of potassium ions results in the influx of calcium ion, which in turn activate phospholipases. Phosphatidylcholine-specific phospholipase C (PC-PLA-2) facilitates lysosomal exocytosis and secretion of IL-1 (Dinarello, 2005).

1.3.1.3 IL-1 signal transduction pathways

When IL-1β binds IL-1R1, IL-1R-AcP associates to the receptor constituting an active heterodimer that results in the formation of a signaling complex responsible for the activation of NFkB and MAPK pathways (Brikos et al., 2007; Dinarello, 1996; Wesche et al., 1997). The cytoplasmic domains of IL-1R1 and IL-1R-AcP are known as Toll/IL-1R

(TIR) domains since they are also found in the cytoplasmic tail of TLRs. The formation of IL-1R1 and IL-1R-AcP complex recruits the myeloid differentiation factor 88 (MyD88) an adaptor protein that possesses a C-terminal TIR domain which interacts with TIR domain of IL-1RI and N-terminal *death domain* (DD) (Burns et al., 1998). MyD88 activates the IL-1R-associated kinases (IRAKs), serine-threonine kinases whose recruitment is mediated by Toll-interacting protein (Tollip), a scaffolding protein that normally inhibits IL-1R1 signalling blocking the phosphorilation of IRAK (Burns et al., 2000; Zhang and Ghosh, 2002). After activation, IRAK interacting with MyD88 is hyperphosphorilated and can dissociate from TOLLIP complex inducing a TOLLIP phosphotylation (Burns et al., 2000; Cao et al., 1996). Thus, IRAK can interact with TNF receptor-associated factor (TRAF)6, another adaptor protein that becomes ubiquinated and forms a complex with TRAF-associated kinase (TAK)-1 (Cao et al., 1996). TAK-1 activated by phopshorylation is involved in the activation of NFkB and MAPK pathways.

1- NFkB-mediated pathway

The pathway leading to NF-κB activation starts with the phosphorylation of the *NF-κB-inducing kinase* (NIK) by TAK-1 (Stancovski and Baltimore, 1997). NIK, in turn, phosphorylates and activates the Inhibitor of NF-κB (IkB) kinase complex (IKK) that is formed by the catalytic subunits α and β and one regulatory subunit NEMO/IKKγ. Finally, the activated kinase IKK phosphorylates IκB which is subsequently ubiquitinated and then degraded by proteosome. The released NF-κB translocates to the nucleus where it exerts its action as transcription factor (Mattson and Camandola, 2001). Following its degradation IkB is resynthesized and acts as NFkB endogenous inhibitor (Ghosh and Karin, 2002).

2- MAP kinase-mediated pathway

Mitogen-activated kinases (MAP kinases) are enzymes that require phosphorylation on threonine and tyrosine subunits in order to become activated. In particular, MKK3 and MKK4 are responsible for p38 phosphorylation that activates the *MAPK-activated protein kinases* (MAPKAP-Ks) 2 and 3, which in turn contribute to phosphorylation of CREB and SRF.

MKK4 and MKK7 are involved in JNK phosphorylation that activates transcription factors such as c-jun and AP-1 (Derijard et al., 1994; Kyriakis et al., 1994).

3- Sphingomyelinase-mediated pathway

The activation of IL-1R1/IL-1R-AcP/MyD88 complex by IL-1β results also in the stimulation of the sphingomyelin signaling pathway (Bankers-Fulbright et al., 1996). Sphingomyelin is present in the outer leaflet of the plasma membrane and can be hydrolyzed by sphingomyelinase to ceramide, a second messenger that diffuses readily through the lipid bilayer. Ceramide has been reported to activate both kinase and phosphatase, suggesting a critical role in regulation of phosphorylation state of a cell following IL-1 stimulation (Kolesnick and Golde, 1994). Two kinds of sphingomyelinase exist. The acidic one is localized to the lysosomal compartment and requires DAG for activation. The neutral sphingomyelinase colocalized with sphingomyelin at the plasma membrane and this particular position makes this enzyme involved in receptor signal transduction (Bankers-Fulbright et al., 1996). Recently, IL-1β-induced sphingomyelin signaling pathway has been described in hypothalamic neurons where IL-1β induces the production of ceramide and the subsequent tyrosine phosporylation of NR2B subunit via the

activation of Src kinases (Sanchez-Alavez et al., 2006). This pathway is activated within minutes by IL-1β application and mediates the IL-1β-induced rapid phase of fever onset in mice (Sanchez-Alavez et al., 2006).

The specific intracellular pathway activated by IL-1β may differ in distinct cell types and may mediate its diverse biological effects (Srinivasan et al., 2004).

A schematic representation of IL-1R1 signal transduction pathways is depicted in Fig. 1.3.

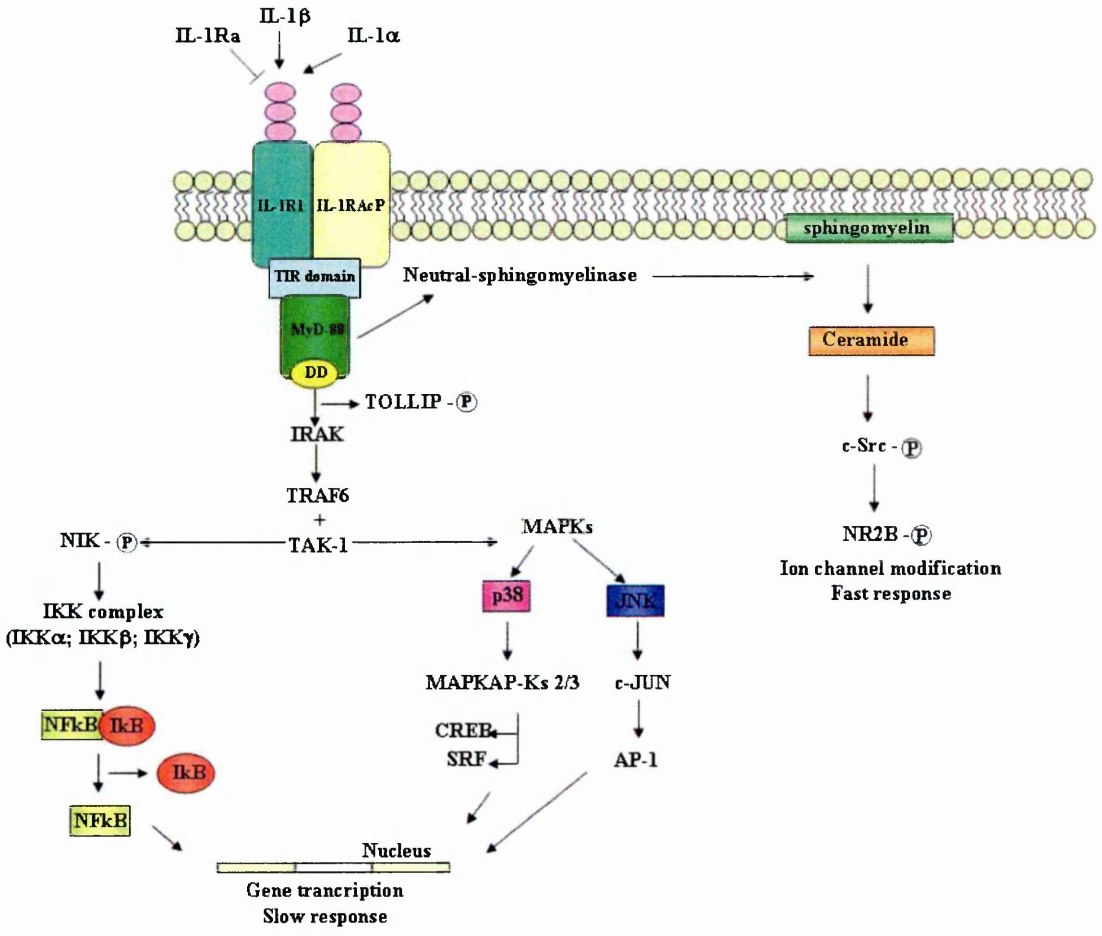


Figure 1.3. Simplified representation of IL-1R1 signalling pathway

1.3.2 TNF-α system

TNF-α is expressed as 26-kDa transmembrane precursor protein that is cleaved by metalloproteinase *TNF-α converting enzyme* (TACE) to constitute the 17-kDa mature form which is subsequently released (Vandenabeele et al., 1995) and is the responsible for the majority of the biological effects of this cytokine. Mature TNF-α is trimeric and the formation of this structure appears to be essential for TNF-α biologic activity.

TNF-α is active either as a membrane-bound or as a soluble form and although it is usually produced in very low amounts in resting cells, it quickly becomes one of the major secreted factors upon cell stimulation and brain injury (Shohami et al., 1999). Indeed, the TNF-α gene is one of the immediate early genes induced by a variety of stimuli, including TNF-α itself and IL-1, and it is known that the activation of p38-MAPK triggered by IL-1 and TNF-α signaling enhances the translational efficiency of TNF-α mRNA (Kotlyarov et al., 1999; Neininger et al., 2002).

Post-translational regulation of TNF-α is accomplished by TACE which has been identified as a metalloproteinase (MMP)-like enzyme (Black et al., 1997; Moss et al., 1997) whose activity can be blocked by MMP inhibitors (Mohler et al., 1994).

TNF-α exerts its biological effects via interaction with two receptors named *TNF receptor type I* (TNFR1) or p55 and *TNF receptor type 2* (TNFR2) or p75 (Vandenabeele et al., 1995). Both receptors are single transmembrane glycoproteins with 28% of homology in their extracellular domain that contains four repeated cysteine rich motifs (MacEwan, 2002a). The intracellular domains of the two receptors, however, are completely distinct indicating that they can lead to different signaling pathway (Vandenabeele et al., 1995). TNFR1 and TNFR2 exist also in soluble forms obtained by the cleavage of their

extracellular domain via metalloproteinases, still preserving the capability to bind, and possibly neutralize, TNF-α (Crowe et al., 1995; Mullberg et al., 1995).

Controversial experimental results have been reported about TNF-α affinity for its receptors (Grell et al., 1998; Tartaglia et al., 1993). Murine TNF activates mouse TNFR1 and TNFR2 equally, whereas human TNF acts on mouse TNFR1 but does not bind mouse TNFR2 (Lewis et al., 1991).

In rodent brain, TNFR1 mRNA was detected mainly in the circumventricular organ, choroids plexus, leptomeninges, ependymal cells, blood vessels (Nadeau and Rivest, 1999), frontal cortex and hippocampus (Bette et al., 2003). TNFR2 mRNA showed a moderate in situ hybridization signal in the hippocampus (Nadeau and Rivest, 1999) and in numerous cells within the brain with microglial-like morphology (Bette et al., 2003). Both receptors can be rapidly upregulated following LPS or TNF-α stimulation.

TNF-α-induced signaling starts with the preassembling of TNFRs on the cell membrane which stabilize in a trimeric structure upon activation by TNF-α binding. The formation of this receptor precomplex is mediated by the extracellular pre-ligand-binding assembly domain (PLAD) that is distinct from ligand binding regions and is present in both receptors (Chan, 2000).

1.3.2.1 TNFR1 signal transduction pathway

Similarly to IL-1R1, the intracellular tail of TNFR1 contains a DD that, in the absence of ligand binding, is bound to a protein known as *silencer of death domain* (SODD) which prevents cytoplasmic DD-containing proteins to interact with TNFR1. SODD is then

removed upon ligand binding, allowing the access to DD receptor module (MacEwan, 2002a; MacEwan, 2002b).

Indeed, the activation of TNFR1 upon TNF-α binding induces the receptor trimerization and the recruitment of an adaptor protein called TNFR1-associated death domain (TRADD) to its cytoplasmic domain. TRADD serves as a platform to recruit three additional signalling proteins, Fas-associated death domain protein (FADD), receptor-interacting protein-1 (RIP-1) and TNFR-associated factor 2 (TRAF2) (Baud and Karin, 2001).

FADD is the major adaptor protein involved in the TNFR-1 pro-apoptotic pathway since it contains a death effector domain (DED) that allows the interaction and activation of caspase-8 followed by activation of caspase-3 and -6 (Chen and Goeddel, 2002). The TRADD/FADD/caspase-8 protein assembly is known as a *death-inducing signaling complex* (DISC) and is dependent on the internalization of activated TNFR1 complex (TNF receptosomes) that regulates the TNFR1 activation and inhibits its long-term action (Schneider-Brachert et al., 2004).

The RIP-1 protein appears to mediate anti-apoptotic activity since it has been shown to be a key effector in the activation of NF-κB by TNFR1 (Liu et al., 1996; Ting et al., 1996) while it does not affect MAPK activation (Kelliher et al., 1998).

TRAF2 is important in order to trigger the activation of NFkB and the MAP kinase cascades which result in both JNK and p38 induction (Baud and Karin, 2001; Devin et al., 2000; Tada et al., 2001). The pathways leading to NF-κB activation can be induced by the recruitment of NIK that associates with TRAF2 and mediates the activation of NF-κB through IKK (Malinin et al., 1997). Moreover, it has been also shown that MEEK1 induces the phosphorylation and degradation of IκBα, resulting in the activation of NF-κB (Hirano

et al., 1996; Lee et al., 1997; Nakano et al., 1998) thus also this enzyme could mediate the TRADD-TRAF2 activation of NF-κB. Other kinases such as apoptosis-stimulating kinase (ASK1), germinal centre kinase (GCK) and MEEK1 are associated with TRAF2 and are implicated in the activation of p38MAPK and c-JNK (MacEwan, 2002a).

Besides the DD domain, TNFR1 has also another functional intracellular region named neutral sphingomielinase activation domain (NSD) (Kolesnick and Kronke, 1998) that recruits the factor associated with neutral sphingomielinase (FAN). FAN activates the neutral sphingomielinase that triggers the degradation of sphingomyelin into ceramide. This second messenger can further activate *ceramide-activated protein kinase* (CAPK) which then phosphorylates cytoplasmic raf-1 inducing the activation of MAPK activity (MacEwan, 2002a; Shohami et al., 1999). Ceramide has also been found to bind the protein kinase c(PKC)-ζ which can activate raf, MEK, MAPK and NFkB transcription (MacEwan, 2002a).

Also *acidic sphingomyelinase* (A-Smase) can be induced upon TNFR1 activation through the induction of *phosphatidylcholine-specific phospholipase C* (PC-PLC) that yields to cleavage of phosphatidylcholine into choline and diacylglycerol (DAG). The last is involved in the activation of *acidic sphingomyelinase* that can in turn induce the ceramide-mediated stress-activated kinase, JNK and p38 and seems to contribute to apoptosis (Shohami et al., 1999).

The activation of p38 induces also the phosphorylation of PLA_2 that results in the generation of the arachidonic acid metabolites, leukotrienes and prostaglandins, which are known to contribute to TNF proinflammatory activities (MacEwan, 2002a).

1.3.2.2 TNFR2 signal transduction pathway

The role of TNFR2 in cellular responses is not fully understood since its activation has been shown to be proliferative and cell protective but it is also known to have a function in regulating TNF-induced apoptosis. TNFR2 contains a so-called TRAF-interacting motif (TIM) in its cytoplasmic domain. Upon TNF-α activation, TNFR2 recruits the TRAF-family members (TRAF2 and TRAF1) that are involved in the activation of signaling pathways such as NFkB, JNK, ERK1/2, p38 and phosphoinositide 3-kinase (Hehlgans and Mannel, 2002). This can result in cytokine production, but also in the expression of anti-apoptotic activity and cell protective effects activating the antioxidant enzyme Mn-superoxide dismutase, the calbindin and anti-inflammatory cytokines (Shohami et al., 1999; Wang et al., 1998).

TNFR2 does not contain the DD as TNFR1, but can induce apoptosis through a so-called ligand-passing mechanism. Thus, TNFR2 binds TNF-alpha, increases its local concentration close to TNFR1 which by accepting TNF ligand by TNFR2 becomes activated and induces the TNFR1 apoptotic machinery (MacEwan, 2002b). TNFR2 is able to signal apoptosis also directly since RIP and FADD are bound to TNFR2 via TRAF2, resulting in the activation of caspase cascade (MacEwan, 2002b). Thus, several factors such as different signaling pathway activated by each receptor (MacEwan, 2002a; Thommesen and Laegreid, 2005), the pattern of TNF-α receptor expression on neurons and glia (Akassoglou et al., 2003; Fontaine et al., 2002), changes in TNFR1/TNFR2 ratio at the plasma membrane (Grell et al., 1995), the regional differences in TNF-α cellular expression across brain regions play a critical role in determining the final outcome on neuronal functions (Sriram and O'Callaghan, 2007).

A schematic representation of TNFR1 and TNFR2 signal transduction pathways is depicted in Fig. 1.4.

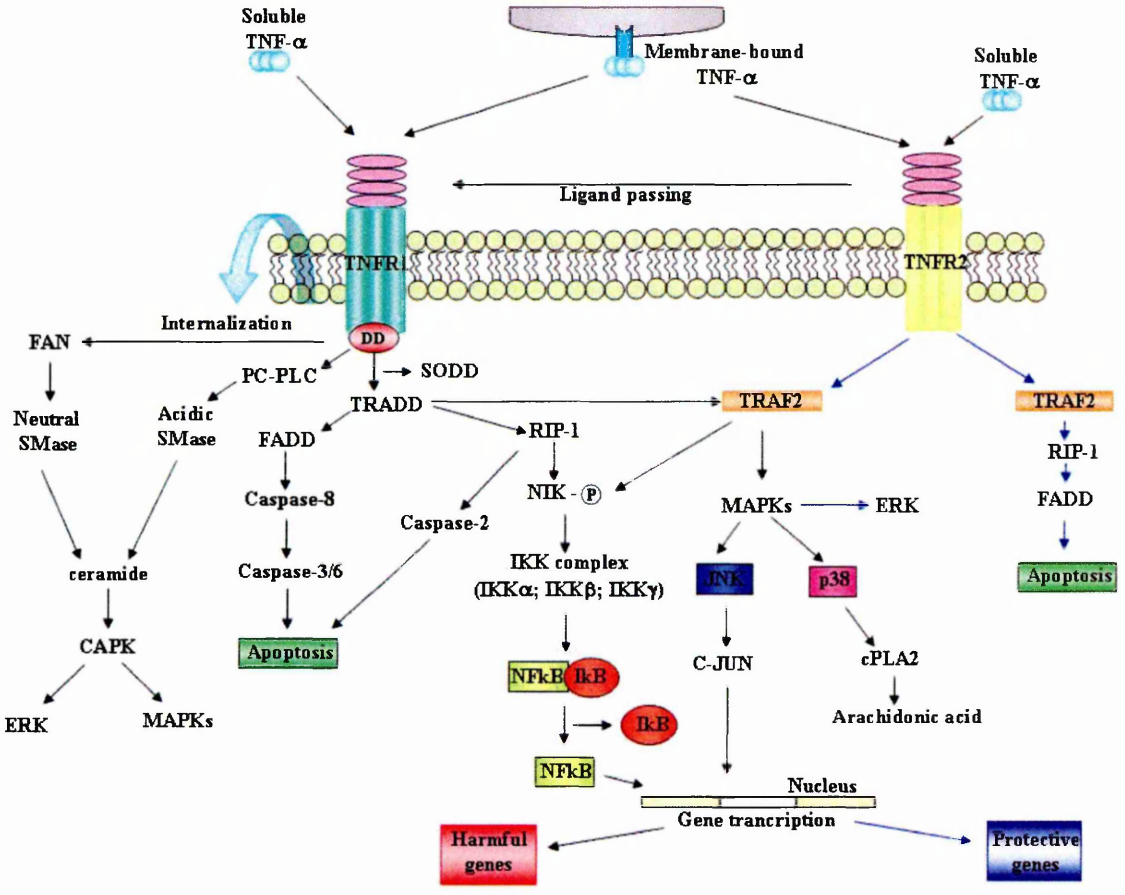


Figure 1.4. Simplified representation of TNFR1 and TNFR2 signaling pathway

1.4 Epilepsy and brain inflammation

1.4.1 Experimental models of epilepsy

Experimental models of epilepsy are necessary to study the pathophysiology of the disease and to find new targets for developing drugs. These aims can be addressed using two distinct animal models of seizures, namely acute and chronic models.

Acute models are represented by seizures induced either chemically or electrically that elapse within minutes (MES, pentylentetrazol) to hours (kainic acid, pilocarpine) from their induction. Seizures can be induced by intrahippocampal application of chemoconvulsant agents such as kainate or bicuculline (Vezzani et al., 1999). The epileptic activity is characterized by discrete ictal episodes followed by spiking activity recurring for about 2 hours from their onset. Epileptic activity in these models is highly reproducible and can be quantified by measuring the onset time to the first ictal event, number and duration of ictal events and interictal activity. These acute models are widely used to study the effect of various pharmacological treatments on the onset and maintenance of ictal activity, and to understand the molecular mechanisms underlying ictogenesis (Jefferys, 2003).

Chronic models are represented by recurrent spontaneous seizures induced by an initial precipitating event. Among the various injuries, status epilepticus is the most widely used experimental stimulus to induce epilepsy and it is responsible for 10% of all acquired human epilepsies (Loscher and Schmidt, 2004; Loscher, 2002). Status epilepticus can be induced by sustained electrical stimulation of hippocampus or amygdala or by administration of chemical convulsants such as pilocarpine or kainate (Buckmaster, 2004; Loscher, 2002). Status epilepticus is followed by a latent period corresponding to epileptogenesis that last few days to weeks depending on the severity of initial insult

(Williams et al., 2007). After this period, spontaneous recurrent seizures can be observed. A pattern of hippocampal sclerosis similar to that found in MTLE, characterized by neuronal cell loss and astrogliosis, is induced in these models.

Chronic models of epilepsy are useful for studying the epileptogenic process including the acute stage of the disease, i.e. status epilepticus, the period of epileptogenesis and the spontaneous recurrent seizures.

1.4.2 Kainic acid-induced seizures

Kainic-acid (KA), isolated from a red alga found in tropical water, is a potent agonist of the ionotropic glutamate AMPA/Kainate subtype receptors. The local or systemic injection of KA was found to induce seizures followed by neuronal cell death in forebrain areas, particularly in the hippocampal subregions CA1, CA3 and in the hilus of dentate gyrus (Ben-Ari and Cossart, 2000; Leite et al., 2002; Oprica et al., 2003; Wang et al., 2005). Induction of seizures by kainate, and the subsequent neurodegenerative pattern, is triggered by activation of high-affinity kainate receptors present in CA3 pyramidal neurons and mossy fiber synapses (Vincent and Mulle, 2008). In particular, the postsynaptic kainate receptors containing GluR6 subunits seem to have an important role in kainite-induced seizure generation since mice deficient in GluR6 subunits did not show the synaptic currents generated in CA3 by the stimulation of mossy fibers, and these mice are less susceptible to systemic administration of kainate than control mice (Ben-Ari and Cossart, 2000). Because of the dense network of recurrent glutamatergic collaterals, the firing of CA3 pyramidal neurons is able to generate synchronized activities that can propagate to other limbic structures (Ben-Ari and Cossart, 2000). The activation of CA3 pyramidal

neurons is followed by release of the endogenous excitatory aminoacids glutamate and aspartate (Ferkany et al., 1982) which in turn activate all subtypes of glutamate receptors favouring the propagation of seizures.

These events are followed by neurodegeneration which appears prominent in hippocampus, entorhinal and piriform cortex, thalamus and amygdala (Sperk et al., 1985). In particular, cell loss mostly affects CA3 pyramidal cells and interneurons in the dentate gyrus (Ben-Ari, 1985) followed by CA1 neurons (Phelps et al., 1991; Sperk et al., 1985). Cell death appears to occur through both necrotic and apoptotic mechanisms (Ferrer et al., 1995; Popescu et al., 2002; Simonian et al., 1996). CA2 pyramidal neurons as well as granule cells are the most resistant to neurodegeneration. This different susceptibility to kainate-induced damage can be related to a different expression of kainate receptors (Malva et al., 1998; Wang et al., 2005). The ability of kainic acid to induce neurotoxicity varies depending on the route of administration, the dose and the animal species and strains (Schauwecker, 2002).

1.4.3 Inflammation in experimental models of epilepsy

Activation of inflammatory pathways resulting in the production of cytokines and related inflammatory mediators have been described in the brain after seizures in various experimental models (De Simoni et al., 2000; Jankowsky and Patterson, 2001; Lehtimaki et al., 2003; Plata-Salaman et al., 2000). This indicates that epileptic activity can stimulate an innate immune response inducing a pattern of inflammatory mediators which in part overlaps with those induced by systemic administration of LPS. However, notable differences between the activation of immune system by endotoxemia or seizures include the time-course of these events and the brain regions and cell populations involved (Rivest,

2003; Turrin and Rivest, 2004). Proinflammatory cytokines are induced transiently by LPS in CVOs and along microvessels and the involvement of parenchymal microglia cells is delayed of several hours and regionally restricted. Neurons do not typically express inflammatory markers after LPS administration (Laflamme and Rivest, 2001). On the other hand, seizure activity rapidly increases proinflammatory cytokines (IL-1β, TNF-α, IL-6) and markers of innate immunity, such as NFkB system, cyclooxygenase-2, prostaglandins, TLRs, complement system, and this induction can be long-lasting and widespread as shown in different experimental models of seizures including status epilepticus, kainic acid, kindling, audiogenic seizures (Gorter et al., 2006; Jankowsky and Patterson, 2001; Turrin and Rivest, 2004). The main source of these proinflammatory mediators in the brain following seizures are microglia and astrocytes. Neurons can also produce cytokines and their related molecules (De Simoni et al., 2000; Ravizza et al., 2008a; Turrin and Rivest, 2004). These inflammatory events involve brain structures such as cortex, amygdala, thalamus, hypothalamus, and hippocampus (Turrin and Rivest, 2004) recruited in the generation and propagation of seizures (Oprica et al., 2003; Ravizza et al., 2008a).

The lasting stimulation of the innate immune response and related inflammatory reactions observed after seizures may eventually promote infiltration of lymphocytes and the establishment of acquired immunity in the CNS. However, it has been reported that markers of adaptive immunity, such as production of IL-12 and IFN-γ by activated T-cells, are undetectable across the brain of pilocarpine-treated mice, at least up to 72 h after seizure induction (Turrin and Rivest, 2004). Accordingly, immunostaining for T-cells, B-cells and NK-cells were rarely found in the brain parenchyma after electrical status epilepticus induction (Ravizza et al., 2008a). Thus, the innate immune response does not appear to be

necessarily associated with adaptive immune and B- or T-cells infiltration in experimental models of TLE.

The role of inflammation in epilepsy raises several questions:

1- whether the inflammatory reactions are only a consequence of seizures and cell damage or contribute to the etiopathogenesis of seizures.

2- whether inflammation mediates structural and molecular changes during the epileptogenic process that can influence the progression of the disease.

The current hypothesis is that an exagerated and lasting immune response, as occurs after seizures, can be detrimental to CNS because of sustained production of cytokines in specific brain areas. This may contribute to establish a pathological substrate (cell death, neuronal hyperexcitability, BBB damage) playing a role in epileptogenesis and in the recurrence of seizures (Vezzani and Granata, 2005).

1.4.4 Expression studies of IL-β, TNF-α and their receptors in experimental models of seizures

After the characterization of the time-course profile of specific proinflammatory events occurring in rodent brain following the induction of seizures, preclinical investigations have focused on the brain regional distribution and cell-specific expression of proinflammatory molecules and their signaling. This experimental approach allows an understanding of which cells are involved in the production of, or are targeted by these proinflammatory molecules. We focused on IL-1β and TNF-α that are rapidly upregulated following the induction of seizures in rodents.

1.4.4.1 IL-1β and its receptor

In physiological condition, IL-1 system is expressed at low levels and seems to implicate in modulation of synaptic plasticity. It has been reported that IL-1β can contribute to long term potentiation (Schneider et al., 1998), a process considered to underlie certain forms of learning and memory. Indeed, IL-1ra inhibited the maintenance phase of long term potentiation suggesting that endogenous IL-1 is involved in this phenomenon (Schneider et al., 1998). There is also evidence that endogenous IL-1β influences sleep patterns, because IL-1ra reduces slow-wave sleep even in the absence of injury (Opp et al., 1991) and IL-1R1 receptor knockout mice have been reported to sleep less compared with their corresponding wild-type controls (Kruger et al., 1998).

The increase in mRNA and protein levels of IL-1β is very rapid (≤30min) and long-lasting reaching the maximal increase within 6h after the induction of electrically-induced status epilepticus (De Simoni et al., 2000). IL-1β is still upregulated 60 days after the induction of status epilepticus in rats with spontaneous seizures (De Simoni et al., 2000). Recent immunohistochemical studies confirmed that IL-1β is rapidly upregulated after the induction of SE and this increase persists also during epileptogenesis and in chronic epileptic tissue (Ravizza et al., 2008a). In particular, during the acute phase of status epilepticus, IL-1β is produced by both microglia and astrocytes whereas only astrocytes showed enhanced immunostaining during epileptogenesis, suggesting that astrocytes are the main source of IL-1β during the latent phase. In the epileptic tissue, the cell expression of the cytokine correlates with the frequency of seizures: rats previously treated with pilocarpine which developed chronic epilepsy with a relatively low seizure frequency

(1seizures/3days) showed an enhanced IL-1β staining only in astrocytes while rats with higher frequency of seizures (1seizure/1day) produced IL-1β in astrocytes, microglia and scattered neurons. This evidence suggests that the severity of epileptic activity can influence the extent of inflammation (Ravizza et al., 2008a).

IL-1R1, which mediates the biological responses to IL-1β, is barely detectable in control brain, but is rapidly upregulated in hippocampal neurons within 2h from status epilepticus, and a late wave of expression is observed during epileptogenesis also in astrocytes. This pattern of neuronal and astrocytic IL-R1 expression persists in chronic epileptic tissue (Ravizza et al., 2008a; Ravizza and Vezzani, 2006). Thus, IL-1β, produced and released by glial cells during the initial injury, may interact with its neuronal IL-1R1 (Vezzani et al., 1999; Vezzani et al., 2002). IL-1R1 expression in astrocytes indicates that IL-1β has autocrine and paracrine effects establishing functional communication between glia and neurons. Recent findings support that cytokines released by glia have a neuromodulatory action on neurons by affecting ionic conductance (Viviani et al., 2003) and synaptic plasticity (Bezzi et al., 2001b).

Strong IL-1β and IL-1R1 immunoreactivity was found during epileptogenesis in perivascular astrocytic endfeet impinging on blood vessels and in endothelial cells of microvasculature. This increased expression is associated with enhanced BBB permeability to serum albumin (Ravizza et al., 2008a; van Vliet et al., 2007) that can contribute to neuronal hyperexcitability (Oby and Janigro, 2006; Seiffert et al., 2004).

The production of pro-inflammatory cytokines is accompanied by the concomitant synthesis of anti-inflammatory mediators apt to modulate the inflammatory response and to prevent the occurrence of deleterious effects. In particular, IL-1ra, an endogenous competitive

antagonist of IL-1β receptor type 1, is released after seizures to a similar extent of IL-1β and with a delayed time-course (De Simoni et al., 2000). This pattern of induction differs from the peripheral immune response where IL-1ra is produced 100-1000 fold in excess and together with IL-1β (Dinarello, 1996), suggesting that the brain is less effective to induce a crucial mechanism that inhibits the deleterious effects of IL-1β.

1.4.4.2 TNF-α and its receptors

Pilocarpine-induced seizures in mice increase mRNA levels of TNF-α in the cerebral cortex, amygdala, thalamus and hippocampus within 6h from status epilepticus induction (Turrin and Rivest, 2004). TNF-α mRNA and protein can be induced in the brain as early as 2h following electrically-induced seizures (De Simoni et al., 2000; Godlevsky et al., 2002; Plata-Salaman et al., 2000) and also in blood serum (Shandra et al., 2002). Evidence of increased production of TNF-α in hippocampus has been also reported after kainic-acid induced seizures (de Bock et al., 1996; Lehtimaki et al., 2003). The increase of TNF-α level is reversible since it returns to control levels after 3 days (De Simoni et al., 2000; Vezzani et al., 2002; Lehtimaki et al., 2003)) suggesting that the upregulation of TNF-α is time-locked to ongoing epileptic activity.

Immunohistochemistry and in situ hybridization analysis of TNF-α mRNA demonstrated that both parenchymal and perivascular microglia are the main cellular source of this molecule in the brain of pilocarpine treated mice (Turrin and Rivest, 2004). Bruce at al. (1996) has demonstrated that mice lacking TNF receptors show a reduced microglial activation following kainate injection indicating that TNF-α signaling plays an important

role in microglial response to injury (Bruce et al., 1996). TNF-α immunoreactivity was found in glia in the hippocampus 18-48h after electrically-induced seizures (De Simoni et al., 2000; Vezzani et al., 2002). Immunostaining was also elevated in neuronal cells, especially in CA1 and CA3 regions 4h after seizures induced by application of kainic acid in mouse hippocampus (Bruce et al., 1996).

TNF-α can bind two different receptors, p55 and p75 and little is known about the regulation of TNF-α receptors during epileptic activity: TNF-α receptors are barely detecteble in normal brain tissue but they are rapidly upregulated in neurons and astrocytes during seizures (Vezzani and Granata, 2005). These data indicate that TNF-α, as IL-1β, can act as a soluble mediator of functional glio-neuronal communication (Beattie et al., 2002; Bezzi et al., 2001b; Stellwagen et al., 2005). TNF-α can induce the upregulation of its own receptors as demonstrated after LPS administration (Bette et al., 2003; Nadeau and Rivest, 1999).

1.4.5 Effect of IL-1β and TNF-α on seizures

Two main experimental approaches were used to investigate the role of inflammation in epilepsy: 1- proinflammatory cytokines were injected in the rodent brain before convulsive stimuli to mimic a pro-inflammatory state 2- a chronic inflammation was induced using transgenic mice overexpressing cytokines in glia.

1.4.5.1 IL-1β and seizures

Experimental models of seizures have shown that IL-1β significantly exacerbates seizures. The pre-application of IL-1β in rodent brain, using concentrations endogenously produced during seizures, increases the duration of electrographic and behavioural seizures induced by intracerebral application of kainic acid or bicuculline methiodide (Vezzani et al., 1999; Vezzani et al., 2000). Furthermore the intracerebroventricular (icv) injection of IL-1ra has powerful anticonvulsant activity (Vezzani et al., 2002) and transgenic mice overexpressing IL-1ra in astrocytes show a reduced susceptibility in seizures (Vezzani et al., 2000). The impairment of endogenous production of IL-1β in mice with a deletion of the caspase-1 gene significantly reduces seizures (Ravizza et al., 2006b). These findings suggest that an endogenous increase of IL-1β has proconvulsive effects and contributes to the maintenance of seizures.

Recent studies have also addressed the possibility of the IL-1β involvement in fever-related seizures. Icv injection of IL-1β reduces the seizure threshold in 14-day old mice subjected to hyperthermia while IL-1β receptor-deficient mice were resistant to these seizures (Dubè et al., 2005). Moreover, in another experimental model of febrile seizures induced in 14-old rats by LPS followed by subconvulsant dose of kainic acid, icv injection of IL-1β increased the number of seizing animals while IL-1ra administration had anticonvulsant properties (Heida and Pittman, 2005). These data indicate that IL-1β system may play an important role in fever-induced hyperexcitability inducing febrile seizures (Vezzani and Baram, 2007; Vezzani and Granata, 2005).

However, one report showed that IL-1β retarded the acquisition of amygdala kindling in rats but this study used daily icv injections of IL-1β at doses 100 times lower than those used to exacerbate seizures (Sayyah et al., 2005). Thus, the concentration of IL-1β in brain tissue is a crucial factor to determine the consequences on neuronal excitability. In particular, high doses of IL-1β such as those produced during epileptic activity seem to mediate the proconvulsant and deleterious effects of this cytokine.

1.4.5.2 TNF-α and seizures

Little is known about the functional role of TNF-α and its receptors on seizures. The available data are apparently controversial and indicate that TNF-α effects likely depend on its brain levels and the activated receptor subtypes. In experimental models of seizures with focal onset it is reported that intraperitoneal injection of human TNF-α, which selectively activates the p55 receptor, prolonges behavioural seizures in amygdala-kindled rats (Shandra et al., 2002). However, a protective role of TNF-α on seizures has been recently proposed using mice with a genetic deletion of the p55 receptor, since these mice were more susceptible to kainic acid-induced seizures and displayed enhanced neurodegeneration and glial activation compared to wild-type mice (Lu et al., 2008).

The effect of TNF-α on seizures was also studied in experimental models of infection-related seizures. Blockade of TNF-α converting enzyme, which reduces the soluble form of TNF-α, attenuated seizure activity and cortical brain damage in a rat model of bacterial meningitis (Meli et al., 2004). Yuhas and colleagues (2003) have shown bidirectional concentration-dependent effects of TNF-α in pentylentetrazole-induced seizures related to

Shigella dysenteriae. In particular, high concentrations of TNF-α prevent enhanced seizure susceptibility caused by Shigella infection while lower concentrations were proconvulsive (Yuhas et al., 2003).

High expression of TNF-α in transgenic mice induces neurodegenerative changes and sporadic spontaneous seizures in an age-dependent manner, suggesting that chronic high levels of TNF-α may be deleterious on brain functions (Akassoglou et al., 1997; Probert et al., 1995; Stalder et al., 1998).

These data indicate that chronic inflammation can enhance the predisposition of brain tissue to develop seizures and neuronal cell loss. This concept was also demonstrated by the systemic administration of LPS to mice that reduced the threshold to pentylentetrazole-induced seizures and this effect was blocked by anti-inflammatory drugs (Sayyah et al., 2003). Repetitive LPS administration in a genetically epileptic rat strain increased the number of spike-wave discharges (Kovacs et al., 2006). Moreover, a first exposure to seizures in 15-old rats was associated with chronic glial cells activation and the development of a lower threshold for seizure induction in adulthood (Somera-Molina et al., 2007). Proinflammation state induced by LPS administration can increase also seizure-related neuronal damage (Auvin et al., 2007).

1.4.6 Anti-inflammatory drugs in experimental models of seizures

Experimental models of seizures have also been used to evaluate the effects of anti-inflammatory drugs on epileptic activity. Various pharmacological studies reported inhibition of penicillin-induced seizures using nonsteroidal anti-inflammatory drugs such as ibuprofen, paracetamol and indomethacin (Wallenstein, 1987), and an anticonvulsant effect

was achieved by injection of nimesulide or rofecoxib in mice treated with bicuculline or picrotoxin (Dhir et al., 2006). Chronic administration of celecoxib, a cyclooxygenase-2 inhibitor, reduced the frequency and the duration of spontaneous recurrent seizures induced by pilocarpine in rats. This treatment prevents neuronal cell death, microglia activation and abnormal neurogenesis during the latent period (Jung et al., 2006) suggesting that COX-2 induction may have an important role during the epileptogenesis.

Aspirin protected mice from maximal electroshock test and pentylenetetrazole-induced seizures and potentiated the anticonvulsant action of diazepam and sodium valproate (Srivastava and Gupta, 2001). On the other hand, pretreatment of rats with indomethacin, aspirin, nimesulide enhanced kainic-acid induced seizures (Baik et al., 1999; Kunz and Oliw, 2001). These conflicting data on the effect of COX-1 and 2 inhibitors on seizures can be explained by considering that prostaglandins can either reduce or increase seizures. The final outcome on neuronal excitability and excitotoxicity may depend on the types of prostaglandins that are produced during seizures and which kind of prostaglandins receptor is activated (Vezzani and Granata, 2005).

It is interesting to consider that sodium valproate (VPA) and carbamazepine (CBZ) have also anti-inflammatory actions. VPA inhibits the LPS-induced activation of NFkB and the production of TNF-α and IL-6 in monocytes and glioma cells; CBZ inhibits the LPS-induced production of PG in rat glial cells (Ichiyama et al., 2000; Matoth et al., 2000).

Glucocorticoids have potent antiinflammatory effects inhibiting the transcription of genes encoding for proinflammatory molecules. The inhibition of glucocorticoid signaling increased the inflammatory reactions induced by LPS in the brain because the inhibition of cytokines production by glucocorticoids was lost (Nadeau and Rivest, 2003). However

prolonged exposure of the tissue to glucocorticoids can have detrimental effects on neuronal survival and enhance kainic acid induced seizures (Lee et al., 1989), likely due to the ability of glucocorticoids to induce a catabolically vulnerable state to neurons by inhibiting glucose uptake and glycogen and protein synthesis (Dinkel et al., 2003).

Immunoglobulins injection protected cats against generalized seizures induced by electrical stimulation of amygdala (Hirayama et al., 1986). This effect can be in part due to their antiinflamamtory effects (Ichiyama et al., 2004).

1.4.7 Pro-inflammatory cytokines and seizure-associated neuronal cell death

Neurodegeneration within the hippocampus is a hallmark of MTLE, a condition linked to neurological dysfunctions such as memory impairment in adult patients and a risk for developing learning disorders in children.

The activation of microglia and astrocytes with subsequent release of cytokines and other potential neurotoxic compounds such as nitric oxide, reactive oxygen species and glutamate are common features of both acute and chronic neurodegenerative diseases, suggesting a potential role for proinflammatory cytokines in the occurrence of neuronal cell death (Allan and Rothwell, 2001; Allan et al., 2005).

A role of inflammation in seizure-induced cell damage is supported by experimental findings showing that proinflammatory molecules produced during epileptic activity are present in the same brain regions involved in neurodegeneration and their increased expression precedes the occurrence of neuronal degeneration (Oprica et al., 2003; Ravizza and Vezzani, 2006; Rizzi et al., 2003; Schultzberg et al., 2007; Vezzani et al., 1999). Sustained inflammation induced in immature rat brain by LPS affects neuronal damage

induced by convulsant, but non neurotoxic, doses of kainate or pilocarpine (Lee et al., 2000; Sankar et al., 2007). Moreover, hippocampal injection of a nonlesional convulsant dose of bicuculline induced inflammation to a lesser extent than in kainic acid-treated rats where seizures are associated with CA3 pyramidal cell loss (Vezzani et al., 1999). This evidence suggests that the extent of inflammatory reactions is an important factor for determining cell survival or death. In this respect, the extent of damage in the hippocampus after kainic acid-induced seizures correlates significantly with the antecedent inflammatory cell infiltration (granulocytes and macrophages) and activation of microglia, indicating that these cells, which are the main source of cytokines, may be a cause rather than a consequence of neuronal cell loss (Choi and Koh, 2008; Dinkel et al., 2003).

It has been proposed that kainic acid-induced seizures promote the formation of a molecular scaffolding complex that includes TNFR1 receptors, TNFR-associated death domains and apoptosis signal-regulating kinase-1. This complex can promote cell death suggesting that the TNF-α-induced activation of TNFR1 may be a crucial event in neuronal cell death associated with seizures (Henshall et al., 2003; Shinoda et al., 2003). In contrast, the stimulation of TNFR2 induced a persistent NF-kB activation that increased the expression of neuronal apoptosis inhibitor protein (NAIP); systemic administration of kainic acid enhanced the expression of NAIP in hippocampus and this increase did not occur in mice lacking both TNF receptors which show increased susceptibility to kainic acid-induced brain injury compared to wild type mice (Thompson et al., 2004).

Cytokines acting on glial receptors can induce the production and release of either cytotoxic or neurotrophic molecules which may contribute to determine whether cells survive or degenerate in hostile conditions. In this respect, IL-1β or TNF-α can exacerbate or reduce

the excitotoxic neuronal damage induced by AMPA in organotypic slice cultures depending on their extracellular concentrations, the period of tissue exposure to these cytokines during injury and which receptor types are activated (Bernardino et al., 2005).

1.4.8 Mechanisms of hyperexcitability and seizure-associated neuronal cell death

It has been shown that the effects of IL-1β and TNF-α on neuronal excitability are related to at least three phenomena, namely an increase of glutamatergic neurotransmission; an enhancement of extracellular glutamate concentration; a reduction of GABAergic neurotransmission.

In particular, recent data have demonstrated that IL-1β produced by glial cells enhances NMDA-mediated inward Ca^{2+} currents in rat hippocampal pyramidal neurons (Viviani et al., 2003; Viviani et al., 2006). This effect is mediated by IL-1R1 that colocalizes with NMDA receptors on dendrites of pyramidal neurons in the post-synaptic density (Viviani et al., 2006), and it is mediated by tyrosine phosporylation of NR2B subunit via the activation of Src kinases (Viviani et al., 2003). The increase of NMDA receptor-mediated Ca^{2+} influx into neurons may play a role in promoting seizure generation and the maintenance of epileptic activity (Vezzani and Baram, 2007). This hypothesis is supported by data indicating that the proconvulsant actions of IL-1β can be inhibited by CPP or ifenprodil, selective NMDA antagonists (Vezzani et al., 1999; Balosso et al., 2008).

IL-1β can also increase the extracellular glutamate concentration by reducing the astroglial glutamate reuptake by a mechanism involving nitric oxide production (Hu et al., 2000; Ye and Sontheimer, 1996) and by inhibiting the astroglial glutamine synthetase, the enzyme that transforms glutamate to glutamine (Huang and O'Banion, 1998). Moreover, IL-1β can

increase the release of glutamate from microglia and astrocyte through the induction of iNOS (Casamenti et al., 1999; Hewett et al., 1994). The increase in glutamate level can facilitate the activation of both ionotropic and metabotropic glutamate receptors, thus playing a role in the generation and spread of epileptic activity. In this respect, it has been recently reported that the astrocytic glutamate release has a role in the genesis or strength of seizure-like events (Fellin et al., 2006; Tian et al., 2005).

IL-1β functionally interacts with GABAergic transmission since at pathophysiological concentrations this cytokine decreases GABA-mediated Cl^- fluxes reducing inhibitory transmission and possibly contributing to hyperexcitability (Wang et al., 2000; Zeise et al., 1997).

TNF-α can also affect glutamate neurotransmission by interacting with AMPA receptors. Beattie et al. (2002) has shown that astrocytic TNF-α, interacting with p55 receptor, induces a rapid increase in neuronal synaptic surface expression of AMPA receptors. This effect leads to a significant increase in the mean frequency of AMPA-induced miniature excitatory postsynaptic currents indicating an enhancement in synaptic efficacy (Beattie et al., 2002). The newly expressed AMPA receptors lack the GluR2 subunit, thus they have a molecular conformation which favors Ca^{2+} influx into neurons. Additionally, TNF-α simultaneously induced endocytosis of $GABA_A$ receptors reducing the inhibitory synaptic strength (Stellwagen et al., 2005). TNF-α-mediated upregulation of surface Ca^{2+}-permeable AMPARs and the decrease of inhibitory neurotransmission may play a role in increasing neuronal excitability (Hermann et al., 2001; Leonoudakis et al., 2004; Yu et al., 2002) and promoting seizure generation.

TNF-α increases the extracellullar glutamate concentration by inhibiting the astroglial glutamate reuptake (Hu et al., 2000; Zou and Crews, 2005) and induces glutamate release from both activated astrocytes (Bezzi et al., 2001a) and microglia (Takeuchi et al., 2006). The increased Ca^{2+} influx into neurons, mediated by the functional interaction between cytokines and glutamate receptors, and the enhancement of extracellular glutamate concentration induced by the cytokines may explain the potentiation of seizures and the neuronal cell damage during a proinflammatory state (Auvin et al., 2007; Choi and Koh, 2008; Dinkel et al., 2003; Lee et al., 2000; Sankar et al., 2007; Somera-Molina et al., 2007). Other mechanisms by which TNF-α and IL-1β participate to seizure-induced brain dysfunction may involve the transcription of proinflammatory mediators such as prostanoids, reactive oxygen species and iNOS with the subsequent production of reactive nitrogen species (Gosselin and Rivest, 2007).

1.4.9 Clinical evidence of brain inflammation in human epilepsy

Clinical evidence supports an activation of the cytokine system and downstream inflammatory events, in the brain, blood and CSF in pediatric and adult patients with epilepsy of various etiologies (Choi and Koh, 2008; Vezzani et al., 1999).

IL-6 is the cytokine consistently found to be significantly enhanced in plasma and CSF of epilepsy sufferers with recent tonic-clonic seizures (Peltola et al., 2000) as well as in children with febrile seizures (Ichiyama et al., 1998; Ichiyama et al., 2008). The increased production of IL-6 plasma levels correlates with the severity of epileptic activity (Lehtimaki et al., 2007).

Contrasting results are reported for IL-1β in CSF of children with febrile seizures where either no increase or significant elevation was measured (Haspolat et al., 2002; Ichiyama et al., 1998). These opposite findings can be explained by differences in time elapsed from the sample collection and the last seizure or by different sensitivity of the assay used for cytokine measurements (Peltola et al., 2000).

Clinical studies carried out on adult patients did not detect any changes of IL-1β in CSF, while increased IL-1ra concentration as well as enhanced IL-6 production were measured (Peltola et al., 2000). The upregulation of IL-1ra and IL-6 levels in CSF can be considered as an indirect evidence of the activation of IL-1 system in human epilepsy because IL-1β can induce the production of both IL-1ra and IL-6 (Dinarello, 1996).

In specimens of focal cortical dysplasia and glioneuronal tumor that are recognized causes of intractable epilepsy, IL-1β and its receptor are highly expressed by glia (astrocytes and microglia) and neurons, while IL-1 receptor type 2 and IL-1ra are expressed to a lesser extent in the same cells suggesting that the human brain does not activate efficiently the mechanisms apt to block the IL-1β actions as previously shown in experimental studies. The number of neurons expressing IL-1β and IL-1R1 positively correlates with the frequency of seizures prior to surgery while the number of IL-1Ra positive neurons and astrocytes negatively correlated with the duration of epilepsy, suggesting that this system contributes to the intrinsic and epileptogenicity of these developmental lesions (Ravizza et al., 2006a). Moreover, hippocampi obtained from patients affected by medically intractable MTLE showed overexpression of IL-1β and IL-1R1 in astrocytes, microglia and neurons. Increased immunoreactivity of IL-1β and related molecules was found also in perivascular astrocytic endfeet impinging on blood vessels and in endothelial cells in brain areas

associated with BBB damage (Ravizza et al., 2008a) suggesting that IL-1β induces an impairment in BBB integrity. These changes can result in chronic neuronal hyperexcitability as demonstrated previously in rodents (Seiffert et al., 2004; van Vliet et al., 2007).

Hippocampi from patients with MTLE showed also an overexpression of NFkB in reactive astrocytes, surviving pyramidal neurons and dentate granule cells (Crespel et al., 2002).

Reactive gliosis, characterized by activation and proliferation of both microglia and astrocytes, has been demonstrated in surgically resected epileptogenic lesions from patients with intractable epilepsy (Choi and Koh, 2008). The duration of epilepsy and frequency of seizures prior to surgical resection significantly correlate with the density of activated microglia as shown in focal cortical dysplasia and glioneuronal tumor associated with epilepsy (Aronica et al., 2005; Boer et al., 2006).

Increased serum or brain levels of proinflammatory cytokines and markers of immune system activation have been described in patients with West syndrome (Liu et al., 2001) and tuberous sclerosis (Maldonado et al., 2003), two neurologic disorders associated with epilepsy. In particular, molecules related to cytokine signaling such as ICAM-1, NFkB and TNF-α are increased in astrocytes, dysplastic neurons and giant cells in tubers (Maldonado et al., 2003).

Finally, one of the best indications of an involvement of inflammatory and immune reactions in the pathogenesis of human CNS disorders associated with epilepsy comes from studies on Rasmussen's encephalitis. Neuropathological examinations of brain specimens from patients affected by Rasmussen's encephalitis and active seizures revealed an increase in the expression of inflammation-related genes such as IL-1β and TNF-α and the presence

of microglia nodules in close apposition to neurons (Baranzini et al., 2002). Cells of the adaptive immune system such as lymphocytes have been described in the lesional tissue (Baranzini et al., 2002). These data suggest that the inflammation associated to Rasmussen's encephalitis differs from that activated in TLE patients. Cells of adaptive immunity such as T and B cells and NK cells were not found in brain specimens resected from patients affected by medically intractable MTLE (Ravizza et al., 2008a).

A role of inflammation in epilepsy is also demonstrated by the antiepileptic activity of immunomodulatory drugs with anti-inflammatory and immunosuppressor actions such as steroids and adrenocorticotropic hormone (Vezzani and Granata, 2005).

These clinical findings suggest that inflammatory processes that might be chronically active or transiently re-induced by recurrent seizures, or both, can take place in human epilepsy. In particular the induction of inflammation in epilepsy disorders that have not an inflammatory pathophysiology such as TLE and tuberous sclerosis suggest that inflammation is a common factor contributing and/or predisposing to the occurrence of seizures and cell death in various forms of epilepsy of different etiologies (Vezzani and Granata, 2005).

Table 1.1 summarizes the data available on inflammatory markers and anti-inflammatory treatments in human epileptic disorders.

Table 1.1. Inflammation in human epilepsies and convulsive disorders

Epileptic syndrome Convulsive disorder	Inflammatory markers		Antinflammatory treatments
	Plasma or CSF	Brain tissue	
Rasmussen encephalitis	GluR3 Ab, Munc-18 Ab	Glur3 Ab $CD\text{-}8^+$ lymphocytes GrB; MAC; cytokines	ACTH, steroids, IVIg PEX,PAI, immunosuppressant
West syndrome	IFN-α, TNF-α, IL2	n.d.	ACTH, steroids, IVIg
Lennox-Gastaut syndrome	n.d.	n.d.	ACTH, steroids, IVIg
Landau Kleffner syndrome	n.d.	n.d.	ACTH, steroids, IVIg
Febrile seizures	IL-1β, IL-1Ra, IL-6, IL-10, TNF-α	n.d.	n.d.
TLE	IL-6, IL-1β, IL-1Ra,	IL-1, NFkB*	n.d.
Tonic-clonic seizures	IL-6, IL1-α, IL-1β n.d.	n.d.	n.d.
Tuberous Sclerosis	n.d.	CD-68 macrophages ICAM-1, TNF-α, NFkB, MAPK	n.d.
Focal Cortical Dysplasia		IL-1β, IL-1Ra	n.d.

**Only in patients with MTLE, n.d., not determined; PEX; plasma exchange; PAI; protein A immunoabsortion; IVIg, intravenous immunoglobulin; MAPK, mitogen activated protein kinase. Adapted from Vezzani and Granata, 2005*

2.1 Aim of the thesis

The identification of brain inflammatory reactions in epileptic tissue raises the possibility that inflammation may contribute to the establishment of a pathological substrate (i.e. neurodegeneration, neuronal hyperexcitability, blood-brain barrier damage, etc) playing a role in epileptogenesis and in the acute manifestation or reinforcement of seizures. In this study, we investigated whether IL-1β and TNF-α, which are rapidly upregulated following epileptic activity, have a role in the pathophysiology of seizures, altering neuronal excitability and cell survival.

In particular, we first studied whether seizures can be effectively inhibited by blocking the brain production of IL-1β, using selective inhibitors of interleukin converting enzyme (ICE/caspase-1). We used pharmacological approaches to study the ictal activity in models of acute and chronic seizures.

Then, we investigated the molecular mechanisms involved in the proconvulsant actions of IL-1β. We tested the hypothesis that the proconvulsive effects of IL-1β were mediated by the activation of a novel non-transcription dependent pathway that leads to ion channel modifications. We specifically studied the involvement of a sphingomyelinase-dependent pathway, the consequent ceramide-induced Src-dependent phosphorylation of NR2B subunit of the NMDA receptor.

We also evalueted whether an inflammatory state may affect the excitotoxic neuronal damage associated with seizures. We set up an *in vitro* model of brain inflammation characterized by the release of IL-1β from activated microglia after transient exposure to

inflammatory stimuli, and we studied the consequences of microglia activation and cytokine release on neuronal susceptibility to a subsequent excitotoxic insult.

Finally, we studied whether molecular and functional interactions between TNF-α receptors and ionotropic glutamate receptors could contribute to the changes in seizure susceptibility mediated by this cytokine.

CHAPTER 3

Materials and methods - general procedures

3.1 Experimental animals

Animals were housed at constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light-dark cycle and free access to food and water.

Procedures involving animals and their care were conducted in accordance with the ethically approved institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec.12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Animals were purchased from Charles River (Calco, Italy).

3.2 Justification of the choice of in vivo models

We have used two models of provoked seizures, namely a model of acute seizures and a model of spontaneous recurrent seizures.

In particular, the acute model induced by intrahippocampal administration of kaininc acid in mice and rats has been widely used to study the effect of various pharmacological treatments on the onset and duration of ictal activity (Balosso et al., 2005; El Bahh et al., 2005; Moneta et al., 2002; Vezzani et al., 1999), and it is sensitive to modulation by inflammatory mediators (Vezzani et al., 1999; Vezzani et al., 2002). Intrahippocampal application of low doses of kainate in mice (7 ng in 0.5 μl) and rats (40 ng in 0.5 μl) induces hippocampal and cortical EEG seizure activity which is highly reproducible and can be quantified by measuring well established parameters such as the onset time to the first ictal event, the number of ictal events and their duration, and interictal activity. These doses of kainate have been titrated in previous experiments to produce a pattern of seizure

activity that can be either reduced or enhanced by pharmacological treatments or genetic manipulations (El Bahh et al., 2005; Moneta et al., 2002; Vezzani et al., 1999). Both in mice and rats, kainic acid induces degeneration of CA3 pyramidal neurons in the injected hippocampus, as assessed one week after seizure generation.

The chronic model of spontaneous seizures has been extensively described by Bouilleret et al (1999) and Riban et al (2002) (Bouilleret et al., 1999; Riban et al., 2002). Spontaneous chronic seizures develop in mice after an average latency of 2 weeks following non-convulsive status epilepticus induced by unilateral intrahippocampal application of a high amount of kainate (200 ng in 50 nl). Spontaneous seizures recur for up to 8 months. Importantly, the baseline of spontaneous seizures in each mouse is stable and reproducible, as assessed by measuring the frequency and duration of ictal events 8 weeks after induction of status epilepticus. This feature is instrumental to carry out reliable pharmacological studies on the effect of the treatments on spontaneous seizure events.

Spontaneous seizures are refractory to various antiepileptic drugs thus providing a valuable model for "drug resistant epilepsies" (Riban et al., 2002). Histopathological changes in the hippocampus are similar to those observed in human MTLE including neuronal loss, granule cell dispersion and sprouting (Bouilleret et al., 1999; Kralic et al., 2005; Riban et al., 2002; Suzuki et al., 2005).

3.3 EEG-related techniques

3.3.1 Placement of cannula and electrodes for EEG recordings

Mice and rats were surgically implanted with an injection guide cannula and recording electrodes under deep Equithesin anestesia (1% phenobarbital and 4% chloral hydrate; 3ml/kg, intraperitoneally, i.p) using stereotaxic guidance (El Bahh et al., 2005; Vezzani et al., 1999; Vezzani et al., 2000).

In mice and rats: a ground lead was positioned over the nasal sinus and two screw electrodes were placed bilaterally over the parietal cortex. Bipolar nichrome wire insulated electrodes (60μm) were implanted bilaterally into the dorsal hippocampus (septal pole) at the following coordinates from bregma: rats; mm, nose bar –2.5, AP –3.5, L ±2.4 and 3.0 below dura mater (Paxinos and Watson, 1986); mice; nose bar 0; anteroposterior –1.9, lateral 1.5 and 2.0 below dura mater (Franklin and Paxinos, 1997). A guide cannula was unilaterally positioned on top of the dura and glued to one of the depth electrodes for intrahippocampal injection of kainic acid or drugs. An additional guide cannula was unilaterally positioned on top of the dura mater for intracerebroventricular (icv) injection of drugs (in mm from bregma, rats; nose bar –2.5; AP –1; L +1.5; mice: nose bar 0; anteroposterior 0.0, lateral 1.0 and 3.0 below dura mater).

The electrodes were connected to a multipin socket and, together with the injection of cannula, secured to the skull by acrylic dental cement (Fig. 3.1).

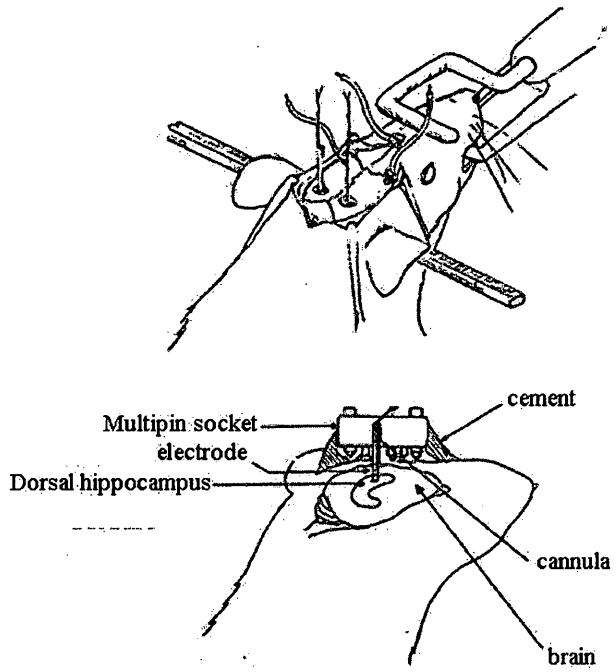


Figure 3.1. A schematic represention of the cannula and recording electrodes implant in rats using stereotaxic guidance

3.3.2 Model of acute seizures

The procedures for intracerebral injection of drugs in freely-moving rats or mice have been previously described (El Bahh et al., 2005; Moneta et al., 2002; Vezzani et al., 1999; Vezzani et al., 2000). Drugs were injected during 60 sec unilaterally through an injection needle connected to a 10 µl Hamilton microsyringhe via PE20 tubing.

Kainic acid (Sigma-Aldrich, St Louis, USA) was dissolved in 0.5 µl phosphate-buffered saline (PBS, pH 7.4) and injected in the mouse (7 ng) or rat (40 ng) dorsal hippocampus unilaterally (0.5 µl/min), using a needle protruding 2.0 or 3.0 mm respectively from the guide cannula.

This dose of kainic acid was chosen in order to induce recurrent EEG epileptic activity composed of ictal episodes (EEG seizures) and spiking activity in 100% of animals without mortality.

The correct positioning of the injection needle and electrodes was verified by Nissl staining of 40 μm cryostat coronal brain sections in all the animals, 24 h after kainic acid injection. The unspecific damage restricted to the insertion of injection needle and the electrode tracks was similar in control and experimental animals.

All pharmacological experiments were carried out between 9.00 am and 1.00 pm.

3.3.3 EEG recordings: assessment and quantification of acute seizures

Recording of EEG seizures induced by intrahippocampal injection of kainic acid in freely-moving mice or rats respectively has been previously described (El Bahh et al., 2005; Moneta et al., 2002; Vezzani et al., 1999).

A 30 min recording was done before kainic acid injection to assess the basal EEG pattern. The EEG recordings were made continuously for 180 min after kainic acid injection. At least a 30 min EEG recording similar to baseline was required before stopping the recording. Ictal episodes are characterized by high-frequency and/or multispike complexes and/or high-voltage synchronized spikes simultaneously occurring in the injected and contralateral hippocampi. Spiking activity is typically observed after seizures subside. The EEG recording of each animal was analyzed visually by two independent investigators to detect any activity different from baseline. Seizure activity was quantified by reckoning the time elapsed from kainic acid injection to the occurrence of the first EEG seizure (onset) and the total number and total duration of seizures (reckoned by summing up the duration

of every ictal episode during the EEG recording period). Interictal activity was reckoned by summing up the time spent in EEG spiking. Seizures occurred with an average latency of about 10 min from kainic acid injection, and then recurred for about 90 min (mice) or 150 min (rats) from their onset. In rats, kainic acid induced stereotyped behaviors such as sniffing and gnawing. "Wet dog shakes" were often observed shortly after kainate injection and during seizures.

Representative EEG tracings of freely moving rats (Fig. 3.2 A) or mice (Fig. 3.2 B) injected unilaterally in the left dorsal hippocampus with kainic acid are depidted below.

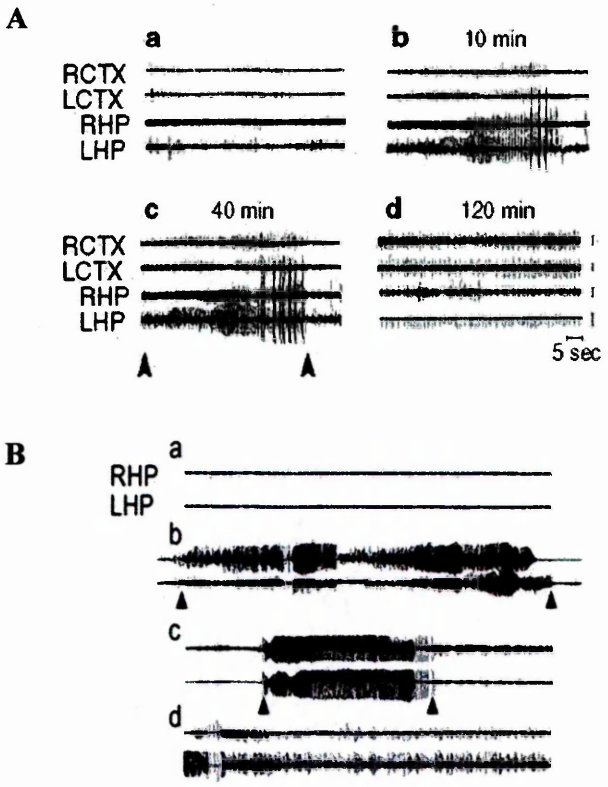


Figure 3.2. EEG seizures induced by intrahippocampal injection of kainic acid in rats (A) or mice (B)

(a) Baseline recording before kainic acid injection; arrowheads in (b) and (c) include representative ictal episodes recorded in the EEG after kainic acid; tracings in (d) depict spiking activity in the EEG after termination of seizures. RCTX and LCTX are right and left cortex, respectively; RHP and LHP are right and left (injected) hippocampus, respectively.

3.3.4 Mouse model of spontaneous seizures

Male C57BL6 mice (~60 days old, 25g) were stereotaxically injected under deep Equithesin anestesia with kainic acid (200 ng in 50 nl) unilaterally into the left septal hippocampus at the following coordinates from bregma: mm, nose bar 0; AP –1.8, L 1.7 and 1.9 below dura (Franklin and Paxinos, 1997). Kainate was injected over 1 min using a thin injection needle connected to a 0.5 µl Hamilton microsyringhe via PE20 tubing trough a CMA/100 pump. At the end of the injection, the needle was mantained *in situ* for an additional 2 min to limit back flow along the injection track.

Immediately after intrahippocampal injection, mice were implanted with EEG electrodes as described above. After surgery, mice treated with kainate were continuously EEG recorded for up to 10 hours to monitor the occurrence of non-convulsive status epilepticus. In accordance with previous studies (Riban et al., 2002), 24 h after kainate application status epilepticus elapses and mice do not show ictal events in their EEG for about 2 weeks (epileptogenesis) after which time they develop chronic spontaneous EEG seizures localized in the hippocampi.

For pharmacological experiments, we used only mice developing chronic spontaneous recurrent seizures starting 8 weeks after the induction status epilepticus as assessed by continuous EEG monitoring (Nervous EEG Recording System connected with a Nervus magnus 32/8 Amplifier, Taugagreining, Iceland) from 9 am to 11 am and from 4 pm to 6 pm for at least three days. These recordings provide the average number of ictal events and their duration occurring in each mouse within the time interval of our pharmacological experiments, thus allowing us to establish if a stable baseline of spontaneous seizures was reached before starting the treatments. Table 3.1 shows the number of spontaneous seizures

and their total duration in 2-h observation period in different epileptic mice, 8 weeks after induction of status epilepticus. Spontaneous seizure quantification clearly shows that the baseline of spontaneous seizures in each mouse is stable. In this model, spontaneous EEG seizures do not occur in clusters; this is the advantage to use the present model rather than systemic kainate or pilocarpine-induced spontaneous seizures in rats where clustering of seizures indeed occurs. Only mice showing a stable baseline of spontaneous seizures (90 %) has been included in the study protocol for pharmacological testing.

Table 3.1. Baseline recordings in spontaneous epileptic mice

9 am-11 am

I.D. mouse number	Number of seizures	Time in seizures (min)
1	50.0 ± 3.0	36.0 ± 5.7
5	48.0 ± 10.0	25.5 ± 7.0
7	53.0 ± 5.0	33.8 ± 1.4
9	55.0 ± 3.0	34.7 ± 3.5
11	30.0 ± 3.0	15.3 ± 2.4
8	99.0 ± 8.0	34.7 ± 1.4
12	80.0 ± 3.0	35.5 ± 3.5
6	107.0 ± 4.0	35.3 ± 3.0
4	56.0 ± 5.0	63.2 ± 1.7
13	36.0 ± 1.0	12.5 ± 1.3

4 pm-6 pm

I.D. mouse number	Number of seizures	Time in seizures (min)
1	48.0 ± 5.0	35.0 ± 2.1
5	48.0 ± 4.0	23.6 ± 2.2
7	58.0 ± 5.0	36.3 ± 2.7
9	57.0 ± 6.0	31.7 ± 4.0
11	32.0 ± 5.0	15.0 ± 2.5
8	92.0 ± 8.0	36.1 ± 2.0
12	69.0 ± 6.0	28.4 ± 1.9
6	108.0 ± 7.0	39.5 ± 5.8
4	61.0 ± 5.0	62.2 ± 1.3
13	37.0 ± 2.0	14.3 ± 1.5

3.4 Immunohistochemistry

3.4.1 Immunohistochemical studies from in vivo preparation

Mice were deeply anaesthetized using Equithesin and perfused via ascending aorta with 50 mM cold PBS, pH 7.4 followed by chilled 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS. The brains were post-fixed for 90 min at 4°C, and then transferred to 20% sucrose in PBS for 24 h at 4°C. The brains were immersed in -50°C isopentane for 3 min and stored at -80°C until assayed. Serial cryostat coronal sections (40 μm) were cut from all brains throughout the septo-temporal extension of the hippocampus

(Franklin and Paxinos, 1997) and collected in 100 mM PBS for IL-1β, IL-1R1, p55 and p75 receptors immunohistochemistry.

3.4.2 IL-1β

Slices were incubated at 4°C for 10 min in 70% methanol and 2% H_2O_2 in Tris-HCl-buffered saline (TBS, pH 7.4), followed by 30 min incubation in 10% fetal calf serum (FCS) in 1% Triton X-100 in TBS. Then, the slices were incubated overnight with the primary antibody against IL-1β (1:200, Santa Cruz Bio., CA, USA) at 4°C in 10% FCS in 1% Triton X-100 in TBS. Immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vector Labs, USA). The sections were reacted using diaminobenzydine (DAB), and the signal was amplified by nickel ammonium. No immunostaining was observed by incubating the slices with the primary antibody preabsorbed with the corresponding peptide (1μM, 24h at 4°C) or without the primary antibody (negative control) as previously described (Vezzani et al., 1999).

3.4.3 IL-1R1

IL-1R1 immunostaining was carried out as previously described (Ravizza and Vezzani, 2006). Briefly, sections were incubated at 4°C for 10 min in 0.3% H_2O_2 in 0.3% Triton X-100 in PBS. After three 5 min washes in 0.3% Triton X-100 in PBS, slices were incubated at 4°C for 60 min in 10% FCS in 0.3% Triton X-100 in PBS. Then the slices were incubated with the primary antibody against IL-1RI (6 μg/ml, R&D System, Minneapolis, USA) for 72 h at 4°C in 4% FCS in 0.3% Triton X-100 in PBS. Immunorectivity was tested as described for IL-1β (see before).

No immunostaining was observed by incubating the slices with the primary antibody preabsorbed with the corresponding peptide (30μg/ml, 24h at 4°C) or without the primary antibody (negative control) as previously described (Ravizza and Vezzani, 2006).

After DAB incubation, three 5-minute washes were done with Tris-HCl–buffered saline, then sections were mounted onto gelatin-coated slides and dried overnight at room temperature. They were dehydrated and coverslipped the next day.

The primary antibodies and experimental procedures have been previously demonstrated to determine a specific immunohistochemical signal of the protein of interest in rodent brain slices (Ravizza and Vezzani, 2006; Ravizza et al., 2008a; Vezzani et al., 1999).

3.4.4 p55 and p75 receptors

Free-floating sections were rinsed for 10 minutes in 0.3% H_2O_2 in 0.3% Triton X-100 (Sigma Labs, St. Louis, MO) in 100 mM PBS at 4°C (Triton was omitted in the whole procedure when assessing p75 receptors), followed by three 5-minute washes in 0.3% Triton X-100 PBS. The slices then were incubated at 4°C for 60 minutes in 10% fetal calf serum diluted in 0.3% Triton X-100 PBS. The primary antiserum was diluted in 0.3% Triton X-100 PBS containing 4% fetal calf serum, and slices were incubated at 4°C for 72 hours with biotinylated monoclonal antibody to human p55 or p75 (1:30; Hbt, Uden, The Netherlands). After three 5-minute washes in PBS, immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The sections then were reacted by incubation with 0.4mM 3,3-diaminobenzidine (DAB; Sigma) in 50mM Tris-HCl–buffered saline, pH 7.4, and 0.01% H2O2, and the signal was amplified using nickel ammonium. After DAB incubation, three 5-minute

washes were done with Tris-HCl–buffered saline, then sections were mounted onto gelatin-coated slides and dried overnight at room temperature. They were dehydrated and coverslipped the next day. Control slices were prepared omitting the primary antibody. The specificity of the p55 or p75 antibodies was also confirmed by the lack of staining obtained in slices from p55 or p75 knock-out mice, respectively.

3.4.5 Double-immunostaining

Two brain slices in each mouse for each cell type marker were randomly chosen to identify the cells expressing IL-1β, IL-1R1, p55 and p75 receptors. After incubation with the primary antibodies, slices were incubated in biotinylated secondary anti-goat (for IL-1β) and anti-hamster antibodies (Il-1R1) (1:200, Vector Labs), then in streptavidin–horseradish peroxidase and the signal was revealed with tyramide conjugated to Fluorescein using TSA amplification kit (NEN Life Science Products, Boston, MA, USA). Sections were subsequently incubated with the following primary antibodies: mouse anti-GFAP (Chemicon, Temecula, Ca, USA; 1:2500), or rat anti-mouse CD11b (Serotec, Oxford,UK; 1:1000), or mouse anti-NeuN (Chemicon; 1:1000).

Fluorescence was detected using anti-mouse or anti-rat secondary antibody conjugated with Alexa546 (Molecular Probes, Leiden, The Netherlands). Slide-mounted sections were examined with an Olympus Fluorview laser scanning confocal microscope (microscope BX61 and confocal system FV500; Hamburg, Germany) using dual excitation of 488 nm (Laser Ar) and 546 nm (Laser He–Ne green) for Fluorescein and Alexa546, respectively. The emission of fluorescent probes was collected on separate detectors. To eliminate the

possibility of bleed-through between channels, the sections were scanned in a sequential mode.

3.4.6 Immunohistochemical studies from in vitro preparation

Organotypic hippocampal slices were fixed by immersion in 4% paraformaldehyde (Merck, Darmstadt, Germany) for 30 min, cryoprotected in 30% sucrose and stored at –20°C until staining. For immunohistochemical studies, 20 µm coronal slices were sectioned in a cryostat.

After thawing at room temperature (RT), sections were rinsed in 0.15 M phosphate-buffered saline solution (PBS, pH 7.4) containing 0.3% Triton X-100. Slices were subsequently incubated for 30 min with 10% foetal bovine serum (FBS), to reduce unspecific labelling, followed by 24 h incubation at 4°C with primary rat anti-mouse CD11b antibody (CD11b; 1:1000; Serotec, United Kingdom), rabbit polyclonal anti-active caspase-3 antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-GFAP antibody (1:100; Sigma) or rabbit polyclonal anti-MAP2 antibody (1:200; Santa Cruz). Thereafter, sections were rinsed in PBS and incubated for 1 h at RT with a secondary anti-rat or anti-rabbit Alexa Fluor 488 antibody (1:200; Molecular Probes, Oregon, USA), respectively. After rinsing with PBS, slices were incubated with a Hoechst 33342 solution (15µg/ml in PBS containing 0.25% BSA) for 5 min at RT to visualize nuclei. Finally, sections were mounted using Dakocytomation fluorescent medium (Dakocytomation Inc., California, USA) and fluorescent images were examined with an Olympus Fluorview laser scanning confocal microscope (microscope BX61 and confocal system FV500; Hamburg, Germany).

3.5 Western blot

The hippocampi were dissected out at 4° C and homogenized in 20 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA, 5 mM EGTA, 1 mM Na-vanadate, 2 μg/μl aprotinin, 1 μg/μl pepstatin, 2 μg/μl leupeptin (30 mg tissue/150 μl homogenization buffer). Total proteins (150 μg per lane for rats and 70 μg per lane for mice; Bio-Rad Protein Assay, Bio-Rad Labs, Munchen, Germany) were separated using SDS-PAGE, 10% acrylamide and each sample was run in duplicate. Proteins were transferred to Hybond nitrocellulose membranes by electroblotting. For immunoblotting, we used specific antibodies that recognize the analyzed protein. Densitometric analysis of immunoblots was done to quantify the changes in protein levels (AIS image analyzer, Imaging Research Inc., Ontario, Canada) using film exposures with maximal signals below the photographic saturation point. Optical density values in each sample were normalized using the corresponding amount of actin.

3.6 In vivo microdialysis

Vertical dialysis probes were prepared with a copolymer of acrylonitrile-sodium methallyl sulphonate (AN 69, Hospal SpA, Italy; 0.31 mm outer diameter, with more than 44,000 Da M.W. cutoff), essentially as described by Robinson and Whishaw (1988) (Robinson and Whishaw, 1988). The length of the exposed membrane was 2.5mm. Mice were anesthetized with Equithesin, placed on a stereotaxic frame and dialysis probe was implanted in the ventral hippocampus. The stereotaxic coordinates were: bar nose 0.0; AP -

2.6; L + 2.6; V -3.4 from bregma and dura surface, according to Franklin and Paxinos (1997). About 20 h after surgery, the probes were perfused with artificial cerebrospinal fluid (aCSF composition in mM: NaCl 140, $CaCl_2$ 1.26, KCl 3, $MgCl_2$ 1, Na_2HPO_4 1.2, glucose 7.2, pH 7.4 with 0.6 M NaH_2PO_4) at 1 µL/min with a CMA/100 pump (CMA/Microdialysis, Stockholm, Sweden). Samples of dialysate were collected every 20 min and stored at 4°C.

During the phase of stable glutamate (GLU) and GABA output, defined as three consecutive baseline samples not differing by more than 20%, 60 mM of KCl (prepared as follow: NaCl 87; KCl 60; $CaCl_2$ 1.26; $MgCl_2$ 1, in ultra pure water, buffered at pH 7.4 with 2 mM sodium phosphate buffer) was perfused through the probe for 20 min to stimulate the exocytotic release of GABA and GLU.

3.6.1 HPLC detection of GABA and GLU into dialysate samples

The concentrations of GABA and GLU in dialysate samples were determined by high-performance liquid chromatography (HPLC) with fluorometric detection after pre-column derivatization with o-phthalaldehyde/β-mercaptoethanol (Sigma-Aldrich, Milan, Italy) reagent according to Donzanti and Yamamoto (1988) (Donzanti and Yamamoto, 1988). Stock derivatizing reagent was prepared by dissolving 27 mg OPA in 1 mL methanol, followed by 5 µL β-mercaptoethanol and 9 mL 0.1 M sodium tetraborate buffer (pH 9.3) prepared by dissolving 0.62 g boric acid in about 80 mL ultrapure water (MilliQ, Millipore, USA). pH was adjusted to 9.3 with 2-3 mL 5 M NaOH and the final volume brought to 100 mL with water. Stock reagent solution was maintained at room temperature in a darkened bottle for one week. Derivatizing reagent was prepared by diluiting stock

solution 1:4 with 0.1 M borate buffer, 24 h before use. 5 μL of derivatizing reagent were added to 5 μL sample to measure GLU or to 15 μL to measure GABA, thoroughly mixed and immediately injected into the HPLC.

GABA and GLU were separated through a 4.6 x 80 mm C18 reverse-phase column (HR-80, ESA, Chelmsford, MA). New Guard RP-18 guard column (3.2 x 15 mm; Perkin-Elmer, USA) was used to protect the analytical column. The mobile phase for GABA was as follows: 0.05 M Na_2HPO_4, 35% methanol, pH 6.25 with 85% phosphoric acid, pumped at 1.2 mL/min with a LC10-Advp HPLC pump (Shimadzu, Milan, Italy).

The mobile phase for GLU separation contained 0.05 M Na_2HPO_4, 28% methanol, pH 6.4 with 85% phosphoric acid at a flow rate of 1 mL/min with a LC10-Advp HPLC pump (Shimadzu, Milan, Italy).

Another mobile phase consisting of methanol:water (80:20), was used to wash out late eluting peaks. This was connected to HPLC pumps through a three-way valve (Biggs et al., 1995; Piepponen and Skujins, 2001). Immediately after the GABA or GLU peak was recorded (R_t 10.5 min and 3.8 min, respectively), the valve was manually switched for 2 min (wash-out step). The whole step-gradient run (from injection to injection) took less than 15 min in total for GABA and 8 min for GLU.

GABA and GLU were measured by a fluorescence detector (F-1080; Merck-Hitachi, Germany). Excitation and emission wavelengths were 335 and 450 nm for both aminoacids. Assays were calibrated daily by injecting 0.4 pmol/20 μL GABA or 5 pmol/5 μL GLU, made up freshly in aCSF. Detection limits were 0.025 pmol/20 μL for GABA and 0.1 pmol/5 μL for GLU (signal-to-noise ratio = 2). Extracellular levels of GABA and GLU,

uncorrected for *in vitro* recovery of the probe, were expressed as pmol/20 µL and pmol/5µL, respectively.

3.7 Organotypic hippocampal slice cultures

Hippocampal slice cultures were prepared from 6-8-day-old C57BL/6 mice, according to the interface culture method (Stoppini et al., 1991), previously described in detail (Bernardino et al., 2005). Mice were killed by decapitation, their brains removed under sterile conditions and the two hippocampi isolated and cut in 350 µm coronal sections using a McIlwain tissue chopper. Individual slices were placed in ice cold Gey's balanced salt solution (Biological Industries, Israel) supplemented with 25 mM D-glucose (Merck, Germany), before being placed on porous insert membranes (Millipore Corp., Bedford, Ma, U.S.A.). Six slices were put onto each membrane and the inserts were transferred to a 6 well culture trays (Corning Costar, Corning, N.Y., U.S.A.). Each well contained 1 ml culture medium, composed of 50% Opti-MEM, 25% heat-inactivated horse serum and 25% Hank's Balanced Salt Solution (all from Gibco B.R.L., Life Technologies Ltd., Scotland) supplemented with 25 mM D-glucose. The culture trays were placed in an incubator with 5% CO_2 and 95% atmospheric air at 33°C and the medium was changed twice a week for 2 weeks. At the start of the experiment, the culture medium was replaced with 1 ml of serum-free Neurobasal medium containing 1 mM L-glutamine and 20 µl B27 supplement (Gibco BRL). No antibiotics or antimitotics were used throughout these procedures.

3.7.1 Determination of cell death by propidium iodide

Spontaneous and induced cell death in slice cultures was assessed by monitoring the cellular uptake of the fluorescent dye propidium iodide (PI; 3,8-diamino-5-(3-(diethylmethylamino) propyl)-6-phenyl phenanthridinium diiodide; Sigma, St. Louis, MO). PI is a stable fluorescent dye adsorbing blue-green light (493 nm) and emetting red fluorescence (630nm). As a polar substance, PI enters only dead or dying cells with a damaged or leaky cell membrane, and interacts with DNA to yield a bright red fluorescence. PI is not toxic to cells and has been used widely as an indicator of neuronal membrane integrity and cell damage (Kristensen et al., 2001; Noraberg et al., 2005). Three hours before exposure to drugs, 2 μM PI was added to the medium for determination of basal cellular uptake, and the same concentration of PI was added during all subsequent medium changes. Cellular uptake of PI was recorded by fluorescence microscopy (microscope Olympus BX61; Hamburg, Germany) using a rhodamine filter and digital camera (F-View 2, Olympus) with 100 ms exposure time. Digital fluorescent micrographs were taken before drug exposure (basal PI uptake) and at fixed time points after drug exposure.

For quantitative assessment of neuronal damage, the region of interest was delineated using NIH Image 1.62 analysis software (NIH, Bethesda, MD, USA) and used for densitometric measurements of the PI uptake. The effect of treatments was assessed by subtracting the basal PI-uptake value measured at 0 d from the value recorded at the end of the treatment (in the same slice).

3.7.2 In vitro model of inflammation

Hippocampal slice cultures were exposed to inflammatory-like stimuli using two distinct experimental protocols. Hippocampal slice cultures were exposed to LPS alone (10 ng/ml; *Escherichia coli* serotype 055:B5, Sigma, St Louis, MO, USA) for 6 h or to LPS alone for 3h followed by 3h co-incubation with 1 mM ATP (Sigma, St Louis, MO, USA). This protocol was previously used to study post-translational processing of murine IL-1β in monocytes/macrophages (Laliberte et al., 1999; Verhoef et al., 2003) or cultured microglial cells (Bianco et al., 2005; Brough et al., 2002; Sanz and Di Virgilio, 2000).

We used hippocampal slice cultures since, as compared to dispersed cell cultures, they reproduce the complex *in vivo* organization of cell network including neurons, astrocytes and microglia (Noraberg et al., 2005). This network is instrumental for studying the consequences of inflammation which are known to depend not only on the type of inflammatory stimulus and the phenotype characterizing microglia reaction but also on the concurrence of several factors related to the brain microenvironment (Lai and Todd, 2008; Minghetti et al., 2005; Schwartz et al., 2006).

3.7.3 ELISA

Hippocampal slices were homogenized in 0.5 ml ice-cold PBS (pH 7.4) containing a complete protease inhibitor cocktail tablet (Roche Biochemical, Indianapolis, IL, USA), and 2% of NP-40 (Sigma). IL-1β was measured in tissue and medium by ELISA (R&D System, Minneapolis, MN, US), accordingly to manufacture's suggested protocol. An affinity polyclonal antibody specific for mouse IL-1β was pre-coated on the ELISA 96-well

polystyrene microplates. After washing with RD1-14 assay diluent, the plates were incubated with the samples and the standards (standards ranging from 500 pg/ml to 7.8 pg/ml) for 2h at RT, washed again four times with wash buffer solution, and a polyclonal anti-mouse IL-1β antibody conjugated to the horseradish peroxidase was applied for further 2h at RT. After a wash to remove any unbound antibody-enzyme reagent, a mixture of stabilized hydrogen peroxide and chromogen (tetramethylbenzidine) was added for 30 min at RT. The colour reaction was stopped by 1M HCl and the absorbance was readed at 450nm (ELISA reader, Infinite M200, Tecan, Italy). The detection limit was <3 pg/ml.

CHAPTER 4

Inactivation of caspase-1 in rodent brain:

a novel anticonvulsive strategy

4.1 Summary

We investigated whether acute and chronic seizures can be effectively inhibited by blocking the brain production of IL-1β, using pralnacasan or VX-765, two selective inhibitors of interleukin converting enzyme (ICE/caspase-1).

Caspase-1 inhibitors reduced the release of IL-1β in organotypic slices exposed to inflammatory conditions.

Intracerebroventricular injection of 50 μg pralnacasan or intraperitoneal administration of VX-765 (25-200 mg/kg) to rats decreased by up to 50% the number and the time spent in seizures and significantly delayed by ~2-fold the seizure onset time. These effects were associated with blockade of seizure-induced production of IL-1β in the hippocampus. Intraperitoneal administration of VX-765 (200 mg/kg) to mice reduced up to 75% the number and the time spent in spontaneous recurrent seizures.

These data indicate that the inhibition of caspase-1 represents an effective and novel anticonvulsive strategy which acts by selectively reducing the brain availability of IL-1β.

4.2 Experimental procedures

4.2.1 Pharmacological treatments

Acute and chronic kainic acid-induced seizures were induced and quantified as previously described in "Materials and methods - general procedures" section (p. 81-85).

Pralnacasan and VX-765. Pralnacasan is a prodrug of the potent, selective, non-peptide competitive inhibitor RU36384, and was the first caspase-1 inhibitor to enter clinical development. VX-765 is a prodrug with improved oral bioavailability and more active than pralnacasan (Braddock and Quinn, 2004; Wannamaker et al., 2007).

Pralnacasan (Vertex Pharmaceuticals, Inc., Cambridge, MA, US) was dissolved in 20% cremophor (25 μg in 4 μl, the maximal concentration soluble in the injected volume) and injected icv in rats (n=8), 45 min and 10 min before intrahippocampal injection of kainic acid. In preliminary experiments, pralnacasan was also administered in a single dose (12.5 or 25 μg in 4 μl), 45 min before kainic acid.

VX-765 (25, 50, 200 mg/kg; Vertex Pharmaceuticals, Inc) was dissolved in 20% cremophor and injected ip in rats (n=8) once a day for 3 consecutive days. On the fourth day, rats received VX-765 45 min and 10 min before intrahippocampal injection of kainic acid. This dosing schedule was shown to reduce disease severity and the expression of inflammatory mediators in models of rheumatoid arthritis and skin inflammation (Braddock and Quinn, 2004; Wannamaker et al., 2007). Respective controls were similarly injected with vehicle before kainic acid.

VX-765 (200 mg/kg) was injected ip in mice (n=10) with spontaneous recurrent seizures twice a day (9 am and 4 pm) for 4 consecutive days followed by 3 days wash-out period

starting from the last drug administration. Controls are mice injected with PBS (50 nl) and chronically implanted with EEG electrodes.

4.2.2 Western blot analysis

Different groups of rats (n = 3 in each treatment group) were treated icv with pralnacasan or its vehicle given alone, or before intrahippocampal application of kainic acid (see above). Ninety min after the onset of EEG seizures, when pralnacasan effect on seizures was evident (see Results), experimental rats and their controls were decapitated. The injected hippocampi were dissected out at 4° C and homogenates as described in "Materials and methods - general procedures" section (p. 92).

For immunoblotting, we used anti-rat IL-1β (0.2 μg/ml, Abcam Limit., Cambridgeshire, UK) or anti-mouse IL-18 (1:250, kindly provided by Dr Dinarello) rabbit polyclonal antibodies. Immunoreactivity was visualized with enhanced chemiluminescence (ECL, Amersham, UK) using peroxydase-conjugated goat anti-rabbit IgG (1:2000; Sigma, St Louis, MO, USA) as secondary antibodies.

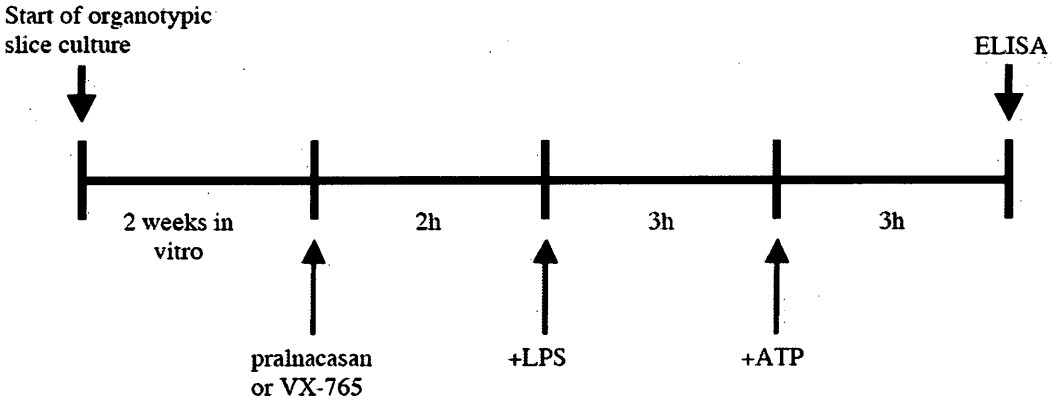
4.2.3 Immunohistochemistry

Two months after kainic acid administration, spontaneously epileptic mice and their controls (n=5) were perfused as previously described (see "Materials and methods - general procedures" section (p. 87)). Serial cryostat coronal sections were used for IL-1β and IL-1R1 immunohistochemestry following the protocol previously described (p. 88).

4.2.4 Organotypic hippocampal slice cultures

Technical details related to organotypic hippocampal slice cultures and ELISA have already been reported in "Materials and methods - general procedures" section (p.95; 97).

To investigate the effect of caspase-1 inhibitors on IL-1β production and subsequent release, slice cultures were preexposed to 0.1-10 μM pralnacasan or VX-765 for 2h followed by coexposure with LPS for 3 h and LPS+ATP for further 3 h (protocol is depicted below). These doses of pralnacasan and VX-765 were shown to inhibit the LPS-induced IL-1β release from peripheral blood mononuclear cells (Braddock and Quinn, 2004; Stack et al., 2005).



4.2.5 Statistical analysis of data

Data are represented as the means ± SE (n= number of individual samples). The effects of treatments were analyzed by two-way ANOVA followed by Fisher's test (for western blot data), or by one-way ANOVA followed by Bonferroni's test (for organotypic cultures), or by one-way ANOVA followed by Tukey's test (for seizure analysis).

4.3 Results

4.3.1 Effects of caspase-1 inhibition on hippocampal IL-1β

In vitro evidence. Fig. 4.1A shows that ATP is required for inducing a massive IL-1β release in the presence of LPS in organotypic hippocampal slice cultures, as previously shown in human monocytes (Mehta et al., 2001). Thus, LPS alone increased IL-1β by ~20-fold ($p<0.01$), while addition of ATP increased IL-1β release into the culture medium by 60-fold compared to control untreated slices ($p<0.01$ vs LPS alone). Two hours pre-incubation with pralnacasan or VX-765 reduced in a dose-dependent manner the LPS+ATP mediated release of IL-1β in the medium, reaching the levels observed when slices were incubated with LPS alone (Fig.4.1A). Maximal 70% inhibition was reached at 1 μM pralnacasan or VX-765. In the corresponding tissue extracts, LPS or the co-exposure to LPS+ATP induced similar increases in IL-1β levels ($p<0.01$ vs control slices; Fig. 4.1B) and pralnacasan or VX-765 did not affect LPS+ATP induced increase (Fig. 4.1B). The basal levels of IL-1β in the medium and in the tissue of control slices were barely or not detectable and did not differ from those measured in the presence of ATP, pralnacasan or VX-765 alone. Although the ELISA method used to measure IL-1β in the tissue and in the medium does not permit to distinguish between pro-IL-1β and the mature 17 kDa cytokine, it is well established that more than 90% of the releasable form of IL-1β consists of the 17 KDa mature form, whereas the tissue content is mostly represented by pro-IL-1β (Dinarello, 1991).

In vivo evidence. Fig. 4.1C-D show the effect of pralnacasan on IL-1β levels induced in the rat hippocampus by seizures. Western blot analysis allowed to distinguish between the immature (pro-IL-1β, 30 kD) and the mature and releasable form of IL-1β (17kD). Seizures increased pro-IL-1β by 84% and the mature and releasable form of IL-1β by 20%. Anticonvulsant doses of pralnacasan (see below) fully reversed the effect of kainic acid-induced seizures on the mature, releasable form of IL-1β ($p<0.01$ vs kainic acid) producing a total reduction of 50% below basal levels; consequently, pro-IL-1β was increased by ~50% above the levels induced by seizures ($p<0.01$ vs kainic acid). In control hippocampi, pralnacasan alone did not significantly affect pro-IL-1β while reducing mature IL-1β levels by 30% below basal values.

IL-18 immunoreactivity was also assessed using western blot since this cytokine is a substrate of caspase-1 (Culhane et al., 1998). However, no signal was detected in hippocampal homogenates either in basal conditions or during seizure activity. The lack of signal was not due to failure of the Ab, since our Ab detected a specific band of 18kD when tested against 1 μg of human or rat recombinant IL-18.

These data demonstrate that both pralnacasan and VX-765 blocked the release of IL-1β induced by activation of caspase-1 in organotypic hippocampal slice cultures and by kainic acid-induced seizures; moreover, an anticonvulsant dose of pralnacasan led to a 50% reduction in the hippocampal levels of the 17 kDa form of IL-1β.

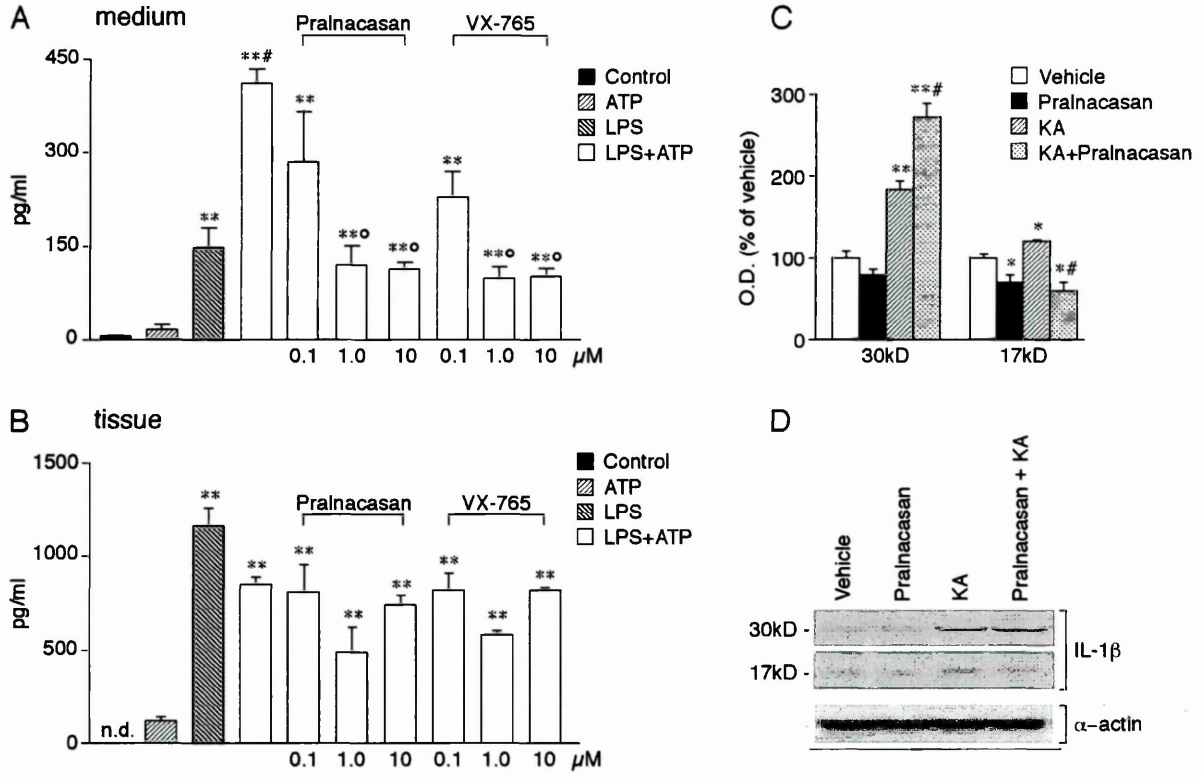


Figure 4.1. Effects of pralnacasan on hippocampal IL-1β levels

*Panels A and B: Bargrams show IL-1β levels (mean ± SE, n=6; two separate experiments) in mouse organotypic hippocampal slice culture media (A) and extracts (B), as assessed by ELISA. **p<0.01 vs control; #p<0.01 vs LPS; °p<0.01 vs LPS+ATP by one-way ANOVA followed by Bonferroni's test.*

*Panels C and D: Bargrams show the IL-1β levels (mean ± SE, n= 3) measured in the rat hippocampus in the various experimental conditions, as assessed by western blot. Panel C shows the quantification of the optical density (OD) of the IL-1β protein bands of 30 kDa (pro-IL-1β) and 17 kDa (mature IL-1β) normalized to the corresponding α-actin levels (depicted in panel D). Data are expressed as % of normalized OD values measured in vehicle-treated rats. *p<0.05; **p<0.01 vs vehicle; #p<0.01 vs KA; proIL-1β (30 kD): F (1,10)=53.8; IL-1β (17 kD): F (1,10)=10.6 by two-way ANOVA followed by Fisher's test.*

Panel D depicts representative western blots of pro-IL-1β (30 kDa) and mature (17 kDa) IL-1β in the rat hippocampus in the various experimental conditions. Each sample was run in duplicate (150 μg protein).

4.3.2 Effects of caspase-1 inhibition on kainic acid-induced acute seizures

Pralnacasan. Fig 4.2A shows the effect of two consecutive icv injections of pralnacasan on kainic acid-induced seizures in freely-moving rats (25 μg in 4 μl given 45 and 10 min before kainic acid injection). Pralnacasan significantly delayed the time to seizure onset by 2-fold ($p<0.01$), reduced the number of seizures by 20% ($p<0.01$) and the total time spent in EEG seizures by 40% ($p<0.01$). The average duration of seizures was also significantly reduced by 25% by pralnacasan (Vehicle, min, 1.6 ± 0.09; Pralnacasan, 1.2 ± 0.07**, n= 8; **$p<0.01$ by Student's t-test). Pralnacasan did not affect seizures when given in a single administration (12.5 or 25 μg in 4 μl given 45 min before kainic acid) or at doses lower than 25 μg (12.5 in 4 μl; data not shown). Pralnacasan inhibited seizure duration by 52% and 38% of control values during the first and second hour of EEG recordings respectively, while its effect faded away during the third hour of recording. Pralnacasan did not affect spiking activity (not shown).

VX-765. As described for pralnacasan (see Fig.4.1A), 2h pre-incubation with VX-765 significantly reduced in a dose-dependent manner the LPS+ATP-mediated release of IL-1β in the slice culture medium (Fig. 4.1A). Maximal inhibition of ~70% was achieved with 1 μM VX-765. In the corresponding tissue extracts, VX-765 did not affect LPS+ATP induced increase (Fig. 4.1B). VX-765 alone did not affect IL-1β concentrations.

Fig. 4.2B shows the effect of systemic administration of VX-765 on kainic acid-induced seizures. VX-765 at the doses of 25, 50 and 200 mg/kg significantly delayed the time to seizure onset by 1.5 to 2-fold ($p<0.01$), reduced the number of seizures by 40% ($p<0.01$)

and the total time spent in EEG seizure activity by 30 to 50% ($p<0.01$). No differences in spiking activity were observed (not shown).

These findings demonstrate that caspase-1 inhibition using pralnacasan or VX-765 significantly delayed seizure onset and reduced the time spent in seizures. This anticonvulsive effect was achieved either after intracerebral or systemic administration of selective caspase-1 blockers, and was associated with the ability of these drugs to prevent the seizure-induced increase of the biologically active form of IL-1β.

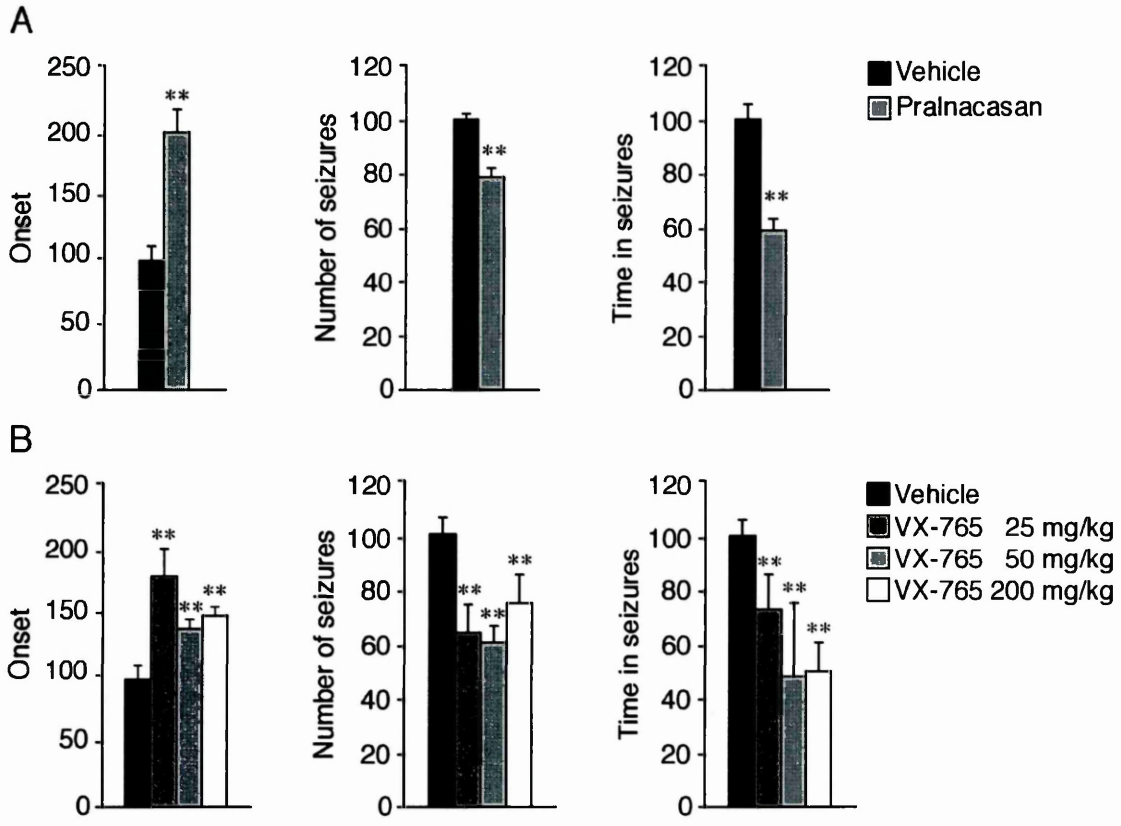


Figure 4.2. Effects of caspase-1 inhibition on kainic acid-induced seizures in rats
*Data represent the mean ± SE of the various seizure parameters expressed as % of control values measured in vehicle-treated rats. Vehicles in pralnacsan or VX-765 treated rats were pooled in a unique control group since they did not differ significantly [Onset (min): 7.9 ± 0.7; Number of seizures: 32 ± 1; Time in seizures (min): 44.0 ± 2.0, n= 22]. **p<0.01 vs vehicle by one-way ANOVA followed by Tukey's test; statistical analysis was done on absolute values.*

4.3.3 IL-1β and IL-1R1 expression in the epileptic mouse hippocampus.

Immunohistochemical analysis of epileptic mouse hippocampus, 2 months after intrahippocampal injection of kainic acid, showed that IL-1β immunoreactivity was strongly enhanced in glial-like cells (Fig. 4.3B) while IL-1R1 was enhanced in neurons (Fig. 4.3D and inset). These data are in accordance with our previous findings showing the induction of IL-1β and IL-1R1 by chemically- or electrically-induced seizures (Ravizza et

al., 2008a). IL-1β and IL-1R1 (Fig. 4.3A,C) were not detectable in control hippocampus, as previously shown (Ravizza et al., 2008a; Ravizza and Vezzani, 2006; Vezzani et al., 1999; Vezzani et al., 2000).

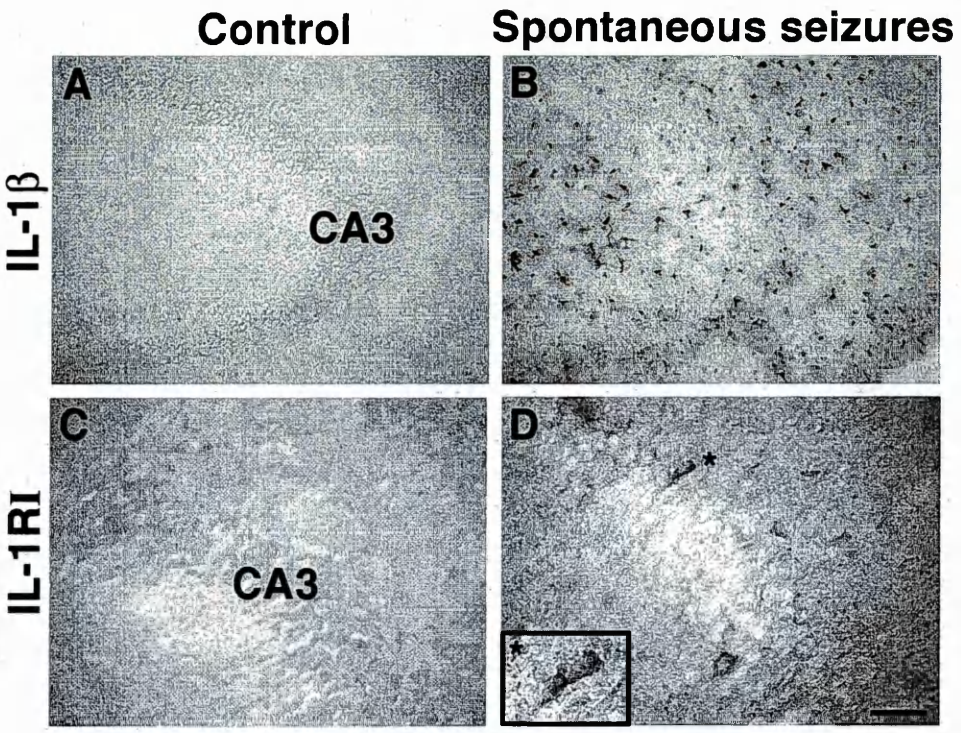
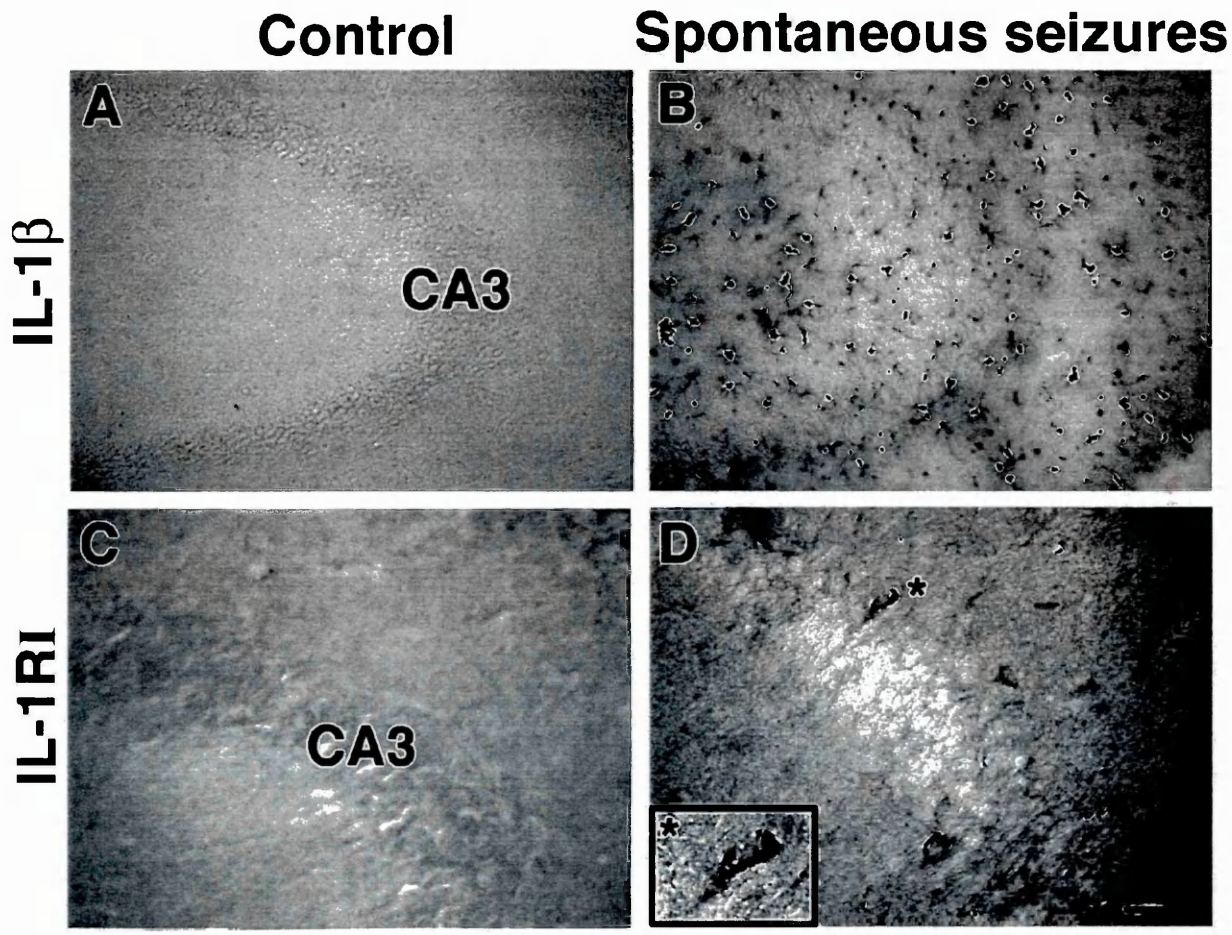


Figure 4.3. IL-1β and IL-1R1 expression in the epileptic mouse hippocampus
Representative photomicrographs of IL-1β (A-B) and IL-1R1 (C-D) immunoreactivity in the CA3 area of the hippocampus, 2 months after seizures induced by intrahippocampal injection of kainic acid (B,D) and in vehicle injected C57BL6 mice (A,C). IL-1β (A) and IL-1R1 (C) immunostaining was not detectable in control hippocampus. After chronic seizures, IL-1β immunoreactivity was strongly enhanced in glial-like cells (B), while IL-1R1 was enhanced in neurons (D and inset). Scale bar: A-D 100 μm; inset 50 μm.



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4.3.4 Effects of caspase-1 inhibition on kainic acid-induced spontaneous recurrent seizures.

Figure 4.4 shows the effect of systemic administration of VX-765 on kainic acid-induced spontaneous recurrent seizures. From the second day of treatment, 200 mg/kg VX-765 significantly reduced the number of spontaneous seizures by 40% and the time spent in seizures by 50%. The maximum effect was reached at fourth day of treatment where VX-765 reduced the number and the total time spent in seizure activity up to 75%. This effect was maintained for 24 h after the drug withdrawal. After 3 days wash-out period starting from the last drug administration, the spontaneous seizures pattern recovered to baseline values.

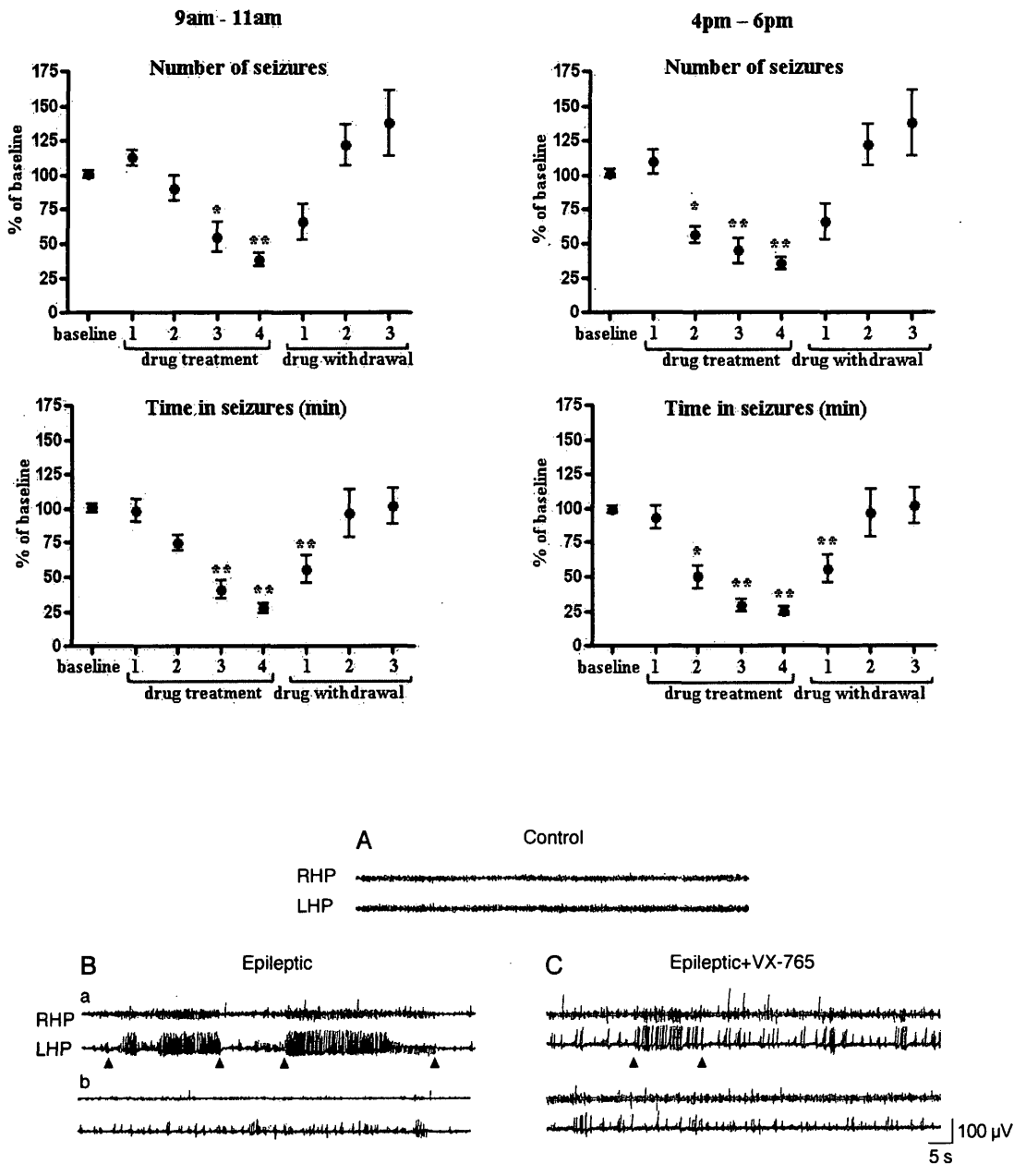


Figure 4.4. Effect of VX-765 on chronic spontaneous seizures

*Data represent the mean ± SE of the various seizure parameters expressed as percentage of baseline of spontaneous seizures measured in mice during 2 hour EEG recording before the first day of treatment. *p<0.05, **p<0.01 vs baseline by one-way ANOVA followed by Tukey's test.*
Panel A: baseline recording from a naïve mouse; Panel B: spontaneous seizure activity recorded 2 months after kainic acid administration; Panel C: effect of VX-765 on spontaneous seizure activity as assessed by 2 hours EEG analysis starting at the time of treatment in the afternoon treatment session. (a) typical ictal episodes (b) spiking activity; RHP right hippocampi; LHP left hippocampi.

These findings demonstrate that IL-1β up-regulation is not only a transient consequence of acute seizures, but it persists in chronic epileptic tissue. VX-765 by inhibiting caspase-1 reduces up to 75% the frequency and duration of spontaneous seizures that are resistant to the treatment of classical antiepileptic drugs.

In summary, this study has shown that:

1. Caspase-1 inhibition reduces up to 70% the release of IL-1β induced by proinflammatory stimuli.
2. Caspase-1 inhibition blocks the brain production and the release of the mature, biologically active form of IL-1β induced by seizures.
3. IL-1β and IL-1R1 are chronically expressed in epileptic tissue.
4. Caspase-1 inhibition by pralnacasan and VX-765 reduces the number and duration of acute and spontaneous seizures.

4.4 Discussion

The main finding of this work is that inhibition of caspase-1 in the brain provides significant protection from acute and spontaneous recurrent seizures induced by intrahippocampal kainic acid administration in rodents. This anticonvulsive effect was achieved either after intracerebral or systemic administration of selective caspase-1 blockers, and was associated with the ability of these drugs to prevent the seizure-induced increase of the biologically active form of IL-1β.

Caspase-1, the enzyme responsible for cleavage of proIL-1β to mature biological active form (Fantuzzi and Dinarello, 1999), is significant involved in seizures as demonstrated by experimental and clinical studies (Eriksson et al., 1999; Henshall et al., 2000; Ravizza et al., 2008b). Caspase-1 activation and the subsequent brain production of IL-1β, seem to be involved in seizure generation and maintenance (Vezzani and Baram, 2007) since mice with a deletion of the caspase-1 gene exhibited a delay of the onset time to seizure and a drastic reduction in epileptic activity (Ravizza et al., 2006b). This possibility is also supported by the chronic expression of IL-1β and its receptor type 1 in epileptic mice in brain areas such as the hippocampus, involved in seizure generation and propagation suggesting that the recurrence of spontaneous seizures may contribute to the chronic activation of IL-1β / IL-1R1 system.

In this study, pharmacological inhibition of caspase-1 was achieved in rodent brain using pralnacasan or VX-765, representing a new class of protease inhibitors which specifically inhibit caspase-1. Pralnacasan is a prodrug of the potent, selective, non-peptide competitive inhibitor RU36384, and was the first caspase-1 inhibitor to enter clinical development.

VX-765 is a prodrug with improved oral bioavailability and more active than pralnacasan (Braddock and Quinn, 2004; Wannamaker et al., 2007). These drugs show anti-inflammatory effects in patients with rheumatoid arthritis, without ensuing signs of toxicity, as reported to date (Braddock and Quinn, 2004; Randle et al., 2001; Stack et al., 2005).

Our biochemical evidence indicates that both pralnacasan and VX-765 blocked the release of IL-1β induced by activation of caspase-1 in organotypic hippocampal slice cultures, thus establishing a proof-of-concept that these drugs should block also the production and subsequent release of mature IL-1β *in vivo*. In our experimental model of seizures, we

indeed found that an anticonvulsant dose of pralnacasan led to a 50% reduction in the hippocampal levels of the 17 kDa mature form of IL-1β induced by seizures (De Simoni et al., 2000; Eriksson et al., 1999; Oprica et al., 2003; Vezzani et al., 1999) suggesting that this effect is responsible for the anticonvulsant effect of this drug. However, residual IL-1β still remained in the hippocampus in the absence of caspase-1 activity suggesting the existence of a pool with a slow turnover rate. Moreover, other enzymes such as matrix metalloproteinases, elastase, cathepsins, trypsin or chymotrypsin, are able to process to some extent pro-IL-1β, and this might also be the reason for the residual IL-1β levels in pralnacasan-treated rats (Fantuzzi and Dinarello, 1999; Schonbeck et al., 1998).

We cannot exclude that these caspase-1 inhibitors also reduced the production of IL-18 in the brain. However, we could not detect measurable levels of this cytokine in hippocampal homogenates either in control conditions or during the acute phases of seizures.

Pralnacasan or VX-765, by inhibiting caspase-1 activity, significantly reduced ictal activity without changing interictal spiking; this effect was observed also after intrahippocampal injection of IL-1ra or in mice overexpressing IL-1ra in astrocytes (Vezzani et al., 2000; Vezzani et al., 2002). It is possible that a reduction in IL-1β actions impairs the transition between interictal and ictal events, although some level of hippocampal hyperexcitability still persists. Similarly, classical antiepileptic drugs such as phenytoin and carbamazepine provide seizure control without modifying interictal epileptiform activity in limbic structures (Bazil and Pedley, 1995).

The most powerful anticonvulsant effect was achieved after peripheral administration of VX-765 in an experimental model of TLE. The inhibition of caspase-1 and, thus the reduction of IL-1β levels, induced a 70% reduction in the number and duration of

spontaneous seizures that are refractory to the treatment with classical antiepileptic drugs (Riban et al., 2002).

These novel findings open the perspective of a clinical use of selective caspase-1 inhibitors for the treatment of seizure disorders, including the cases that are not responsive to the available antiepileptic drugs.

CHAPTER 5

A novel non-transcriptional pathway mediates the proconvulsive effects of IL-1β

5.1 Summary

We investigated the molecular mechanisms involved in the proconvulsant actions of IL-1β. We show here that EEG seizures induced by intrahippocampal injection of kainic acid in C57BL6 adult mice were increased by 2-fold on average by pre-exposure to IL-1β and this effect was blocked by 3-O-methylsphingomyelin (3-O-MS), a selective inhibitor of the ceramide-producing enzyme sphingomyelinase. C2-ceramide, a cell permeable analog of ceramide, mimicked IL-1β action suggesting that ceramide may be the second messenger of the proconvulsive effect of IL-1β. The seizure exacerbating effects of either IL-1β or C2-ceramide were dependent on activation of the Src family of tyrosine kinases since they were prevented by CGP76030, an inhibitor of this enzyme family.

The proconvulsive IL-1β effect was associated with increased Tyr^{418} phosphorylation of Src-family of kinases indicative of its activation, and Tyr^{1472} phosphorylation of one of its substrate, the NR2B subunit of the NMDA receptor, which were prevented by 3-O-MS and CGP76030. Finally, the proconvulsive effect of IL-1β was blocked by ifenprodil, a selective NR2B receptor antagonist.

These results indicate that the proconvulsive actions of IL-1β depend on the activation of a sphingomyelinase- and Src-family of kinases-dependent pathway in the hippocampus which leads to the phosphorylation of the NR2B subunit, thus highlighting a novel, non-transcriptional mechanism underlying seizure exacerbation in inflammatory conditions.

5.2 Experimental procedures

5.2.1 Pharmacological treatments

Acute kainic acid-induced seizures were induced and quantified as previously described in "Materials and methods - general procedures" section (p. 81-83).

Human recombinant (hr)IL-1β (R&D system, Minneapolis, USA) was dissolved in 0.1 M PBS supplemented with 0.1% bovine serum albumin (BSA) and injected intrahippocampally (1 ng/0.5 µl) 10 min before kainic acid. This is the same dose causing proconvulsive effects in kainate-injected rats (Vezzani et al., 1999; Vezzani et al., 2002).

C2-ceramide, a membrane-permeable ceramide analogue, and dihydro-ceramide, the biologically inactive form of C2-ceramide (Sigma) (Obeid et al., 1993) were dissolved in 10% dimethylsulfoxide (DMSO) in PBS and injected intrahippocampally (0.25-1.0-2.0 µg in 0.5 µl), 10 min before kainic acid.

3-0-Methylsphingomyelin (3-O-MS, N-Smase inhibitor; BioMol Research Laboratories Inc., PA, USA) (Tsakiri et al., 2008; Zeng et al., 2005) was dissolved in 10% DMSO in PBS and injected intrahippocampally (3 µg/0.5 µl) or icv (15 µg/1µl), 20 min before kainic acid. These doses were shown to block the rapid phase of febrile response to IL-1β after injection into the mouse preoptic area/anterior hypothalamus (pilot study; see Sanchez-Alavez et al., 2006).

CGP76030, a selective Src-family of tyrosine kinase inhibitor which binds to the catalytic SH1 domain, thus preventing substrate phosphorylation (Rucci et al., 2006; Susa et al., 2000; Susa et al. , 2005) was provided as a research tool by Novartis AG, Basel, Switzerland. It was dissolved in 2% DMSO in PBS and injected intrahippocampally (65

ng/0.5 μl) or intracerebroventricularly (icv; 130 ng/1μl), 20 minutes before kainic acid. The dosage of CGP76030 corresponds to the one achieved in mouse brain after oral administration of 30 μmol/kg, as assessed measuring its brain/plasma ratio at 1 and 3h, reaching a highest ratio at 3 h. This dose blocked the electrophysiological effects of IL-1β on the activity of warm-sensitive hypothalamic neurons *in vivo* (pilot study; see also (Sanchez-Alavez et al., 2006).

Ifenprodil, (NR2B-selective NMDA antagonist (Chenard and Menniti, 1999), Sigma) was dissolved in 5% DMSO with 9% Tween80 in PBS and injected intraperitoneally (1mg/kg), 15 min before kainic acid. We choose this dose since it was shown to block NR2B receptors in vivo during tPA facilitation of ethanol withdrawal seizures, although not affecting seizures *per se* (Pawlak et al., 2005).

Control mice were injected with the corresponding volume of vehicles (10% DMSO in PBS; 2% DMSO in PBS; 5% DMSO with 9% Tween80 in PBS; PBS supplemented with 0.1% BSA) before kainic acid.

A schematic representation of experimental protocols is depicted in figure 5.1.

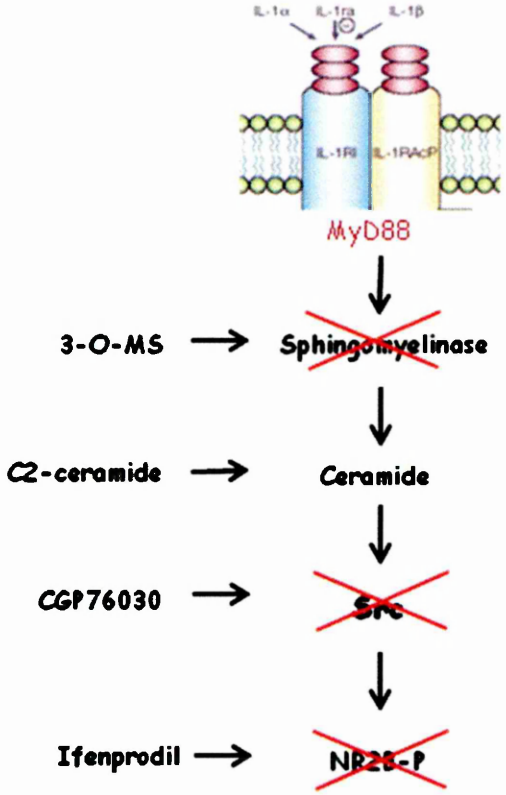


Figure 5.1. Schematic representation of experimental protocols

5.2.2 Immunohistochemistry

At the end of the EEG analysis, mice injected with kainic acid (n=5) and their controls (n= 5), were perfused as previously described (see "Materials and methods - general procedures" section (p. 87)). Serial cryostat coronal sections were used for IL-1β and IL-1R1 immunohistochemestry following the protocol previously described (p. 88).

5.2.3 Double-immunostaining

Two brain slices in each mouse brain for each cell type marker were randomly chosen to identify the cells expressing IL-1β or IL-1R1. The procedures for double-immunostainig is described in "Materials and methods - general procedures" section (p. 90).

5.2.4 Western blot

Different groups of mice (n=10-12 in each group) were implanted with electrodes and cannula and injected with hrIL-1β, kainic acid, or their combination ± 3-O-MS or CGP76030 (see before for injection protocol). Control mice (n=14) were implanted with electrodes and cannula and injected with the corresponding vehicles of the various treatments at the appropriate time interval. One hour after the onset of EEG seizures, experimental mice and their controls were decapitated. This time was chosen in order to provide sufficient EEG activity tracing to evaluate whether mice had the typical EEG pattern induced by kainic acid administration. The dorsal injected hippocampus was dissected out at 4°C and 2 hippocampi obtained from different mice within the same experimental group were pooled and homogenized as described in "Materials and methods - general procedures" section (p. 92).

For immunoblotting, we used an anti-pTyr^{418}-Src which is located in the catalytic domain therefore indicative of Src-family of tyrosine kinases activation (1:750; Sigma) or anti-pTyr^{1472}-NR2B (1:1000; Affinity Bioreagents Golden, CO, USA) rabbit polyclonal antibodies. Total NR2B levels were assessed using goat polyclonal anti-NR2B antibody (1:1000; Santa Cruz). Immunoreactivity was visualized with enhanced chemiluminescence

(ECL, Amersham, UK) using peroxydase-conjugated goat anti-rabbit (1:2000; Sigma) or rabbit anti-goat (1:10000; Sigma) IgGs as secondary antibodies.

5.2.5 Statistical analysis of data

Data are the mean ± SEM (n = number of individual samples). The effects of treatments were analyzed by two-way ANOVA followed by Tukey or Kruskal-Wallis test, or by Student *t*-test.

Data depicted in Figs. 5.2 and 5.3 are expressed as % of vehicle-injected mice; however, statistical analysis was carried out using absolute values.

5.3 Results

5.3.1 Seizure-mediated induction of IL-1β and IL-1R1 in the mouse hippocampus

Immunohistochemical analysis of hippocampal sections 3 h after intrahippocampal injection of kainic acid, showed increased IL-1β immunoreactivity in astrocytes (Fig. 5.2B) while IL-1R1 was enhanced both in neurons and astrocytes (Fig. 5.2D); this activation involved the dorsal and temporal poles of the hippocampus bilaterally. These data are in accordance with our previous findings showing the induction of IL-1β and IL-1R1 by chemically- or electrically-induced seizures in rats (Ravizza et al., 2008a; Ravizza and Vezzani, 2006; Vezzani et al., 1999) or bicuculline-induced seizures in mice (Vezzani et al., 2000). IL-1β and IL-1R1 (Fig. 5.2A,C) were not detectable in control hippocampus, as previously shown (Ravizza et al., 2008a; Ravizza and Vezzani, 2006; Vezzani et al., 1999; Vezzani et al., 2000).

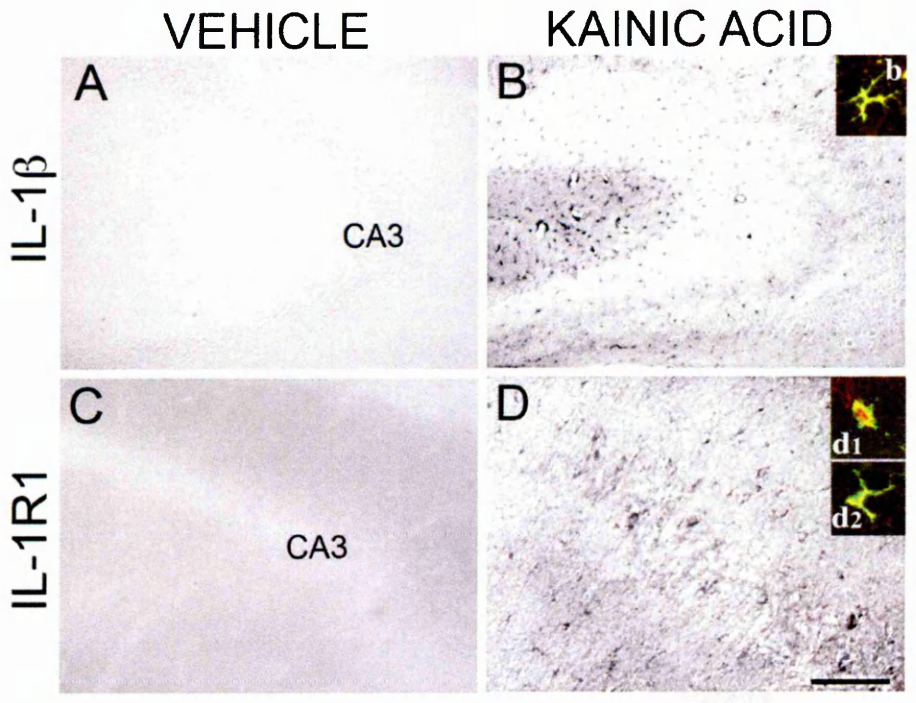


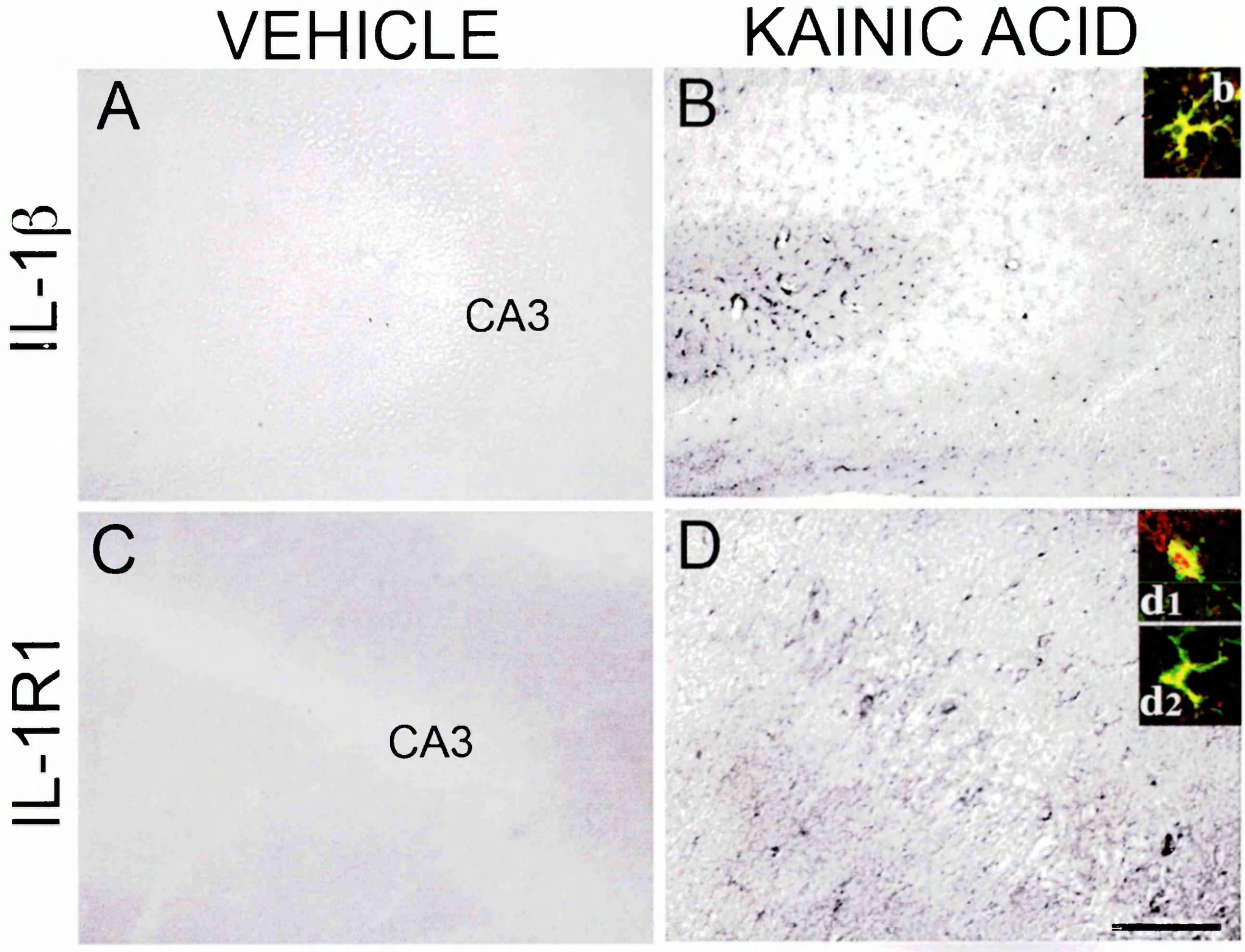
Figure 5.2. IL-1β and IL-1R1 expression in the mouse hippocampus after kainic acid-induced seizures

Representative photomicrographs of IL-1β (A-B) and IL-1R1 (C-D) immunoreactivity in the CA3 area of the hippocampus, 3 h after seizures induced by intrahippocampal injection of kainic acid (B and D) and in vehicle-injected C57BL6 mice (A,C). IL-1β (A) and IL-1R1 (C) immunostaining was not detectable in control hippocampus. After seizures, IL-1β immunoreactivity is strongly enhanced in GFAP-positive astrocytes (B, yellow signal in inset); IL-1R1 staining was enhanced both in neurons (D, yellow signal in d_1) and astrocytes (D, yellow signal in d_2). Scale bar: A-D 100 μm; insets, 25 μm.

5.3.2 N-Smase-Src kinase-NR2B pathway mediates the proconvulsive activity of IL-1β

3-O-MS

We established the role of neutral-sphingomyelinase (N-Smase) in the proconvulsive effect of IL-1β using the selective N-Smase inhibitor 3-O-MS (Tsakiri et al., 2008; Zeng et al., 2005) (Table 5.1). The intrahippocampal injection of hrIL-1β, 10 min before kainate, increased by ~1.8-fold the number of seizures and by ~2.0-fold their duration as compared



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with vehicle-treated mice ($p<0.01$; Table 5.1). Three μg 3-O-MS blocked the proconvulsive effect of hrIL-1β when injected into the hippocampus, 10 min before the cytokine (i.e. 20 min before kainate) since it abolished the increase in the number of seizures ($F_{(1,62)}=2.2$; $p < 0.05$) and time spent in ictal activity ($F_{(1,62)}=5.9$; $p < 0.01$ by two-way ANOVA) induced by hrIL-1β. The latency to the first seizure and the time spent in interictal activity were not affected by these treatments. 3-O-MS injected intrahippocampally (3 μg/0.5 μl) (Table 5.1) or icv (15 μg/1 μl) (not shown) 20 min before kainic acid, did not affect seizure parameters; higher doses could not be used because of the limit of solubility of this compound in the volume of injection.

These data demonstrate that the inhibition of N-Smase blocks the increase in the frequency and duration of seizures induced by IL-1β suggesting that N-Smase activation mediates the proconvulsive actions of this cytokine.

Table 5.1. Effect of inhibition of the N-Smase-Src-NR2B pathway on the proconvulsive effects of IL-1β

	Dose	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Vehicle	-	8.7 ± 0.5	9.0 ± 1.0	5.6 ± 0.5	48.0 ± 3.8
hrIL-1β	1 ng, i.h.	7.6 ± 0.4	16.0 ± 2.0 **	11.8 ± 1.1**	50.0 ± 3.7
3-O-MS	3 μg, i.h.	8.6 ± 1.0	7.0 ± 1.0	4.9 ± 0.6	43.9 ± 6.6
3-O-MS + hrIL-1β		9.9 ± 2.1	8.0 ± 1.0†	5.2 ± 0.6††	48.5 ± 4.6
CGP076030	65 ng, i.h.	8.1 ± 1.0	10.0 ± 2.0	6.3 ± 1.1	53.3 ± 7.5
CGP76030 + hrIL-1β		8.8 ± 0.9	7.0 ± 1.0†	3.8 ± 0.6††	47.4 ± 4.7
Ifenprodil	1 mg, i.p.	9.2 ± 2.1	8.0 ± 1.0	6.4 ± 1.3	42.9 ± 6.6
Ifenprodil + hrIL-1β		8.9 ± 1.7	9.0 ± 1.0†	5.0 ± 0.5††	59.4 ± 4.2

*Data are the mean ± SE. Vehicle (n=23) represents control mice treated with the corresponding vehicles of the various treatments before kainic acid application; since these mice did not differ in seizure parameters they were pooled in a single control group. hrIL-1β (n=28) are mice injected with this cytokine after receiving the corresponding vehicles of the various treatments, then they were treated 10 min later with kainic acid. Since these mice did not differ in seizure parameters, they were pooled in a single group. 3-O-MS, CGP76030 or ifenprodil (n=7-11) were injected alone or with hrIL-1β before kanic acid injection (see Pharmacological treatments for details). **$p<0.01$ vs vehicle; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs hIL-1β by two-way ANOVA followed by Tukey test ($F_{(DF)}$ for each treatment group are reported in the Result section).*

C2-ceramide

Ceramide is produced by N-Smase acting on membrane sphingomyelin and this pathway is rapidly activated in mouse forebrain by IL-1β via IL-1R1 (Nalivaeva et al., 2000). Intrahippocampal injection of C2-ceramide, the cell-penetrating analog of ceramide, 10 min before kainic acid, dose-dependently enhanced the number of seizures and their duration (Fig. 5.3) without changing the time to onset of seizures and the spiking activity (see Table 5.2). No effect on seizures was observed using 0.25 μg C2-ceramide, 1 μg significantly increased seizures duration by ~2-fold ($p<0.05$) while 2 μg significantly increased the number of seizures by 2.0-fold and their duration by 2.5-fold on average ($p<0.01$) vs mice injected with dihydroceramide, a membrane-impermeable ceramide analog (Fig. 5.3A; see Table 5.2). Thus, C2-ceramide mimics IL-1β proconvulsive effects inducing also a similar EEG pattern of seizures (Fig. 5.3B).

These findings demonstrate that C2-ceramide mimicked IL-1β action suggesting that ceramide may be the intracellular second messenger of the proconvulsive effect of the cytokine.

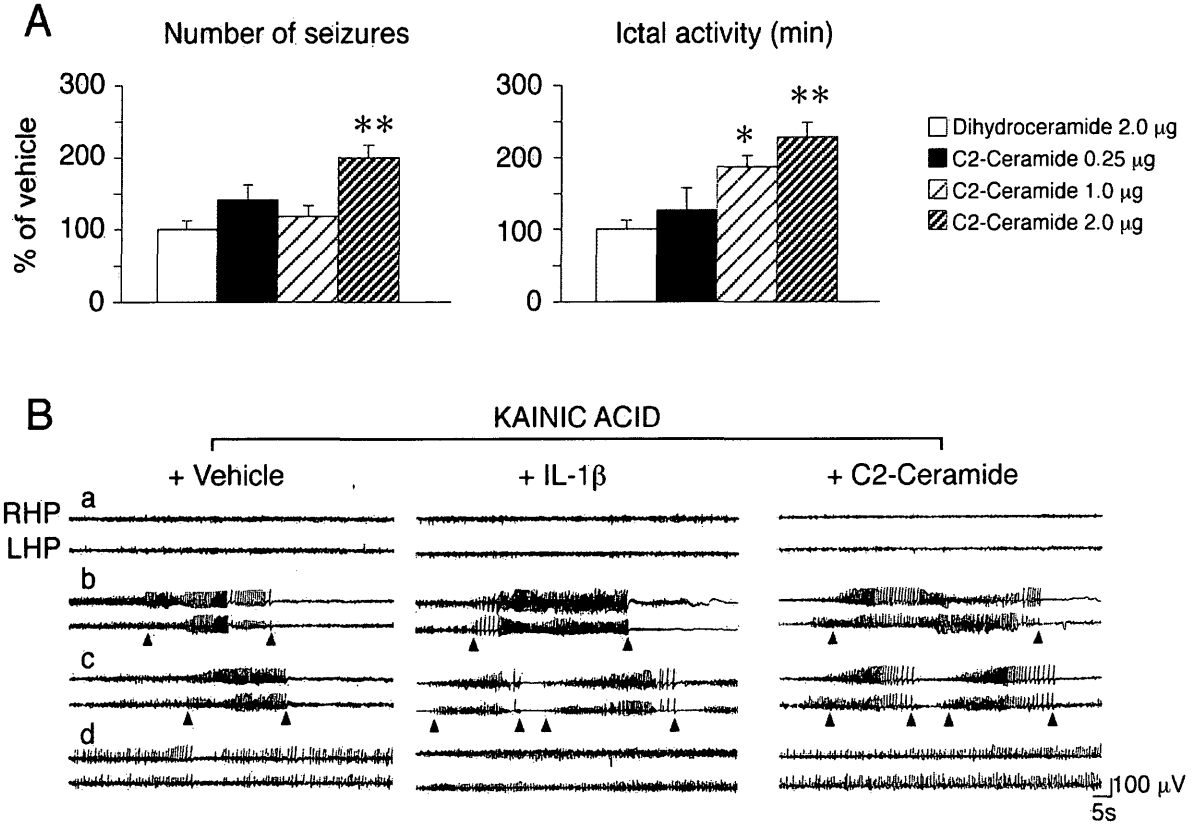


Figure 5.3. Effect of C2-ceramide on seizures

*Panel A. Bargrams represent the mean ± SE (n=12). Significant increases in seizure parameters were observed at 1 and 2 µg C2-ceramide. *p<0.05; **p<0.01 vs dihydroceramide by one way ANOVA followed by Tukey's test.*

Panel B. Representative EEG tracings of freely moving C57BL6 mice injected unilaterally in the hippocampus with kainic acid ± IL-1β or C2-ceramide. Treatments or vehicles were given 10 min before kainic acid. (a) Baseline recording before kainic acid injection; arrowheads in (b) and (c) include representative ictal episodes recorded in the EEG during 90 min after kainic acid injection ± IL-1β or C2-ceramide; tracings in (d) depict spiking activity in the EEG after termination of seizures. RHP and LHP are right and left (injected) hippocampus, respectively.

CGP76030

Tables 5.1 and 5.2 show the effect of intrahippocampal injection of the selective inhibitor of Src-family of tyrosine kinase activity, CGP76030 (Rucci et al., 2006; Susa et al., 2000; Susa et al. , 2005) on the proconvulsant effect of hrIL-1β (Table 5.1) and C2-ceramide (Table 5.2). CGP76030 (65 ng/0.5μl) injected 20 min before kainate, significantly prevented the 2-fold increase in the number of seizures induced by IL-1β ($F_{(1,63)}$=5.9; $p < 0.05$) or C2-ceramide ($F_{(1,28)}$=23,9; $p<0.01$) and in the time spent in ictal activity induced by IL-1β ($F_{(1,63)}$=13.3; $p < 0.01$) or C2-ceramide ($F_{(1,28)}$=30.9, $p < 0.01$ by two-way ANOVA). Intrahippocampal CGP76030 did not affect seizures *per se*; however, when 130 ng/1μl CGP76030 alone was injected icv 20 min before kainate, the number and the duration of seizures were decreased by ~1.8- and 2.0-fold, respectively, as compared with vehicle-treated mice (*Number of seizures*: Vehicle, n=8, 16.0±2.0; CGP, n=8, 9.0±1; $p<0.01$; *Time in ictal activity* (min): Vehicle, 9.9±0.5; CGP, 4.8±0.4; $p<0.01$ by Student *t*-test).

These findings demonstrate that inhibition of Src kinase activity prevents the IL-1β and C2-ceramide proconvulsive effects, thus suggesting that Src kinase activation is a downstream event subsequent to IL-1β activation of N-Smase. Moreover, Src kinase inhibition *per se* reduced seizure number and duration in mice suggesting that Src-family of tyrosine kinases appear to contribute to seizures also in the absence of pro-inflammatory conditions.

Table 5.2. Effect of inhibition of Src-family of tyrosine kinases on the proconvulsive effects of C2-ceramide

	Dose	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Di-hydro-ceramide	2 μg	8.6 ± 0.7	8.0 ± 1.0	5.4 ± 0.5	44.0 ± 3.4
C2-Ceramide	2 μg	8.9 ± 1.4	18.0 ± 2.0**	13.4 ± 1.0**	51.1 ± 3.7
CGP076030	65 ng	8.1 ± 1.0	10.0 ± 2.0	6.3 ± 1.1	53.3 ± 7.5
CGP076030 + C2-Ceramide		8.4 ± 1.6	8.0 ± 1.0††	5.5 ± 0.4††	45.0 ± 3.3

*Data are the mean ± SE (n=7-9 mice in each experimental group). **p<0.01 vs the inactive analog dihydro-ceramide; ††p<0.01 vs C2-ceramide by two-way ANOVA followed by Tukey's test ($F_{(DF)}$ for each treatment group are reported in the Result section).*

Ifenprodil

Ifenprodil (1 mg/kg), a NR2B-specific NMDA antagonist, injected 5 min before hrIL-1β blocked the cytokine-mediated increase in the number ($F_{(1,61)}$=2.2; p<0.05) and total time in seizures ($F_{(1,61)}$=9.0; p<0.01 vs hrIL-1β by two-way ANOVA) (Table 5.1). This dose of ifenprodil did not affect seizures *per se*, although higher doses are known to provide anticonvulsive effects (Kohl and Dannhardt, 2001; Yen et al., 2004) .

These data demonstrate that ifenprodil prevented the proconvulsive effects of IL-1β, thus indicating that NR2B subunit is critically involved in IL-1β action on seizures.

5.3.3 Effect of pharmacological treatments on Src-family of tyrosine kinases activation and NR2B phosphorylation

We assessed by western blot analysis of hippocampal homogenates, the level of tyrosine phosphorylated (p) forms of Src-family of kinases and the NR2B subunit of NMDA receptor, 60 min after seizures onset in kainate ± IL-1β injected mice, as well as in mice receiving IL-1β alone (Fig. 5.4). Densitometric analysis of the specific protein bands showed that either hrIL-1β alone (no seizures) or seizures *per se* increased phosphorylation (p) of Src-family of kinases on Tyr^{418} in the catalytic domain denoting their activation (Papp et al., 2008), by 40% on average as compared to vehicle-treated mice ($p<0.05$ and $p<0.01$, respectevely) while in the same hippocampi the Tyr^{1472} phosphorylation of the NR2B form, causing upregulation of channel gating properties (Salter and Kalia, 2004), was increased by 26% and 47% on average, respectively ($p<0.01$). When hrIL-1β was coinjected with kainic acid, thus producing proconvulsive effects, (p)Src and (p)NR2B levels were increased by 127% and 82% respectively as compared to vehicle-treated mice ($p<0.01$), denoting additive effects of the single treatments. We also evaluated whether inhibition of N-Smase or Src-family of kinases activity affects the increased (p)Src and (p)NR2B levels observed in proconvulsive conditions. 3-O-MS or CGP76030 at the doses which blocked the proconvulsive effects of IL-1β, reversed NR2B phosphorylation by reducing (p)NR2B to the levels of vehicle-injected mice (3-O-MS: $F_{(1,17)}=21.9$; $p<0.01$; CGP76030: $F_{(1,17)}=10.7$; $p<0.01$). In IL-1β+kainate-treated mice, inhibition of N-Smase by 3-O-MS similarly reversed Src-family of kinases phosphorylation ($F_{(1,17)}=30.5$; $p<0.01$), which was not affected by CGP76030 treatment. 3-O-MS or CGP76030 alone did not

change the basal level of (p)Src and (p)NR2B. The total levels of NR2B were not changed by the various treatments (not shown).

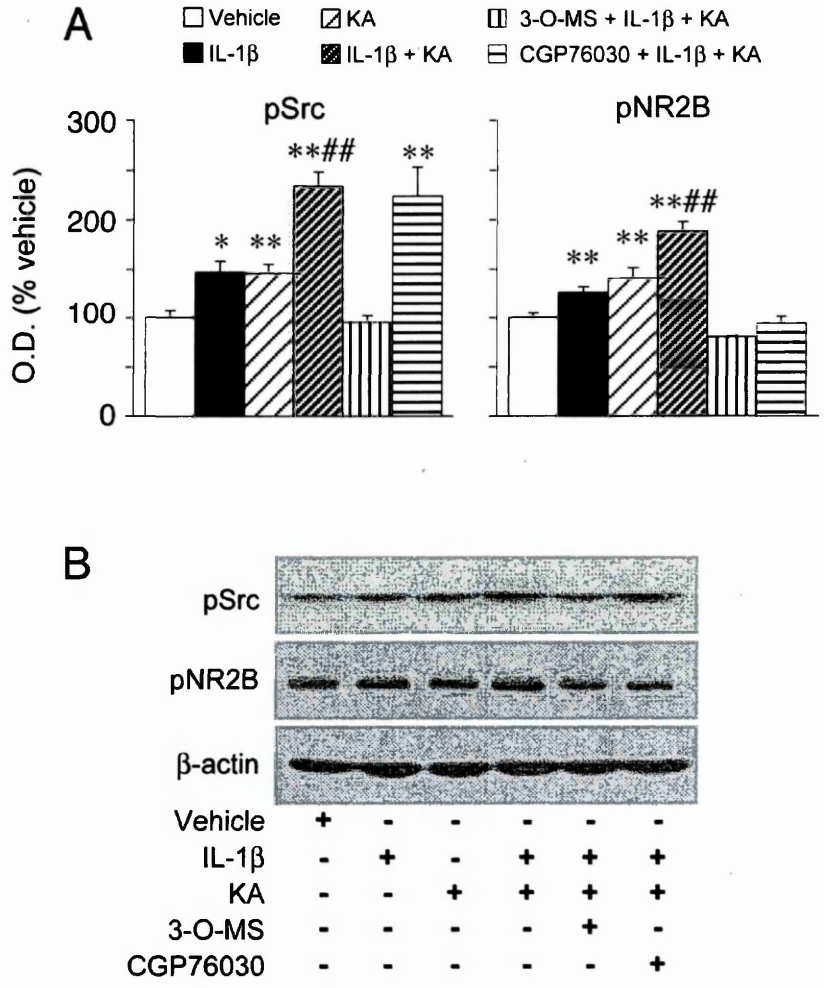


Figure 5.4. IL-1β and seizure induced tyrosine phosphorylation of Src-family of kinases and the NR2B subunit of the NMDA receptor: effect of pharmacological treatments

*Bargrams show densitometry analysis of the Src kinase and NR2B bands corresponding to their phosphorylated (p) forms (Src-Tyr-^{418}; NR2B-Tyr-^{1472}) in the various experimental groups, 60 min after the onset of kainate-induced seizures (~70 min after kainate injection), as assessed by western blot analysis of hippocampal homogenates. Data (means ± SE, n=5-7) are optical density (O.D.) values of the relevant bands (as depicted in the representative western blot), divided by the corresponding β-actin value (internal standard). Data are expressed as % of values measured in corresponding vehicle treated mice. Statistical analysis of data was done on absolute values; *p<0.05; **p<0.01 vs vehicle; ##p<0.01 vs 3-O-MS+IL-1+KA (for pSrc and pNR2B) and vs CGP76030+IL-1+KA (for pNR2B only) by two-way ANOVA followed by Kruskal-Wallis test. Representative western blot bands corresponding to the specific proteins are depicted in B.*

These findings demonstrate that IL-1β or seizures enhanced the levels of the phosphorylated forms of Src-family of tyrosine kinases and the NR2B subunit in the hippocampus and these effects were additive when seizures were increased by IL-1β. Moreover, NR2B phosphorylation induced in proconvulsive conditions was reversed to baseline level in mice treated with N-Smase and Src kinase inhibitors, thus supporting the involvement of these enzymes in the activation of this receptor subunit.

In summary, this study has shown that:

1. The proconvulsant effect of IL-1β involves a non-transcription-dependent pathway that leads to the activation of N-Smase and the consequent ceramide-induced Src-dependent phosphorylation of NR2B subunit.
2. This intracellular signaling highlights a functional interaction between IL-1β and NMDA receptors.

5.4 Discussion

The main finding of this study is that the proconvulsive actions of IL-1β depend on the activation of a sphingomyelinase- and Src-family of kinases-dependent pathway in the mouse hippocampus which leads to the phosphorylation of the NR2B subunit, thus highlighting a novel, non-transcriptional mechanism underlying seizure exacerbation in inflammatory conditions.

The proconvulsant actions of IL-1β occur within a few minutes from the intracerabral application of this cytokine (Vezzani et al., 1999); therefore, this time frame is not compatible with the classic signaling cascade involving activation of genomic transcriptional events (between 30-90 minutes). This consideration raises the hypothesis that the proconvulsant effects of IL-1β involve an alternative, rapid, non-transcriptional intracellular neuronal pathway leading to fast changes in ion channels (Fig. 5.5).

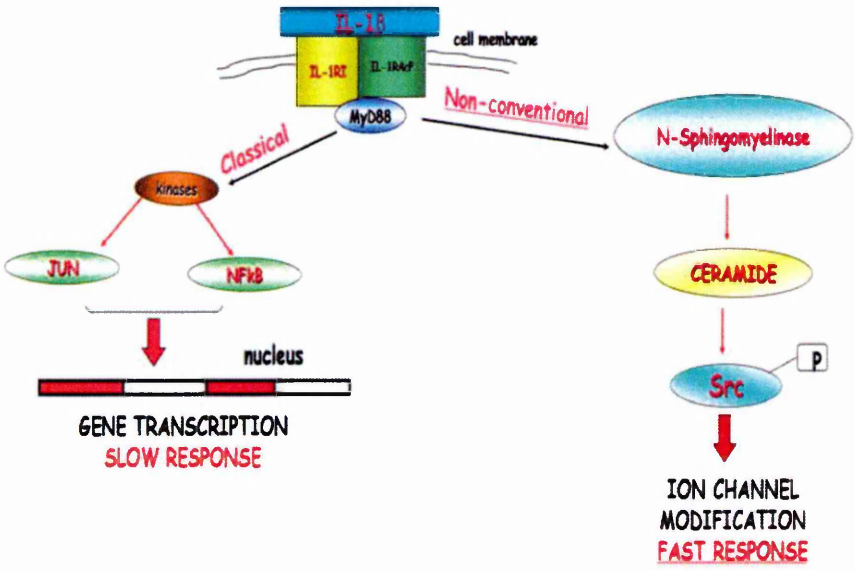


Figure 5.5. Simplified representation of IL-1β non-transcriptional neuronal pathway

Previous reports have shown effects of IL-1β in hippocampal and hypothalamic neurons involving synaptic plasticity and thermoregulation which also appear to be independent of transcriptional events (Davis et al., 2006b; Pickering and O'Connor, 2007; Sanchez-Alavez et al., 2006; Viviani et al., 2007). In particular, the activation of the *IL-1β -N-Smase-Src kinase pathway* has been shown to mediate the *fast actions of IL-1β* on preoptical/anterior warm sensitive hypothalamic neurons underlying the rapid phase of the febrile response to

IL-1β (Sanchez-Alavez et al., 2006). These effects of IL-1β can be mimicked by C2-ceramide, and inhibitors of N-Smase in the brain lower or prevent the response to IL-1β (Sanchez-Alavez et al., 2006). Furthermore, the formed ceramide activates the Src-family of tyrosine kinases in hypothalamic neurons, but not in glia (Davis et al., 2006a; Davis et al., 2006b).

We show here, using a detailed pharmacological approach, that inhibition of N-Smase using 3-O-MS blocks the increase in the frequency and duration of seizures induced by IL-1β, and that C2-ceramide faithfully mimics the IL-1β effect on seizures, thus suggesting that the activation of the N-Smase-ceramide pathway mediates the proconvulsive activity of this cytokine. To elucidate the downstream events which follow N-Smase activation, we addressed the possible involvement of the Src-family of kinases that is known to be activated by ceramide (Kolesnick and Golde, 1994; Saklatvala, 1995). Our data show that the inhibition of the Src-family of kinases activity prevents the IL-1β and C2-ceramide proconvulsive effects similarly, thus suggesting that Src-family of tyrosine kinases activation is a downstream event subsequent to IL-1β activation of N-Smase. This possibility is also supported by the reversal of IL-1β-induced tyrosine phosphorylation of Src-family of kinases during seizures using the N-Smase inhibitor.

Src-family of tyrosine kinases is abundantly expressed in neurons, and one main function of the activated forms of two members of this family, namely c-Src and Fyn, is to upregulate the activity of NMDA receptors via Tyr^{1472}-phosphorylation of the NR2B subunit (Ali and Salter, 2001). We found that ifenprodil, a selective antagonist of NMDA receptors containing the NR2B subunit, prevented the proconvulsive effects of IL-1β, thus indicating

that this subunit is critically involved in IL-1β action on seizures. Our previous findings in primary cultures of hippocampal neurons showed that IL-1β induces the Tyr^{1472}-phosphorylation of the NR2B subunit via Src-family of kinases activation (Viviani et al., 2003), therefore we assessed whether these events occurred *in vivo* when seizures were potentiated by IL-1β. The levels of the phosphorylated forms of Src-family of kinases and the NR2B subunit in the hippocampus were indeed enhanced by IL-1β or seizures, and these effects were additive when seizures were increased by IL-1β. Moreover, NR2B Tyr^{1472}-phosphorylation induced in proconvulsive conditions was reversed to baseline level in mice treated with N-Smase and Src-family of kinases inhibitors, thus supporting the involvement of these enzymes in the activation of this receptor subunit (Yu et al., 1997). Tyr^{1472}-phosphorylation of the NR2B subunit promotes Ca^{2+} influx into neurons (Viviani et al., 2003; Yu et al., 1997), thus resulting in potentiation of NMDA function which is pivotal for evoking neuronal hyperexcitability (Meador, 2007). This molecular event may be the ultimate step of this novel *IL-1β-N-Smase-Src kinase* pathway responsible for the increased neuronal excitability and subsequent proconvulsive effects of this cytokine. Although CGP76030 selectively inhibits Src-family of tyrosine kinases activation, and it was shown to have higher affinity for c-Src vs other Src-family of tyrosine kinase members (Susa et al., 2000; Susa et al. , 2005), no data about the inhibitor IC50 on Fyn activity are available, therefore both c-Src and Fyn may be involved in the IL-1β signaling underlying its proconvulsive effects. The possible specific involvement of c-Src vs Fyn should await the possibility to unequivocally distinguish the involvement of these two kinase activities using pharmacological approaches which are not yet available.

Interestingly, Src-family of tyrosine kinases appears to contribute to seizures also in the absence of pro-inflammatory conditions since Src-family of kinases inhibition *per se* after icv, but not intrahippocampal injection of CGP76030, reduced seizures number and duration in mice. We could not affect seizures by inhibiting the activity of N-Smase using 3-O-MS either intrahippocampally or icv injected. The lack of effect of intrahippocampal application of these drugs on kainate-induced seizures may be due to the local spread of the inhibitors around the injection site as opposed to seizures-induced IL-1β expression throughout the whole hippocampus. Moreover, we should also consider the possibility that the dose of 3-O-MS allowed to be injected by the limited solubility of this compound is not enough to protect from kainate seizures.

The relevance of our findings for the etiopathogenesis of seizures is based on clinical and experimental evidence showing that the IL-1β system is activated in chronic human epilepsy (Vezzani and Granata, 2005; Ravizza et al., 2006a; Ravizza et al., 2008a) and that anti-IL-1β pharmacological treatments result in powerful anticonvulsant effects (De Simoni et al., 2000; Vezzani et al., 2000; Vezzani et al., 2002; Ravizza et al., 2006b). Moreover, several brain injuries in humans are associated with brain inflammation, result in early occurrence of seizures and present a high risk of developing epilepsy (Pitkanen and Sutula, 2002; Vezzani and Granata, 2005). Therefore, the elucidation of this novel IL-1β-activated pathway in the hippocampus adds important insights into the mechanisms of ictogenesis in inflammatory conditions and may allow the development of innovative strategies to block the activation of IL-1β signaling in disease conditions, thus highlighting potential new targets of therapeutic intervention.

CHAPTER 6

Transient inflammatory-like events in hippocampal slice cultures prime neuronal susceptibility to excitotoxic injury: a crucial role of $P2X_7$ receptor-mediated IL-1β release

6.1 Summary

We investigated the consequences of transient application of inflammatory stimuli to hippocampal tissue on microglia activation and neuronal cell vulnerability to a subsequent excitotoxic insult.

Mouse organotypic hippocampal slice cultures were exposed for 3 h to lipopolysaccharide (LPS; 10 ng/ml) followed by 3 h co-incubation with 1 mM ATP, or 100 μM BzATP, a selective $P2X_7$ receptor agonist. This treatment induced a pronounced activation, and apoptotic-like death, of Mac-1 positive microglia associated with a massive release of IL-1β, exceeding that induced by LPS alone. Antagonists of $P2X_7$ receptors prevented these effects.

Transient pre-exposure of slice cultures to a combination of LPS and $P2X_7$ receptor agonists, but not either one or the other alone, significantly exacerbated CA3 pyramidal cell loss induced by subsequent 12 h exposure to 8 μM AMPA. Potentiation of AMPA toxicity was prevented by blocking IL-1β production or its receptor signaling. The same treatments did not prevent microglia apoptosis-like death.

These findings show that transient exposure to specific pro-inflammatory stimuli in brain tissue can prime neuronal susceptibility to a subsequent excitotoxic insult. $P2X_7$ receptor stimulation, and the consequent IL-1β release, is mandatory for exacerbation of neuronal loss. These mechanisms may contribute to determine cell death in acute and chronic neurodegenerative conditions associated with inflammatory events.

6.2 Experimental procedures

6.2.1 In vitro model of inflammation

Technical details related to organotypic hippocampal slice cultures, determination of cell damage by PI, ELISA and immunohistochemical staining have already been reported in "Materials and methods - general procedures" section (p. 91; 95-97).

Organotypic hippocampal slice cultures were maintained in incubator for 2 weeks and resembled the characteristic young/adult brain.

Hippocampal slice cultures were exposed to inflammatory-like stimuli following the protocol previously described (see "Materials and methods - general procedures" (p. 97). The various treatments were applied to the LPS+ATP protocol as depicted in Fig. 6.1 by pre-incubating slice cultures for 2h with the following drugs: 25 μM z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (zVAD-fmk; Sigma, St Louis, MO, USA), a pro-apoptotic caspases inhibitor (Ferrari et al., 1999); 300 μM oxidized ATP (oATP; Sigma;) or 200 nM Brilliant Blue G (BBG; Sigma), purinergic $P2X_7$ receptor antagonists (Choi et al., 2007; Khakh et al., 2001; North, 2002); 1 μM IL-1 receptor antagonist (IL-1ra), a competitive antagonist of IL-1β receptor type 1 (Dinarello et al., 1996; Eisenberg et al., 1990); 100 μM VX-765, a selective ICE/Caspase-1 inhibitor (Ravizza et al., 2006b; Stack et al., 2005). In one set of experiments, incubation with 1 mM ATP was substituted by 100 μM 2'-3'-O-(4-Benzoyl-benzoyl) adenosine 5'-triphosphate triethylammonium salt (BzATP, Sigma), a $P2X_7$ receptor agonist (Bianco et al., 2005; North, 2002). All drugs were dissolved in phosphate-buffered saline (PBS, pH 7.4) except for VX-765 and IL-1ra, respectively dissolved in DMSO and PBS+0.1% BSA. All experiments included control cultures

exposed to drug vehicles only. Each experimental group consisted of 12 to 30 well replicates (resulting from 8 separate experiments) containing 6 slices each well. At the end of the experiments, tissue was pooled from 6 slices per experimental group while the respective culture media were collected separately and samples were stored at –70° C until ELISA measurement.

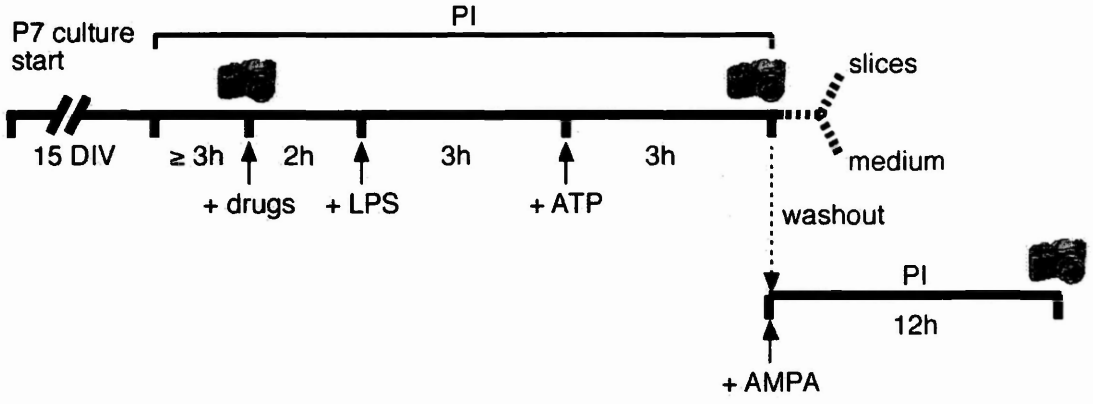


Figure 6.1. Schematic representation of experimental protocols
Digital fluorescent micrographs were taken before drug exposure (basal PI uptake) and at fixed time points after drug treatments. IL-1β protein was measured in hippocampal slice cultures (slices) and respective medium (medium) by ELISA at the end of the LPS±P2X$_7$ agonists/antagonists incubation period. To investigate the consequence of inflammatory-like events on excitotoxic neuronal cell death, hippocampal slice cultures pre-exposed to LPS or LPS+ATP protocols were incubated for 12 h, after medium wash-out, with 8 μM AMPA alone.

6.2.2 AMPA-induced neuronal damage

To investigate the consequence of inflammatory-like events on excitotoxic neuronal cell death, an additional set of hippocampal slice cultures pre-exposed to LPS or LPS+ATP protocols were incubated for 12 h, after medium wash-out, with 8 μM AMPA alone (Tocris; United Kingdom) as depicted in Fig. 6.1. This AMPA concentration induced 50% of maximal PI-uptake in CA1-CA3 pyramidal cell layers in organotypic hippocampal slice cultures as previously reported in detail (Bernardino et al., 2005). All experiments included

series of control cultures exposed to drug vehicles or to each drug separately. Fluorescent micrographs of PI-uptake were taken prior to LPS exposure (basal uptake) and 12 h after the exposure to AMPA (see Fig 6.1).

6.2.3 Statistical analysis of data

The data of densitometry analysis of PI-uptake were expressed as means ± SE. The number of independent wells we used in each experimental condition was used for statistical analysis of data. When evaluating the effect of LPS and ATP on AMPA-induced neuronal cell death, the PI-uptake values induced by 8 μM AMPA in control cultures were set to 100%. Statistical significance was determined by using one-way ANOVA followed by Bonferroni's test for multiple comparisons, with $p<0.05$ considered to represent statistical significance.

6.3 Results

6.3.1 Cellular PI uptake induced by LPS plus ATP

To investigate the consequences of inflammation on cellular viability, we evaluated the PI uptake in organotypic slice cultures exposed to 10 ng/ml LPS ± 1 mM ATP at the end of incubation period (3h LPS + 3 h co-incubation with ATP).

Fig. 6.2 shows representative pictures of PI-uptake in control untreated slices (n=18) and in slices exposed to LPS for 6 h (n=18), PBS (3h) + ATP for 3 h (n=12) or their combination (LPS for 3 h then LPS+ATP for additional 3 h; n=18). In control slices, basal PI uptake was very low and restricted to sparse granule and CA1 pyramidal cells likely reflecting

mechanical damage due to slice preparation procedures (Bernardino et al., 2005). Treatment of the slices with LPS or ATP alone did not affect PI-uptake significantly when compared to control slices (Fig. 6.2, first row). Differently, PI-uptake into cellular elements was highly increased in all slices treated with LPS+ATP (Fig. 6.2, second row).

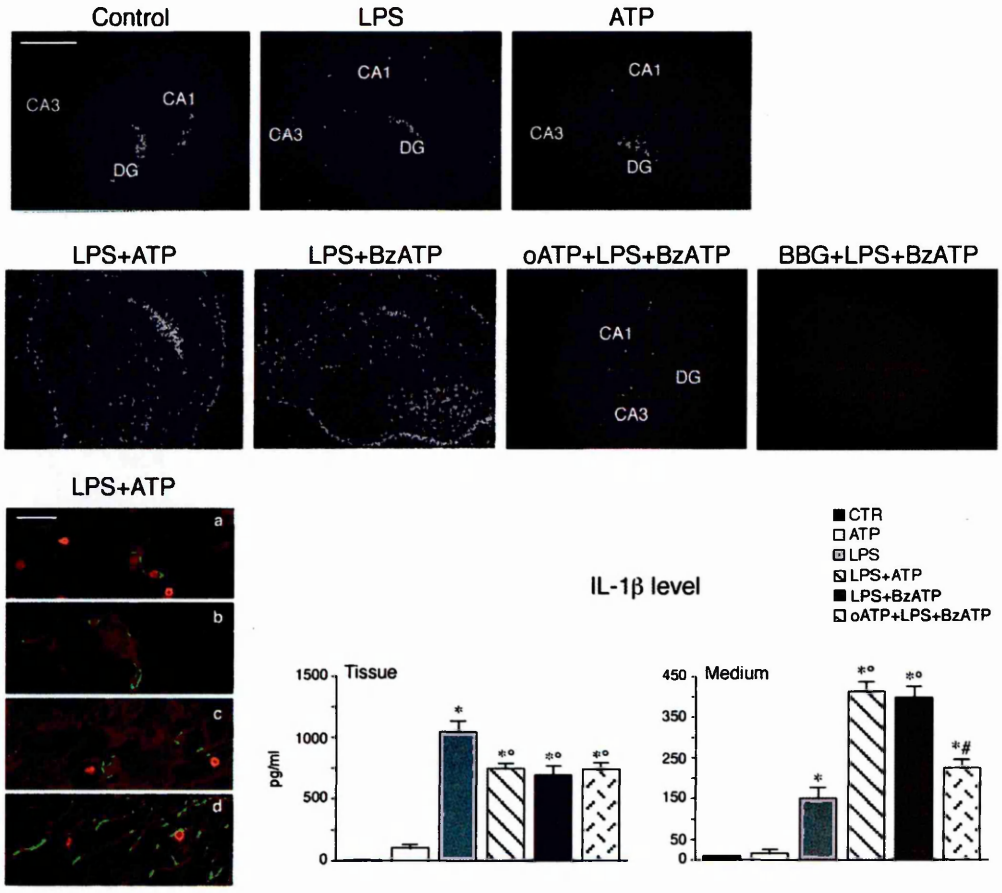


Figure 6.2. Effects of LPS plus ATP on cellular PI-uptake and concomitant production of IL-1β

Representative fluorescence photomicrographs depicting PI-uptake in hippocampal slice cultures. First row: slice cultures were not exposed to drugs (control), or were exposed to LPS or ATP. Low PI uptake is similarly observed in these experimental conditions likely due to mechanical damage during slice preparation.

Second row: slice cultures were exposed to LPS followed by co-exposure to ATP (LPS+ATP) or to BzATP, a $P2X_7$ receptor agonist (LPS+BzATP). Increased cellular PI uptake was observed in

LPS+ATP and LPS+BzATP treated slices. The effect of LPS+BzATP was prevented by 2 h pre- and 6 h co-exposure to oATP (oATP+LPS+BzATP) or BBG (BBG+LPS+BzATP), two $P2X_7$ antagonists. Scale bar 500μm.
Third row: high magnification fluorescent photomicrographs show in panels a,b the co-localization of PI (red nucleus) in Mac-1-positive microglial cells (green cytoplasm). No co-localization was observed between propidium iodide (red nucleus) in GFAP-positive astrocytes (green signal in c) or MAP-2-positive neurons (green signal in d), although PI-positive nuclei may be in proximity of GFAP and MAP-2-positive cells. Please note that the few nuclei which do not appear to co-localize MAC-1 and PI signals are cells not surrounded by cytoplasm or plasma membrane, thus likely representing late apoptotic cells.
*Bargrams show IL-1β concentration in tissue and medium of slice cultures (mean±SE, n=15-52) as assessed by ELISA at the end of the pharmacological experiments. *p<0.01 vs control (untreated slices); °p<0.01 vs LPS alone; #p<0.01 vs LPS+ATP by one-way ANOVA followed by Bonferroni's test for multiple comparison.*

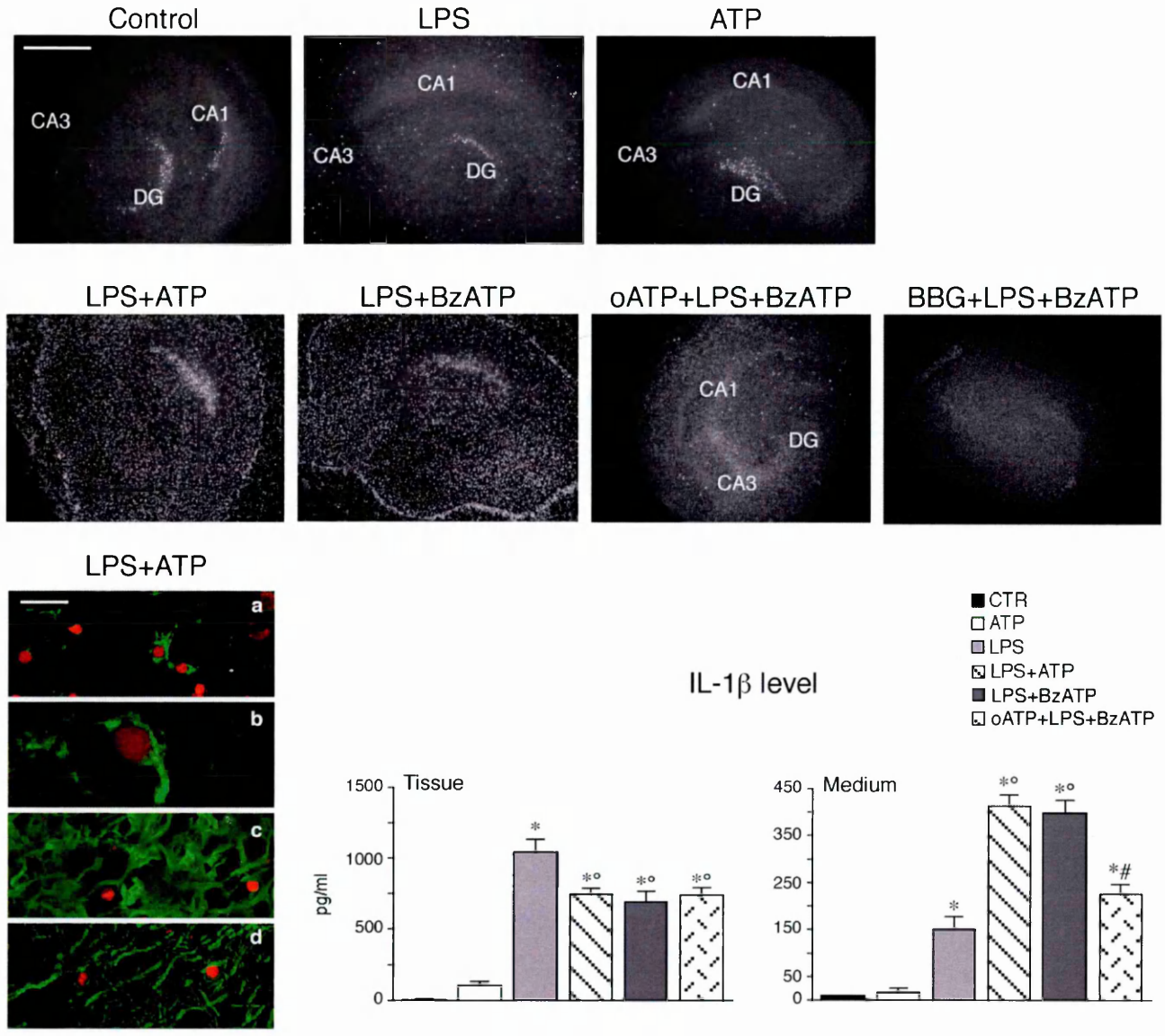
6.3.2 Involvement of $P2X_7$ receptors

Fig. 6.2 (second row) shows the effect of BzATP, a $P2X_7$ receptor agonist, and oxidized (o)ATP or BBG, two $P2X_7$ receptor antagonists, on PI uptake. BzATP (100 μM) mimicked the effect of ATP whereas pre-exposure of the slices to 300 μM oATP or 200 nM BBG prevented the cellular PI-uptake induced by LPS+BzATP (Fig. 6.2, second row) or by LPS+ATP (not shown). The $P2X_7$ agonist or the antagonists alone did not modify basal PI uptake (data not shown).

Propidium iodide co-localized with Mac-1-signal in microglia (Fig. 6.2a,b) but was not found in GFAP-positive astrocytes (Fig. 6.2c) or MAP-2-positive neurons (Fig. 6.2d), in accordance with the evidence that $P2X_7$ receptors are highly expressed by microglia (North, 2002; Sanz and Di Virgilio, 2000).

6.3.3 IL-1β synthesis and release in LPS plus ATP or BzATP exposed slices

The basal levels of IL-1β in tissue and medium of control slices were barely or not detectable and they did not differ from those measured in the presence of ATP (Fig. 6.2) or



Original photo refered to Figure 6.2

BzATP alone (not shown). LPS alone, or co-incubated with ATP or BzATP, induced a prominent increase in tissue levels of IL-1β ($p<0.01$ vs control slices, n=15-39). IL-1β concentration in the culture medium was increased by ~25-fold by LPS alone compared to control slices ($p<0.01$; n=18). This LPS-induced effect was enhanced by ~2-fold in the presence of ATP or BzATP ($p<0.01$ vs LPS alone; n=52), likely reflecting $P2X_7$ receptor-mediated activation of ICE/caspase-1 and consequent production of the mature and releasable form of IL-1β (Fantuzzi and Dinarello, 1999). Accordingly, oATP (Fig. 6.2) prevented the ATP- or BzATP-induced enhancement of LPS-dependent IL-1β release.

6.3.4 Cellular PI uptake reflects microglia activation and apoptotic-like cell death

Cellular PI uptake in slices co-exposed to LPS and ATP occurred in microglia as shown by the co-localization of PI fluorescent signal with the microglia marker Mac-1 (Fig. 6.2a,b and Fig. 6.3, second row). Additionally, Mac-1/PI-positive microglia in LPS+ATP-exposed slices showed Hoechst/PI-positive condensed nuclei (Fig. 6.3, first and second rows; see also panel b) and co-localized with active caspase-3 (panel a) indicating that these cells were undergoing apoptotic-like death.

Sporadic Hoechst/PI-positive condensed nuclei were observed in slices exposed to LPS alone or in control slices (Fig. 6.3, first and second rows). In control slices, microglial cells were in their resting state (Fig. 6.3, second row) showing long and ramified processes; microglia was instead activated in LPS-treated slices as shown by their characteristic ameboid-like morphology (Fig. 6.3, second row). Slices exposed to LPS plus ATP

displayed hyper-reactive microglia with short and thick processes; these cells were localized randomly over the whole slice (Fig. 6.3 first and third rows).

Microglial PI-uptake in slices exposed to LPS+ATP, or to LPS+BzATP (not shown) was abolished by 2 h pre-treatment with 25 μM zVAD-fmk (Fig. 6.3, third row), a pan-caspase inhibitor widely used as an anti-apoptotic agent (Ferrari et al., 1999).

This set of data demonstrates that a transient application of inflammatory stimuli induced a marked activation and apoptotic death of microglia as indicated by typical changes in their morphology, presence of condensed nuclei and colocalization with caspase-3. These phenomena were associated with a massive release of IL-1β that is prevented by antagonists of $P2X_7$ receptors suggesting the involvement of these receptors in the processing of IL-1β release.

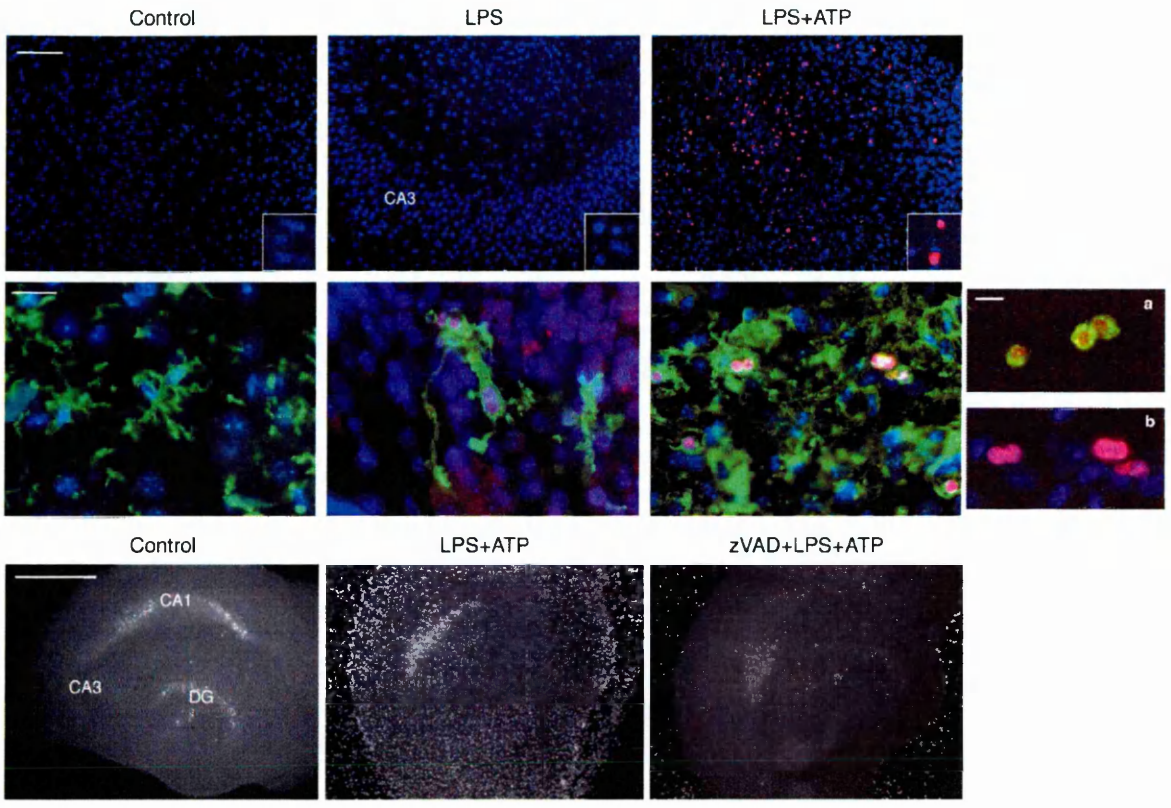
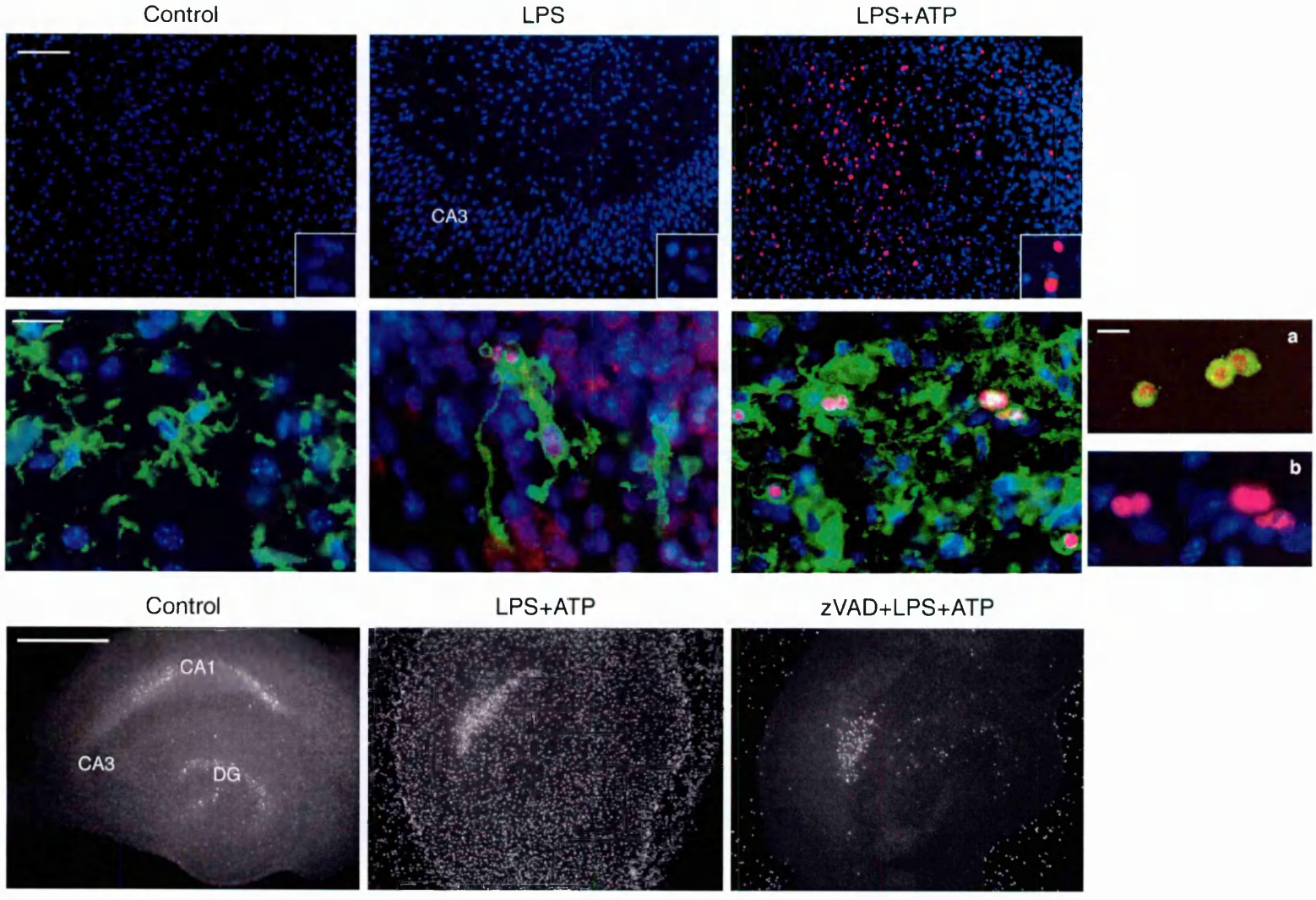


Figure 6.3. Exposure of hippocampal slice cultures to LPS plus ATP induces microglia apoptotic-like death

First row depicts representative fluorescence photomicrographs of PI uptake (red nucleus) and Hoescht staining (blue nucleus) in CA3 pyramidal cell layer. High magnification images are framed within the panels denoting co-localization of the two fluorescent nuclear signals and nuclear condensation in LPS+ATP treated slices only. Scale bar 500 μm; 20 μm in inset.

Second row depicts high magnification fluorescent photomicrographs of Mac-1-positive microglial cells (green cytoplasm), propidium iodide (red nucleus) and Hoescht staining (blue nucleus). Both LPS and LPS+ATP treatments induce changes in microglial cell morphology denoting shifting from resting (control) to activated states. Widespread PI uptake was induced in Mac-1-positive activated microglia by LPS+ATP while sporadic PI uptake was observed in microglial cells exposed to LPS alone. Panel a shows co-localization of active caspase-3 signal (green) with PI signal (red); panel b shows co-localization of Hoescht staining (blue signal) with PI (red signal) denoting condensed nuclei. Scale bar 20 μm; 10 μm in panels a and b.

Third row depicts prevention of LPS+ATP induced PI uptake in microglia by zVAD, a pan caspase inhibitor with anti-apoptotic properties. Similar results were obtained when slice cultures were incubated with BzATP instead of ATP (not shown). Scale bar 500 μm.



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6.3.5 Microglia activation is associated with exacerbation of neuronal excitotoxicity: crucial involvement of $P2X_7$ receptors and IL-1β release

Control cultures (no treatment) showed no, or very low, cellular PI-uptake (Fig. 6.4, first panel). Slices incubated for 12 h with 8 μM AMPA alone showed increased PI-uptake in MAP-2 positive neurons in CA3 subfield, and this effect was significantly enhanced by ~30% after transient pre-exposure to LPS+ATP (Fig. 6.4). PI-positive cells were identified as neurons as demonstrated by co-localization with MAP-2 staining (see inset in Fig 6.4). Slices exposed to LPS+ATP only, showed very low cellular PI-uptake in neurons similar to control slices (see bargram in Fig. 6.4).

In slices exposed to AMPA for 12 h following preincubation with LPS+ATP, PI-positive MAC-1 microglia was still present with a pattern similar to that observed 6 h after LPS+ATP incubation (see fig. 6.2, first panel in second row and panels a,b for comparison).

Quantification of neuronal damage in CA3 area by densitometric analysis of neuronal PI uptake (bargram in Fig. 6.4A) showed that AMPA alone induced ~6-fold increase in PI uptake in pyramidal cell layer (n=30) vs control untreated slices (n=20). When slices were transiently pre-incubated with LPS+ATP, PI uptake induced by AMPA was increased in CA3 by 8-fold on average (n=20) vs control untreated slices (Fig. 6.4A). Thus, AMPA-mediated excitotoxicity in CA3 pyramidal neurons was enhanced by ~30% when slices were transiently pre-exposed to specific inflammatory stimuli.

Exacerbation of AMPA-induced neuronal cell damage was totally abolished when IL-1 receptor type 1-(R1) mediated signaling or pro-IL-1β cleavage, was blocked by 1 μM IL-1ra or 100 μM VX-765 respectively, or when 200 nM BBG, a selective antagonist of $P2X_7$

receptors, was added to the medium for 2h of pretreatment and during the transient exposure to LPS+ATP (bargram in Fig. 6.4A). Co-incubation of BBG and IL-1ra did not provide additive effects in reversing LPS+ATP induced increase in AMPA neurotoxicity (Fig. 6.4A). Treatment of slices with LPS, ATP, IL-1ra, VX-765 or BBG alone did not affect AMPA-induced excitotoxicity (Fig. 6.4B).

IL-1Ra or VX-765 did not affect LPS+ATP-mediated microglial cell death (Fig. 6.5). ELISA quantification of IL-1β concentrations in the tissue and medium after 12 h exposure to AMPA alone did not detect any measurable level of this cytokine similarly to control slices (data not shown); however, when slices were preincubated with LPS+ATP, then the medium was wash-out, and AMPA was added for 12 h, IL-1β concentration in the tissue and in the medium at the 12 h time point were 2.1 ± 0.1 ng/ml and 2.2 ± 0.2 ng/ml (n=30 slices), respectively.

These findings demonstrate that transient inflammatory stimuli prime neuronal vulnerability to a subsequent excitotoxic insult. The chronic presence of critical concentrations of IL-1β is the crucial trigger of increased neuronal vulnerability to AMPA. Thus, the inhibition of IL-1β production or its receptor signaling fully prevented the exacerbation of AMPA-induced neuronal damage. Moreover, antagonism of $P2X_7$ receptors blocks both the release of IL-1β and the enhancement of AMPA-induced neuronal cell death induced by LPS+ATP, therefore demonstrating the crucial involvement of these receptors in the observed effects.

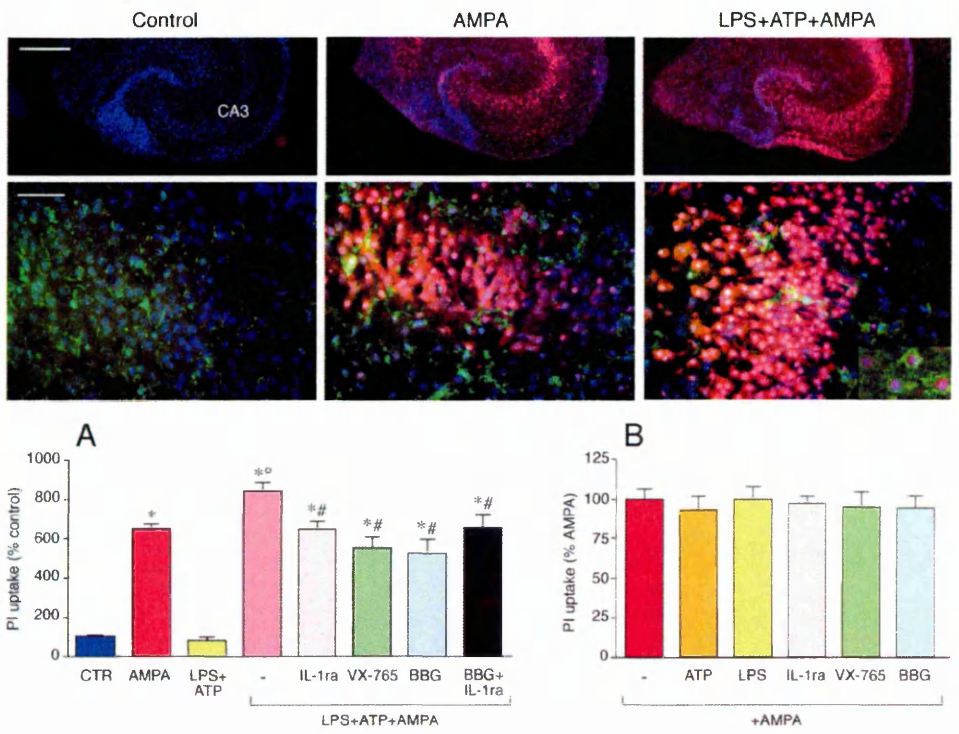


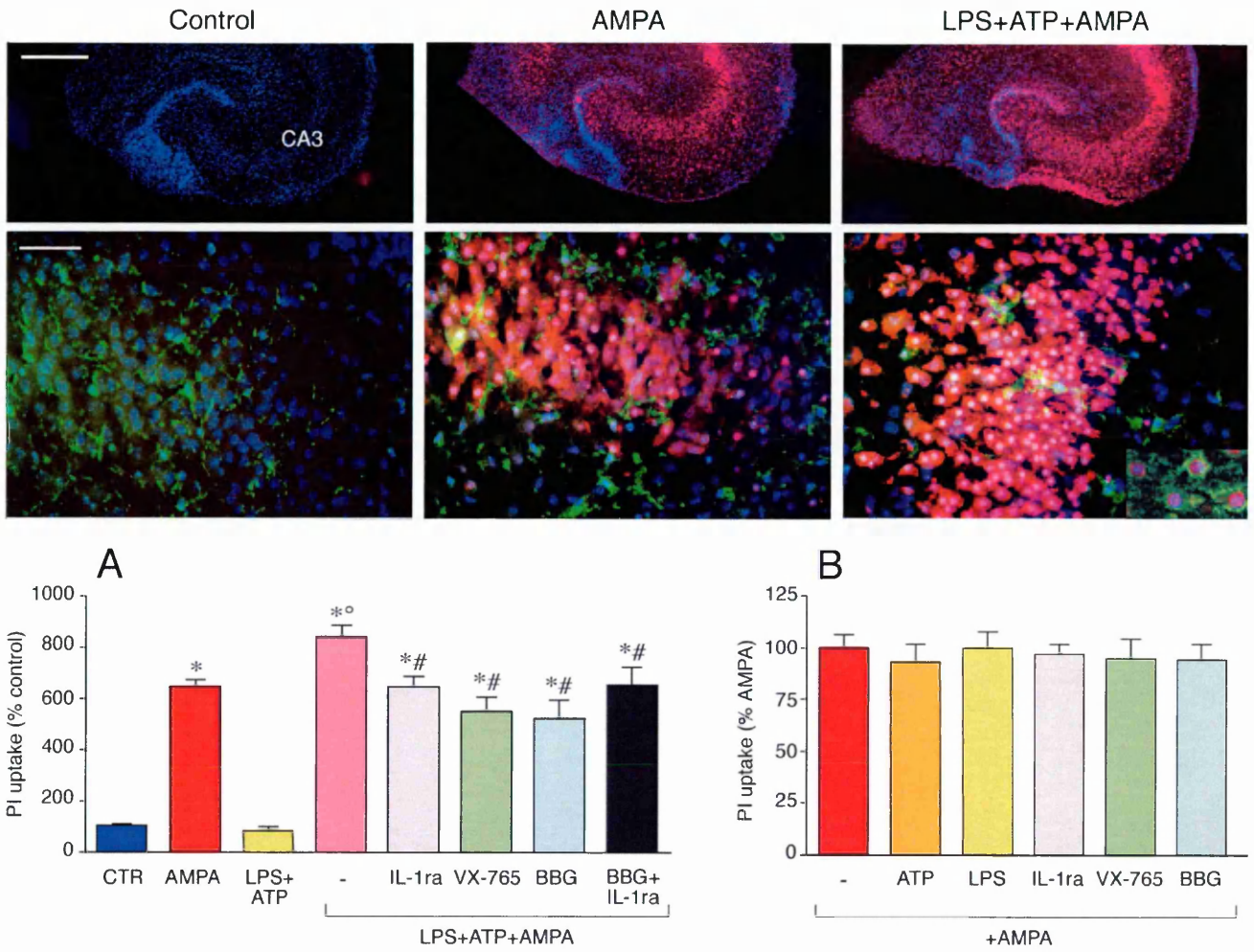
Figure 6.4. Potentiation of AMPA-induced excitoxicity in CA3 pyramidal cells induced by LPS plus ATP is mediated by IL-1β release

First row depicts representative fluorescence photomicrographs of PI uptake (red nucleus) in control culture (no or low PI-uptake) and in culture exposed to AMPA alone or preceded by transient incubation with LPS+ATP. Enhanced PI uptake was observed in CA3 pyramidal cell layer in the presence of AMPA and this effect was significantly increased by pre-incubation with inflammatory stimuli (LPS+ATP). Scale bar 250 μm.

Second row depicts high magnification images of PI-positive CA3 pyramidal cell layer corresponding to panels shown in first row. Mac-1-positive microglia (green signal) with long and slender processes and several thin side-branches, denoting its resting state, was randomly distributed in the whole control slice. In slices exposed to AMPA, with or without pre-exposure to LPS+ATP, microglia with activated morphology was found intermixed to damaged PI-positive neurons. Note that PI signal in AMPA-treated slices was enhanced by transient preincubation with LPS+ATP. Inset shows that PI-positive cells were identified as neurons by co-localization of MAP-2 (green signal) with PI (red) and Hoescht staining (blue). Scale bar 100 μm; 20 μm in last panel of third row.

Bargram in panel A represents densitometry measurements of PI-uptake (mean±SE, n= 15-30; eight separate experiments) in CA3 pyramidal cell layer neurons induced by AMPA alone or applied after pre-incubation with LPS+ATP, with or without addition of anti-IL-1β treatments (IL-1ra, selective antagonist of IL-1R1; VX-765, selective ICE inhibitor) or an antagonist of P2X7 receptors (BBG). Note that LPS+ATP alone did not induce PI-uptake in neurons.

*Bargram in panel B shows no potentiation of AMPA-induced damage when slice cultures were pre-incubated with LPS, ATP, IL-1ra, VX-765 or BBG alone. *p<0.01 vs control slices (CTR); °p<0.05 vs AMPA alone; #p<0.05 vs LPS+ATP+AMPA by one-way ANOVA followed by Bonferroni's test for multiple comparison.*



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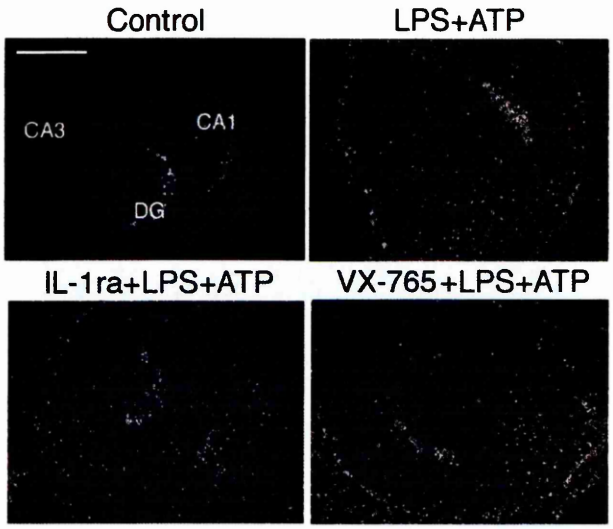
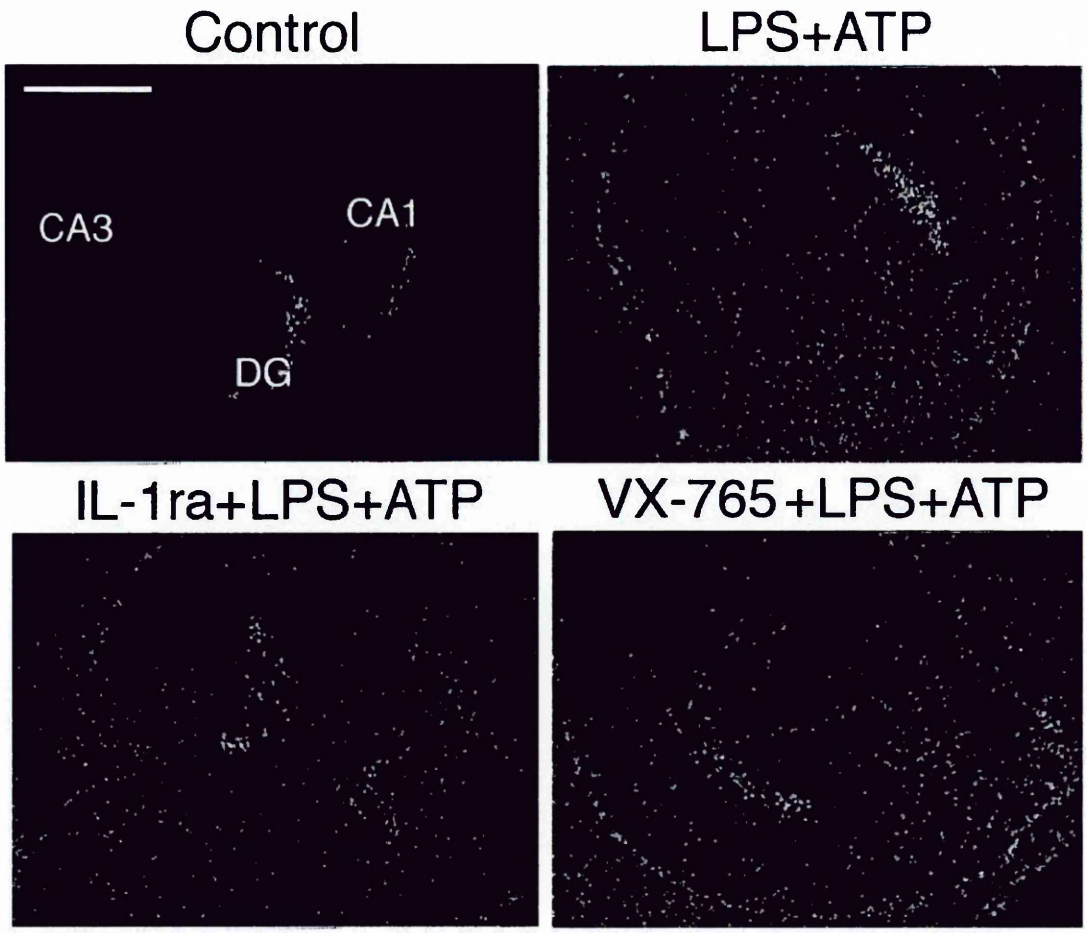


Figure 6.5. Lack of effects of VX-765 or IL-1ra on LPS plus ATP-induced cellular PI-uptake

Representative fluorescence photomicrographs depicting PI-uptake in hippocampal slice cultures. First row: slice cultures were not exposed to drugs (control), or were exposed to LPS+ATP. Second row: The effect of LPS+ATP was not prevented by 2 h pre- and 6 h co-exposure to 1 μM IL-1ra or to 100 μM VX-765. These antagonists did not modify per se PI uptake as compared to control slices (not shown). Scale bar 500 μm.

In summary, this study has shown that:

1. Transient exposure of hippocampal slices to LPS plus $P2X_7$ receptor agonists induces microglia activation and apoptosis-like cell death, accompanied with a sustained release of IL-1β
2. The application of LPS plus ATP, but not either one or the other alone, induced an increase of CA3 pyramidal neuron susceptibility to AMPA-induced neurotoxicity.
3. Activation of microglia-expressing $P2X_7$ receptors and subsequent IL-1β release are involved in the exacerbation of AMPA-induced neuronal cell loss.



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6.4 Discussion

The main finding of this study is that transient exposure of organotypic hippocampal slice cultures to specific stimuli mimicking inflammation primes neuronal vulnerability to a subsequent excitotoxic insult. Importantly, this priming effect fully depends on the activation of $P2X_7$ receptors and the consequent IL-1β release. In particular, we found that co-stimulation with LPS and $P2X_7$ receptor agonists induced a marked activation of microglia as indicated by typical changes in their morphology. Moreover, evidence of apoptosis was observed in MAC-1 activated microglia. These phenomena were associated with a massive release of IL-1β which was required for exacerbation of AMPA-induced neurotoxicity. Thus, the presence in the culture medium of VX-765, a specific caspase-1 inhibitor, or IL-1ra, a selective IL-1β receptor type 1 antagonist, during the transient application of LPS+ATP fully prevented the exacerbation of AMPA-induced neuronal damage. Our previous findings showed that VX-765 treatment reversed the LPS+ATP-mediated release of IL-1β in the medium taking back this level to the one induced by LPS alone (Ravizza et al., 2006b). This evidence demonstrates that IL-1β release is the crucial trigger of increased neuronal vulnerability to AMPA, however other substances, induced or enhanced by IL-1β, such as cytokines, free radicals and prostanoids (Allan et al., 2005) may contribute to exacerbate AMPA-induced neuronal death. Notably, enhanced neuronal susceptibility to AMPA was associated with higher IL-1β concentration in the slice and in the medium, whereas slices incubated with AMPA alone did not produce or release any detectable amount of this cytokine. This evidence suggests that although slices were transiently incubated with LPS+ATP, the tissue was indeed chronically exposed to this

cytokine. The concentrations of IL-1β measured at 6 h after LPS+ATP, and at 12 h after AMPA incubation after extensive LPS+ATP washout (0.4 ng/ml-2.2 ng/ml) are in the range of those previously found to exacerbate AMPA toxicity in organotypic slices after exogenous application of IL-1β (1ng/ml) (Bernardino et al., 2005). Moreover, in accordance with our data, only slices pretreated for 24h with IL-1β and followed by a coincubation with AMPA (IL-1β+AMPA) for other 24h (thus a chronic exposure of tissue to exogenous IL-1β) was effective in exacerbating neuronal cell loss (Bernardino et al., 2005). Although the exogenous application of IL-1β clearly differs from the endogenous production of this cytokine due to inflammatory conditions, these experiments are concordant to demonstrate that the chronic presence of critical concentrations of IL-1β can become detrimental for neuronal survival to a subsequent injury (Viviani et al., 2003).

An important finding is that LPS alone did not affect AMPA-induced neurotoxicity although it significantly activates microglia (while not inducing microglia-like apoptosis) and increases IL-1β release, albeit to a significantly lower extent than in the co-presence of ATP. It seems therefore important that IL-1β reaches a critical extracellular concentration above which this cytokine can become detrimental to neurons. This cytokine released in the extracellular *milieu* at "threatening" concentrations can induce long-lasting priming of neuronal vulnerability by direct effects mediated by IL-1R1 on hippocampal pyramidal neurons (Ravizza and Vezzani, 2006; Viviani et al., 2003) or indirectly by acting on astrocytes (Bezzi et al., 2001a; Hu et al., 2000; Ye and Sontheimer, 1996). A schematic representation of the sequelae of events underlying priming of neuronal vulnerability is depicted in Fig. 6.6.

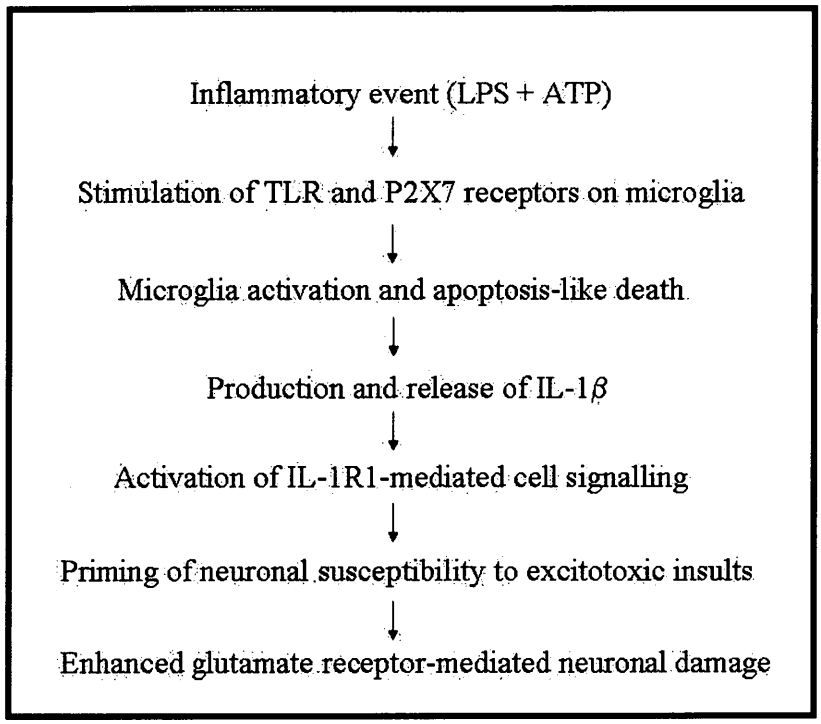


Figure 6.6. Schematic representation of the events underlying priming of neuronal vulnerability to excitotoxic damage

A large body of evidence demonstrates the presence of $P2X_7$ receptors on microglia (Boucsein et al., 2003; Brough et al., 2002; Choi et al., 2007; Ferrari et al., 2006; Le Feuvre et al., 2002b; Sanz and Di Virgilio, 2000) and the activation of these receptors by LPS and ATP triggers microglia release of IL-1β (Brough et al., 2002; Ferrari et al., 2006; Le Feuvre et al., 2002b; Sanz and Di Virgilio, 2000). In accordance with this evidence, we show that antagonism of $P2X_7$ receptors blocks both the release of IL-1β and the enhancement of AMPA-induced neuronal cell death induced by LPS+ATP, therefore demonstrating the crucial involvement of these receptors in the observed effects. Functional and pharmacological data have suggested that $P2X_7$ receptors are also expressed by neurons (Anderson and Nedergaard, 2006), however our data do not support such involvement in

the enhanced neuronal susceptibility to AMPA toxicity. Thus, we found no additivity in the ability of IL-1ra or BBG, when added together, to block the LPS+ATP effect on AMPA neurotoxicity (i.e. the blocking effects of either one of these drugs was similar to the blocking effect observed when the drugs were co-incubated). These results indicate that BBG and IL-1ra are acting along the same IL-1β-dependent pathway and not via independent mechanisms such as direct involvement of neuronal P2X7 receptors.

Our experimental conditions did not permit to elucidate whether IL-1β was specifically released by apoptotic-like microglia or if activated microglia (including both apoptotic and non-apoptotic cells) was the major source. However, apoptosis was not blocked by treatments which prevent neuronal priming to excitotoxicity, such as caspase-1 inhibition or by IL-1ra, indicating that apoptosis *per se* is not sufficient to enhance neuronal susceptibility to injury. This may be due to the fact that microglia apoptosis and neuronal susceptibility to injury are due to distinct causes: $P2X_7$ activation by ATP may induce microglia apoptosis in the presence of LPS while IL-1β release either from apoptotic or non-apoptotic microglia, or both, is responsible for enhanced neuronal damage.

We found that the major consequences of microglia activation on neuronal survival to excitotoxic insult in our experimental conditions are deleterious. In this frame, microglia-apoptosis may either be part of a self-defensive mechanism by which these cells are impaired to prevent exacerbation or perpetuation of inflammation (Chao et al., 1995; Hanisch and Kettenmann, 2007) or result in the loss of microglia-mediated protective mechanisms (Minghetti, 2005; Schwartz et al., 2006).

Our findings also suggest that the mechanism underlying microglia apoptosis are independent on the intracellular production of IL-1β (or IL-18) by caspase-1 activation, in

accordance with previous evidence in cultured macrophages (Brough et al., 2002; Le Feuvre et al., 2002b; Perregaux and Gabel, 1998). Other factors may therefore play a role in determining microglia death in the presence of LPS+ATP such as an altered surface distribution of $P2X_7$ receptors (Gu et al., 2000), their direct co-activation by LPS (Denlinger et al., 2001) or changes in intracellular ionic environment (Le Feuvre et al., 2002a).

In summary, our findings show that neurons can become more susceptible to an excitotoxic insult if brain tissue has been pre-exposed to a specific inflammatory environment, even if transiently. Critical extracellular IL-1β concentrations are required for this phenomenon to occur. Although these findings await validation in appropriate *in vivo* models of inflammation and neurodegeneration, they shed light on the mechanisms by which inflammatory reactions, which are often associated or even precede the onset of neurodegeneration *in vivo*, can become detrimental to neurons (Allan et al., 2005; Block and Hong, 2005; Choi et al., 2007; Minghetti, 2005; Vezzani and Granata, 2005). Since activation of AMPA receptors is a common pathway involved in various acute and chronic CNS injuries (Lipton and Rosenberg, 1994), our findings suggest that pharmacological treatments blocking excessive IL-1β synthesis and release from microglia, such as caspase-1 inhibitors (Ravizza et al., 2006b), may improve neuronal survival in hostile conditions.

CHAPTER 7

TNF-α inhibits seizures in mice via p75 receptors

7.1 Summary

We studied the role of TNF-α and its p55 and p75 receptors in seizure modulation. We found that intrahippocampal injection of murine recombinant TNF-α potently inhibits seizure in mice while human recombinant TNF-α, which shows strong specificity for mouse p55 receptors, was ineffective. p75 receptors were detected in mouse hippocampal neurons, whereas p55 receptors were absent. Both receptors are upregulated in astrocytes following seizures. Transgenic mice with a perturbed TNF-α system showed profound alterations in seizure susceptibility: astrocytic overexpression of TNF-α was associated with reduced seizures, whereas mice lacking TNF-α p75 receptors showed prolonged seizures. Mice deficient in p55 receptors showed reduced seizures and both p75 and TNF receptor–associated factor 2 protein levels were upregulated in their hippocampi. Our findings show that increased brain levels of TNF-α result in significant inhibition of seizures in mice, and this action is mediated by neuronal p75 receptors. This evidence highlights a novel function of TNF-α in brain and indicates a new system for anticonvulsive intervention.

7.2 Experimental procedures

7.2.1 Experimental animals

C57BL6 mice were used to generate homozygous p55 or p75 deficient mice. Generation and detailed characterization of these TNF receptor–deficient mice was reported previously (Peschon et al., 1998).

C57BL6xSJL mice were used for generating transgenic mice with astrocyte-specific expression of murine TNF-α (GT-8 line) under control of the murine glial fibrillary acidic protein promoter (Stalder et al., 1998). This transgenic line is shown to produce TNF-α at levels that did not compromise normal development and breeding viability. These mice did not show overt phenotype and any detectable brain pathology until they were older than 6 months (Stalder et al., 1998).

Control animals were wild-type mice (not genetically modified) of the corresponding genetic background of the TNF receptor knock-out mice, or of the glial fibrillary acidic protein TNF-α transgenic mice of the same age.

7.2.2 Pharmacological treatments

Acute kainic acid-induced seizures were induced and quantified as previously described in "Materials and methods - general procedures" section (p. 81-83).

Murine (m) recombinant TNF-α (specific activity, 1.2 X 10^8 IU/mg; kindly provided by Dr P. Vandenabeele, Gent, Belgium) or human (h) recombinant TNF-α (specific activity, 6.6 X 10^7 IU/mg; R&D system, Minneapolis, USA) were dissolved in 100 mM phosphate-buffered saline (PBS; pH 7.4) devoid of Mg^{2+} and Ca^{2+} and supplemented with 1% fetal

calf serum (vehicle). mTNF-α (1.5, 2.5, or 15 pmol in 0.5 μl) or hTNF-α (30 pmol in 0.5 μl) was injected in the mouse dorsal hippocampus 5 minutes before 10 ng kainic acid dissolved in 0.5μl PBS (Sigma-Aldrich, St. Louis, MO). This mTNF-α dosing schedule was shown to attenuate NMDA-induced excitotoxic injury in the hippocampus (Liu et al., 1999). Control mice were injected with the corresponding volume of vehicle before the convulsant.

7.2.3 Immunohistochemistry

Technical details related to p55 and p75 receptors immunohistochemical staining have already been reported in "Materials and methods - general procedures" section (p. 89).

7.2.4 Double-immunostaining

Two brain slices in each mouse brain for each cell type marker were randomly chosen to identify the cells expressing p55 or p75. The procedures for double-immunostainig is described in "Materials and methods - general procedures" section (p. 90).

7.2.5 Western Blot

Male $p55^{-/-}$ mice and their C57BL6 control mice (n=6) were decapitated. Both hippocampi were dissected out at 4°C and one hippocampus for each animal was homogenized as described in "Materials and methods - general procedures" section (p. 92). For immunoblotting, we used a monoclonal antibody against human TNF receptor 2 (1:500; Hbt), rabbit polyclonal antibody against mouse TNF receptor–associated factor 2 (TRAF-2; 1:1000; Santa Cruz, Santa Cruz, CA), or rabbit polyclonal antibody against neurofilament

M (1:1000;Chemicon). Immunoreactivity was visualized with enhanced chemiluminescent (ECL; Amersham, Buckinghamshire,United Kingdom), using peroxidase-conjugated goat anti–mouse IgG (for TNF receptor 2 detection, 1:2000; Sigma-Aldrich) or peroxidase-conjugated goat anti–rabbit IgG (for TRAF-2 and NF detection, 1:2000; Sigma-Aldrich) as secondary antibodies.

7.2.6 Statistical analysis of data

Data are the mean ± standard error (n = number of animals). The effects of treatments were analyzed by one-way ANOVA followed by Tukey's or Fisher's test or by Student's *t*-test.

7.3 Results

7.3.1 Effect of exogenous acute application of TNF-α

Intrahippocampal injection of mouse mTNF-α in C57BL6 mice 5 minutes before a local application of kainic acid dose-dependently inhibited the number and the duration of seizures without changing their time of onset. No effect was observed using 1.25 pmol mTNF-α, whereas 2.5 and 15.0 pmol reduced by 37% the number of seizures ($p < 0.05$) and by 50%, on average, their duration compared with mice injected with vehicle + kainic acid. No effect of mTNF-α was observed on interictal spiking with any of the doses used (see Table 7.1; Fig. 7.1). Notably, the intrahippocampal application of up to 30 pmol hTNF-α in C57BL6 mice, which is known to have strong specificity for murine p55

receptors (Lewis et al., 1991) did not modify kainic acid–induced seizures (see Table 7.1), suggesting that the anticonvulsant effect of mTNF-α was not mediated by p55 receptors.

Table 7.1. Effect of murine (m) or human (h) TNF-α on seizures

	Dose (pmol)	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Vehicle	-	5.6 ± 0.9	19.0 ± 2.0	24.1 ± 3.1	66.9 ± 5.9
mTNFα	1.25	5.5 ± 1.6	15.0 ± 3.0	19.2 ± 3.2	64.9 ± 11.0
	2.5	6.6 ± 0.8	12.0 ± 2.0*	13.7 ± 2.7*	57.0 ± 5.0
	15.0	6.0 ± 0.6	12.0 ± 2.0*	10.6 ± 1.7**	50.0 ± 5.0
hTNFα	30.0	6.6 ± 1.7	15.0 ± 2.0	19.2 ± 2.6	53.6 ± 6.7

Data are the mean ± SE (n=6-13 mice in each experimental group). $^{*}p < 0.05$; $^{**}p < 0.01$ versus vehicle by one-way ANOVA followed by Tukey's test for multiple comparisons.

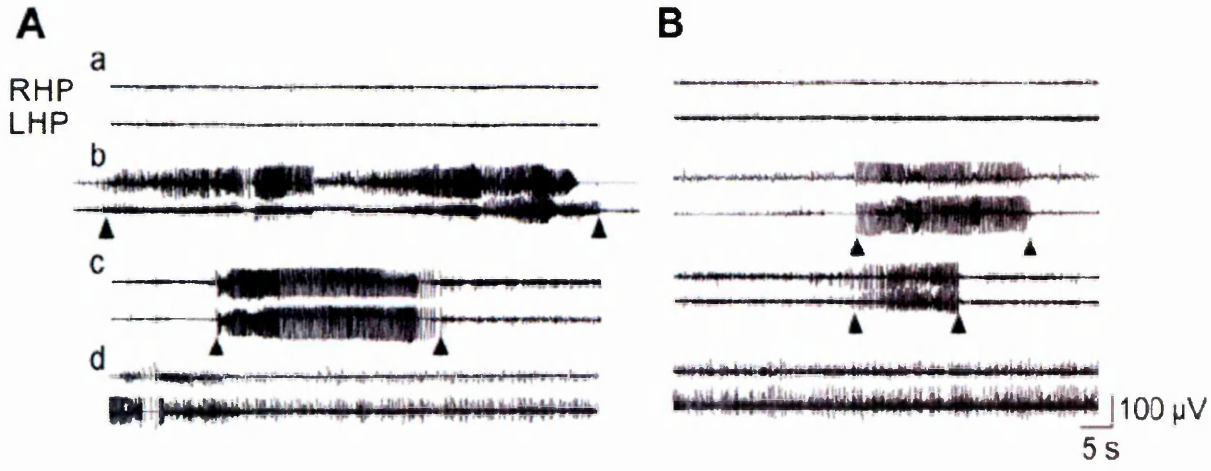


Figure 7.1. EEG seizures induced by intrahippocampal kainic acid in mice: effect of mTNF-α

Representative EEG tracings of freely moving C57BL6 mice injected in the left dorsal hippocampus with 10 ng in 0.5 µl kainic acid + vehicle (A) or kainic acid + 15 pmol mTNF-α (B). Baseline (region a); typical ictal episodes (regions b and c); spiking activity (region d). RHP and LHP indicate right and left hippocampus, respectively. Arrowheads delimit ictal episodes. Note that the duration of ictal events in B is shorter than in A.

7.3.2 Effect of endogenous chronic overexpression of TNF-α on seizures

Transgenic expression of endogenous mTNF-α in astrocytes of C57BL6/SJL mice (GT-8 line) mimicked the anticonvulsant effect of exogenous application of mTNF-α. Thus, the number of seizures and the time spent in seizures were both reduced by ~50% in GT-8 versus wild-type mice ($p < 0.01$), whereas no changes were observed in interictal spiking (see Table 7.2).

Table 7.2. Susceptibility to kainic acid–induced seizures in transgenic mice overexpressing mTNF-α in astrocytes (GT-8)

	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Wild-type	7.1±0.9	11.0±1.0	7.8±0.9	105.5±3.4
GT-8	8.0±0.8	6.0±1.0**	4.4±0.6**	117.8±8.6

Data are the mean ± SE (n=8-9). A significantly lower number of seizures (ictal events) and a reduction in their duration were found in GT-8 mice compared with wild-type controls.
$^{**}p < 0.01$ versus wild type by Student's t test.

This set of data demonstrate that increased brain levels of TNF-α in hippocampus due to exogenous application or overexpression have a powerful inhibitory action on EEG seizures reducing their frequency and duration without changing the time to onset of seizures. These findings suggest that mTNF-α is not involved in the mechanisms by which the first seizure is triggered and it appers to affect seizure maintenance. hTNF-α, which selectively activates p55 receptor subtype in mice, did not affect seizures in wild-type mice suggesting that p55 receptors are not involved in the anticonvulsant effect of mTNF-α.

7.3.3 Role of TNF-α receptor subtypes

To investigate the role of TNF-α receptor subtypes in seizure susceptibility, we used C57BL6 knock-out mice lacking either p75 or p55 receptors and their wild-type control animals. Table 7.3 shows that mice lacking p75 receptors displayed enhanced seizure activity. Thus, the duration of ictal activity was increased by 76% ($p < 0.01$). $p55^{-/-}$ mice

were instead less susceptible to seizures, as indicated by a 38% reduction in the number of seizures and 47% shorter duration of seizures compared with control mice ($p < 0.05$).

Western blot analysis of hippocampal homogenates showed that $p55^{-/-}$ mice had a twofold upregulation of p75 receptors ($p < 0.01$), which was associated with a concomitant 40% increase in TRAF-2 levels (Fig 7.2; $p < 0.01$).

p55 receptors were not detected at measurable levels in wild-type or p75 knock-out mice (not shown).

Table 7.3. Susceptibility to seizures in C57BL6 knock-out mice lacking either p75 ($p75^{-/-}$) or p55 ($p55^{-/-}$) receptors

	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Wild-type	5.9 ± 0.8	13.0 ± 1.0	17.4 ± 2.5	45.9 ± 4.3
$p75^{-/-}$	6.0 ± 0.7	20.0 ± 3.0	30.0 ± 3.7**	47.6 ± 6.3
$p55^{-/-}$	6.3 ± 0.1	8.0 ± 1.0*	9.3 ± 2.0*	34.2 ± 8.8

Data are the mean ± SE (n=9-20). Mice lacking p75 receptors were more susceptible to seizures, whereas mice lacking p55 receptor had a lower number and duration of seizures.
*$p < 0.05$; **$p < 0.01$ versus wild-type by one-way ANOVA followed by Tukey's test for multiple comparisons.*

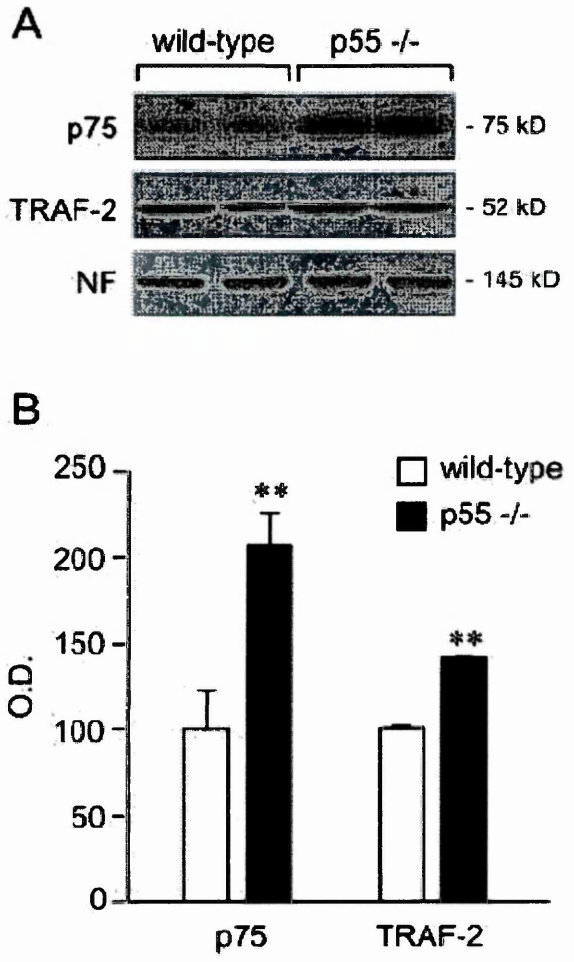


Figure 7.2. Western blot analysis of hippocampal p75 receptor and TRAF-2 protein levels in wild-type and p55$^{-/-}$ mice

*(A) Representative blot showing the increase in p75 receptors and in TRAF-2 protein in duplicate samples from hippocampi of p55$^{-/-}$ mice as compared with wild-type mice (n=6). (B) Bars depict the optical density values of the 75 or 52 kDa bands divided by that of the corresponding neurofilament M (NF;145kDa), which was used as an internal standard in each sample. **p < 0.01 versus wild type by Fisher's test.*

These findings demonstrate that in the absence of p75 receptors, ictal activity is enhanced, clearly indicating a pronounced increase in neuronal excitability when p75 receptors are lacking. Mice deficient in p55 receptors displayed reduced seizure activity after kainate application and showed a marked upregulation of p75 receptors and in the related signaling

pathway, suggesting that reduced seizure activity in $p55^{-/-}$ mice is a consequence of the adaptive increase in p75 receptors.

7.3.4 TNF-α receptor expression

In saline-injected hippocampus, a specific p75 signal was detected in neurons throughout the strata oriens, radiatum (Fig 7.3 A-C; G-I), and molecular layers of the hippocampus proper, as well as in the hilar region (not shown).

Ninety min after kainic acid-induced seizures, p75 was expressed both in neurons and astrocytes (Fig 7.3 D-F) as demonstrated by double immunostaining using a specific astrocytic marker (GFAP; Fig 7.3 J-L).

p55 staining was not detectable in control hippocampus (Fig. 7.4 A), but it is upregulated 90 minutes after seizure onset in astrocytes as shown by the colocalization with GFAP (Fig. 7.4 B-E).

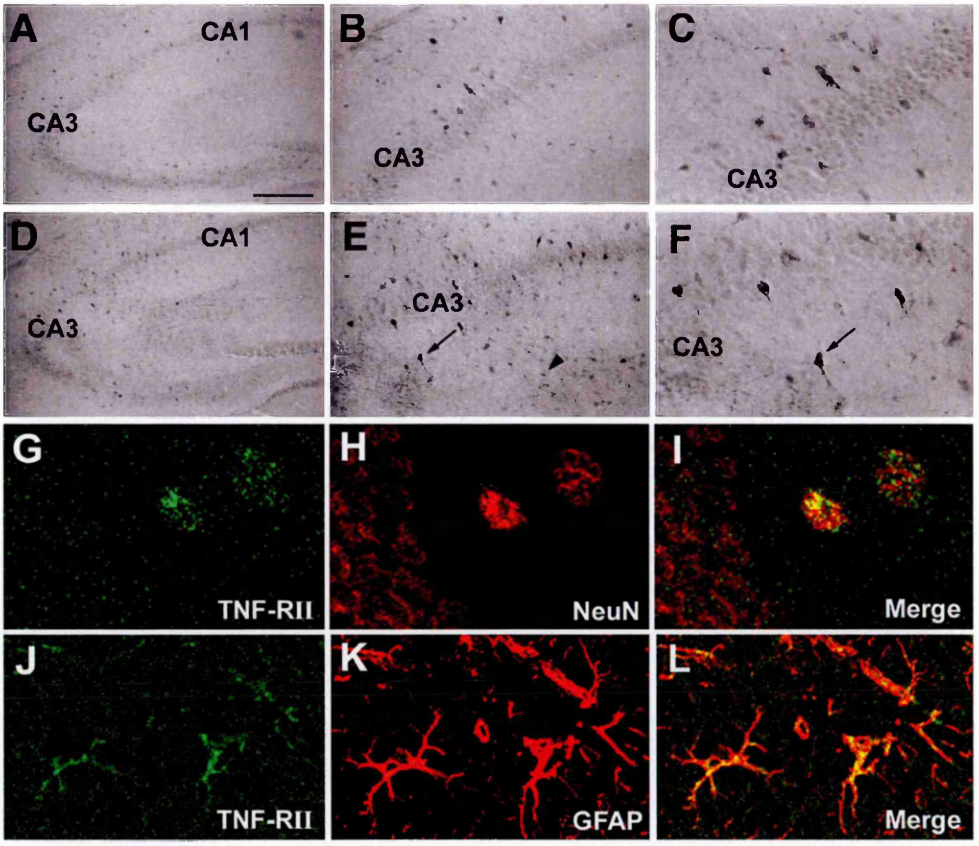
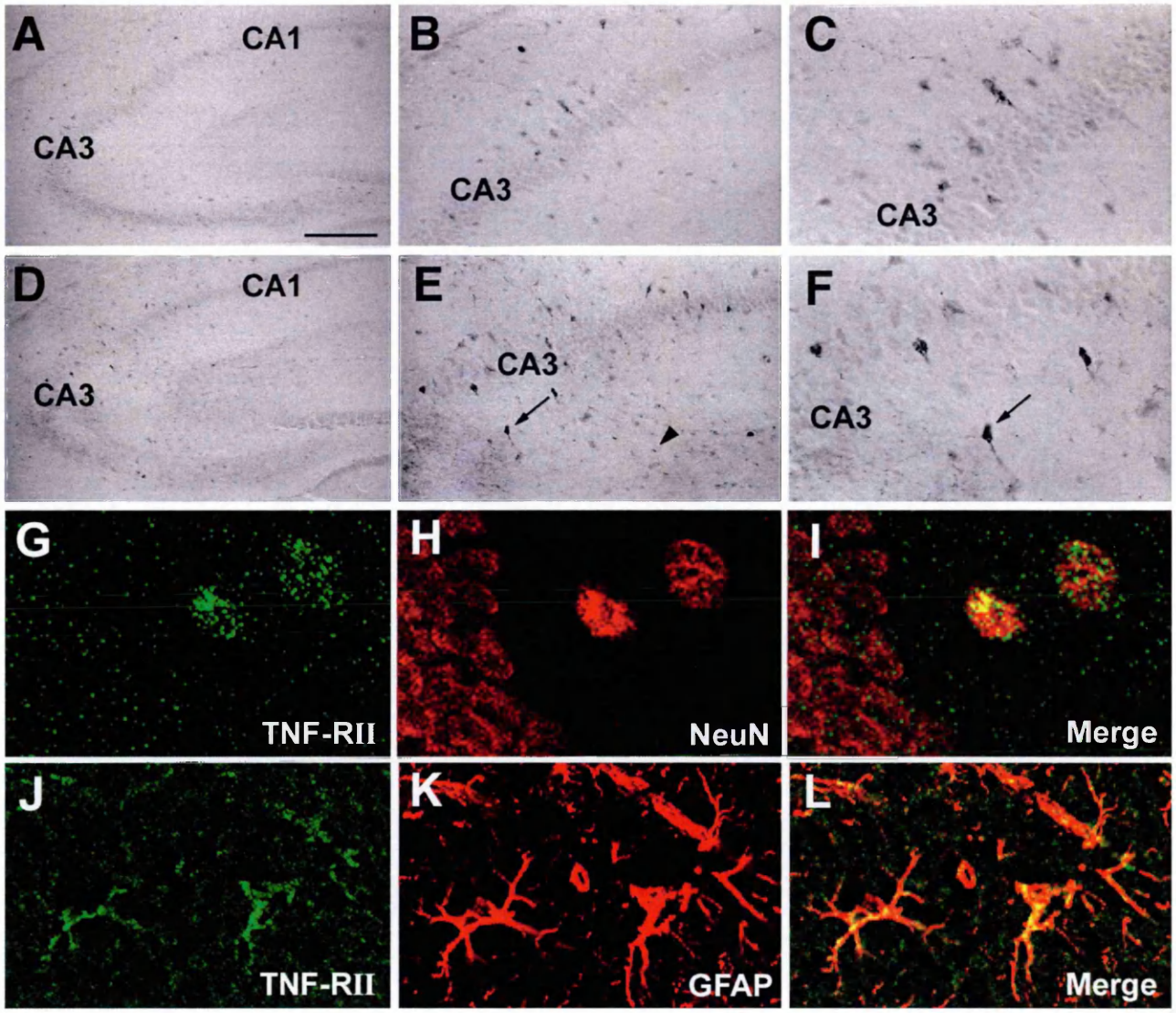


Figure 7.3. p75 expression in the mouse hippocampus after kainic acid-induced seizures

Representative photomicrographs of p75 immunoreactivity in the hippocampus, 90 min after seizures (D-F) and in vehicle-injected C57BL6 mice (A-C). Immunopositive neurons were scattered in strata oriens, radiatum, and molecular layers of the hippocampus proper and in the hilus in vehicle-injected mouse (A-C; G-I). After seizures, p75 staining was enhanced both in neurons (D-F, as indicated by arrows) and astrocytes (D-F; J-L, as indicated by arrowheads and GFAP colocalization). Scale bar: 400 μm (A, D); 300 μm (B, E); 200 μm (C, F); 100 μm (G-I; J-L).



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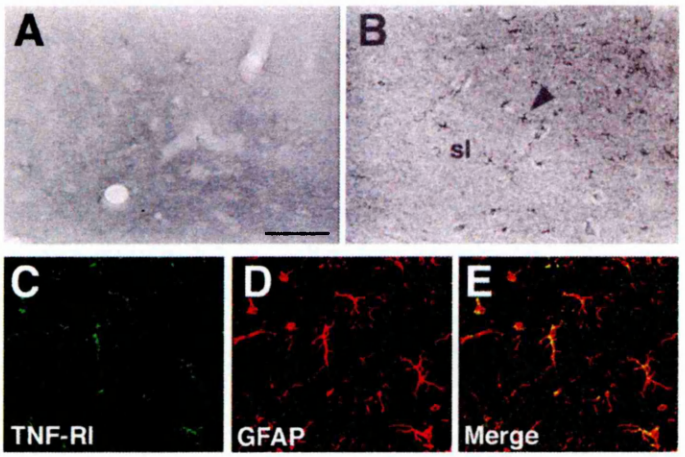


Figure 7.4. p55 expression in the mouse hippocampus after kainic acid-induced seizures

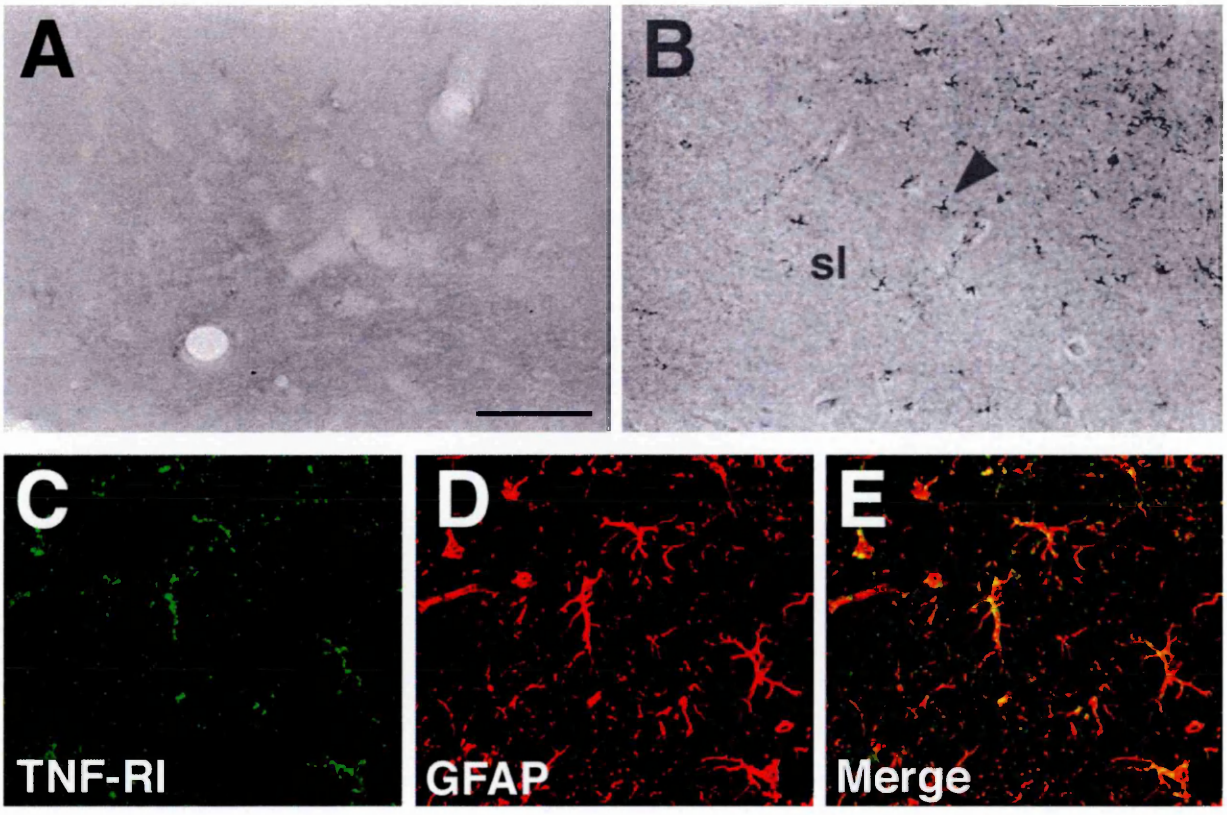
Representative photomicrographs of p55 immunoreactivity in the hippocampus, 90 min after seizures (B) and in vehicle-injected C57BL6 mice (A). p55 immunostaining was not detectable in control hippocampus (A). After seizures, p55 immunoreactivity is strongly enhanced in GFAP-positive astrocytes (B) as demonstrated by double immunostaining using the specific astrocytic marker GFAP (C-E). Scale bar: 300 μm (A, B); 100 μm (C-E).

These findings demonstrate that p75 receptor is the only receptor subtype detected by immunohistochemistry and Western blot analysis in normal mouse hippocampus indicating that the anticonvulsant effect of mTNF-α in wild-type mice is mediated by neuronal p75 receptors and not p55 receptors. p55 protein is not detectable in physiological conditions indicating that the transcription of its mRNA requires a tight control to prevent possible harmful effects of TNF-α mediated by this receptor subtype. Seizures trigger the overexpression of both receptors in astrocytes.

In summary, this study has shown that:

1. mTNF-α potently inhibits seizures in mice by stimulating neuronal p75 receptors

2. accordingly, mice overexpressing mTNF-α in astrocytes are less susceptible to seizures



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3. $p55^{-/-}$ mice are less susceptible to seizures due to an up-regulation of p75 receptors and TRAF2
4. $p75^{-/-}$ mice show increased seizure activity
5. overexpression of TNF-α receptors in neurons and/or astrocytes is induced by seizures

7.4 Discussion

The main finding of this study is that murine TNF-α has a powerful inhibitory action on EEG seizures induced in mice by intrahippocampal application of kainic acid. Murine TNF-α significantly reduced ictal activity without modifying the time to onset of seizures and the duration of interictal spiking. This evidence suggests that TNF-α is not involved in the mechanisms by which the first seizure is triggered, but it does affect seizure maintenance by impairing the transition between ictal and interictal events. Inhibitory effects of TNF-α on neuronal excitability have been reported previously; thus, TNF-α inhibits long-term potentiation in CA1 and dentate gyrus in hippocampal slices (Cunningham et al., 1996; Tancredi et al., 1992) and mediates long-term depression of synaptic transmission in CA1 (Albensi and Mattson, 2000). Modulation of intracellular concentrations of Ca^{2+} has been proposed as a crucial pathway mediating TNF-α actions on neuronal excitability (Bezzi et al., 2001a; McLarnon et al., 2001; Stellwagen et al., 2005). Two lines of evidence indicate that the anticonvulsant effect of murine TNF-α in wild type mice is mediated by neuronal p75 receptors and not p55 receptors. First, p75 receptor is the only receptor subtype detected by immunohistochemistry and Western blot analysis in normal mouse hippocampus, although measurable levels of p55 transcript have been

reported previously (Bette et al., 2003). The apparent absence of p55 protein implies a limited transcription of its messenger RNA in physiological conditions, which may represent a mechanism for limiting the potential harmful effects of TNF-α on neuronal survival that appear to be mediated by this receptor (MacEwan, 2002a). Second, hTNF-α, which is known to specifically interact with p55 receptor subtype in mice (Lewis et al., 1991) did not affect seizures in wild type mice up to 30 pmol in 0.5µl, suggesting that p55 receptors are not involved in the anticonvulsant effect of mTNF-α. Greater doses were not tested because of the limit of the solubility of this peptide in the injected volume.

Ninety minutes after the onset of seizures, both p55 and p75 receptors are expressed in astrocytes, suggesting that glial receptors are not involved in the acute effects of TNF-α on neuronal excitability. Delayed astrocytic expression of TNF-α receptors may play a role in long-term events consequent to seizure activity such as alterations of BBB (Albensi, 2002), neurogenesis (Monje et al., 2003), neuronal survival (Shinoda et al., 2003), which are all critically dependent on glia function.

We next used transgenic mice with a perturbated TNF-α system to investigate the functional consequences on seizures of the chronic brain overexpression of this cytokine. Seizure activity was drastically reduced in GT-8 transgenic mice overexpressing TNF-α in astrocytes, and this effect was similar to that observed after exogenous cytokine application, suggesting that the presence of TNF-α pre-existing pool may contribute to decrease seizure activity. An early report has shown that transgenic mice overexpressing high amounts of TNF-α in astrocytes experience development of neurological deficits within 4 postnatal weeks and show brain damage and sporadic seizures (Akassoglou et al., 1997). The GT-8

mice we used in this study expose mild overexpression of TNF-α (Stalder et al., 1998), no pathological phenotype until they are older than 6 months, and decreased seizure susceptibility. These differences indicate that brain concentration of TNF-α is crucial for determining its effects on brain function (Bernardino et al., 2005; Sriram and O'Callaghan, 2007). Using mice lacking p55 or p75 receptors, we elucidate the role of TNF-α receptor subtypes in seizure brain susceptibility. p75-deficient mice showed *increased* epileptic activity on kainate injection, indicating a pronounced increase in neuronal excitability when p75 receptors are lacking. $p75^{-/-}$ mice did not show adaptive changes in p55 receptors because these receptors were undetectable as in wild type mice (not shown). p55 knock-out mice displayed reduced seizure activity after kainate application. These mice showed a marked upregulation of p75 receptors and in the related signaling pathway (Wajant and Scheurich, 2001). Because p55 receptors do not mediate any effect on seizures in wild type mice after stimulation with a selective ligand and are not represented at measurable levels in normal mouse brain, we suggest that reduced seizures in $p55^{-/-}$ mice is a consequence of the adaptive increase in p75 receptors. The increased seizure activity observed in transgenic mice after deletion of the p75 receptor is in full agreement with the inhibitory role played by this receptor in wild type mice where p75 mediates the anticonvulsant effects of murine TNF-α exogenously applied in the hippocampus. In conclusion, our findings indicate that a TNF-α increase in the brain reduces seizure duration in mice via neuronal p75 receptor subtypes. We did not detect any role of p55 receptors in this model; however, this finding may be because of the scarce density of this receptor subtype in mouse hippocampus. Previous reports have shown that many of the detrimental effects of TNF-α on neurons are mediated by p55 receptors (Fontaine et al., 2002; Galasso et al., 2000; Liu et al., 1994).

Therefore, we cannot exclude that p55 receptors may also be involved in seizures in their sequelae in brain regions other than the hippocampus or in conditions where they are upregulated (Nadeau and Rivest, 1999; Shinoda et al., 2003).

Our evidence suggests that the release of TNF-α by glia during seizures (de Bock et al., 1996; Vezzani et al., 2002) or its pre-existence in brain because of chronic inflammation (Baranzini et al., 2002; Maldonado et al., 2003) can significantly affect the maintenance of epileptic activity.

CHAPTER 8

Molecular and functional interactions between TNF-α, glutamate and GABA in the mouse hippocampus: implications for neuronal excitability and seizure susceptibility

8.1 Summary

We investigated the molecular interactions between TNF-α system and ionotropic glutamate receptors subunits, and the receptor-mediated effects of TNF-α on glutamate and GABA release using mice lacking p55 ($p55^{-/-}$) or p75 ($p75^{-/-}$) receptors.

$p55^{-/-}$ mice showed a decreased susceptibility to kainic acid-induced seizures and displayed a decrease in GluR3 and NR1 glutamate receptor subunits in the hippocampus while GluR1, GluR2, GluR6/7 and NR2A/B were unchanged as compared to wild-type mice. In $p75^{-/-}$ mice which are more susceptible to seizures, GluR2, GluR3, GluR6/7 and NR2A/B glutamate receptor subunits were increased in the hippocampus while GluR1 and NR1 did not change. *S*pontaneous glutamate release measured by microdialysis in freely-moving mice was significantly decreased in $p55^{-/-}$, but not in $p75^{-/-}$ mice. *S*pontaneous GABA release was significantly decreased in both $p55^{-/-}$ and $p75^{-/-}$ mice. No changes were observed in KCl-induced glutamate and GABA release in mice lacking p55 or p75 receptors vs wild-type mice.

Our evidence indicates that there are molecular and functional interactions between TNF-α system and glutamate and GABA neurotrasmission in the hippocampus. The actions of TNF-α on neuronal excitability strictly depend on whether p55 or p75 receptors are preferentially involved and appear to be mediated also by changes in the assembly of glutamate receptor subunits. These novel mechanisms of functional glia-neuronal interactions may be relevant for controlling neuronal excitability in physiological and in pathological conditions.

8.2 Experimental procedures

Technical details related to microdialysis have already been reported in "Materials and methods - general procedures" section (p. 92-93).

8.2.1 Western blot

$p75^{-/-}$, $p55^{-/-}$ and wild type mice (n=5) were decapitated and 2 hippocampi obtained from different mice within the same experimental group were pooled and homogenized as described in "Materials and methods - general procedures" section (p. 92).

To separate cytosol and membrane fractions, the homogenized hippocampi were centrifuged at 45000 rpm at 4°C for 1 h, as previously described (Bendotti et al., 2001).

For immunoblotting, we used an anti-GluR2 (1:750, Chemicon, Temecula USA) and anti-GluR3 mouse monoclonal antibody (1:500, Chemicon), anti-GluR6/7 (1:2000, Chemicon) rabbit monoclonal antibody, anti-GluR1 (1:1000, Chemicon), anti-NR1 (1:250,Chemicon) and anti-NR2A/B rabbit polyclonal antibody (1:750, Chemicon). Immunoreactivity was visualised with enhanced chemiluminescence (ECL) using peroxydase-conjugated goat anti-mouse (1:2000; Sigma) and goat anti-rabbit (1:2000; Sigma) IgGs as secondary antibodies.

Optical density values in each sample were normalized using the corresponding amount of neurofilament M (NF 1:1000, Chemicon) for total homogenized hippocampi and β tubulin (1:5000; Sigma) for the membrane fractions.

8.2.2 Statistical analysis of data

Data are represented as the means ± standard error (n=number of animals). Statistical significance was determined by using one-way ANOVA followed by Fisher's test (for Western blot data) and Dunnet's test (for microdyalisis data).

8.3 Results

8.3.1 Spontaneous and depolarization-induced glutamate release

Fig. 8.1A shows the basal glutamate release in mice lacking TNF-α p55 or p75 receptors and wild type mice. Mice lacking TNF-α p55 receptors showed a ~50% decrease ($p<0.05$) of spontaneous glutamate release compared to wild type mice (Fig.8.1A), and these mice were also less susceptible to kainic-acid induced seizures (Balosso et al., 2005). $p75^{-/-}$ mice did not show significant changes in spontaneous glutamate release (Fig. 8.1A). Fig. 8.1B shows the effect of perfusion of 60 mM KCl on extracellular glutamate concentrations. No significant differences in depolarization-induced glutamate release were observed in mice lacking $p55^{-/-}$ or $p75^{-/-}$ receptors (Fig. 8.1B).

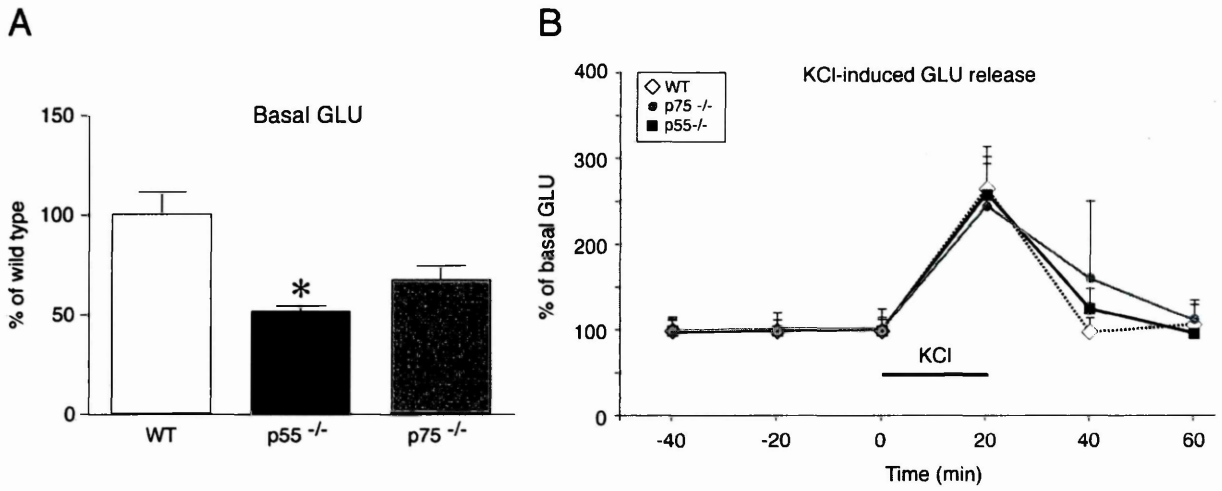


Figure 8.1. Spontaneous and depolarization-induced glutamate release
*Panel A: data represent the means ± SE (n=7-8) of the basal glutamate (GLU) in the ventral hippocampus of wild type (WT), p55$^{-/-}$ and p75$^{-/-}$ mice and are expressed as percentage of wild type. Panel B: data represent the effect of 60 mM KCl on extracellular GLU concentrations in the ventral hippocampus of WT, p55$^{-/-}$ and p75$^{-/-}$ mice and are expressed as percentage of basal levels. *p< 0.05 versus WT by one-way ANOVA followed by Dunnet's test.*

These data demonstrate that p55$^{-/-}$ mice display a decrease in spontaneous glutamate release suggesting that TNF-α, by interacting with p55 receptors, can affect the release of glutamate in physiological condition.

8.3.2 Spontaneous and depolarization-induced GABA release

Fig. 8.2A shows the basal GABA release in mice lacking TNF-α p55 or p75 receptors and wild type mice. Both p55$^{-/-}$ and p75$^{-/-}$ mice show a ~40% decrease ($p<0.05$) of spontaneous GABA release compared to wild type mice (Fig. 8.2A).

Fig 8.2B shows the effect of perfusion of 60 mM KCl on extracellular GABA concentrations. No significant differences in depolarization-induced GABA release were observed in TNF-α receptors deficient mice (Fig. 8.2B).

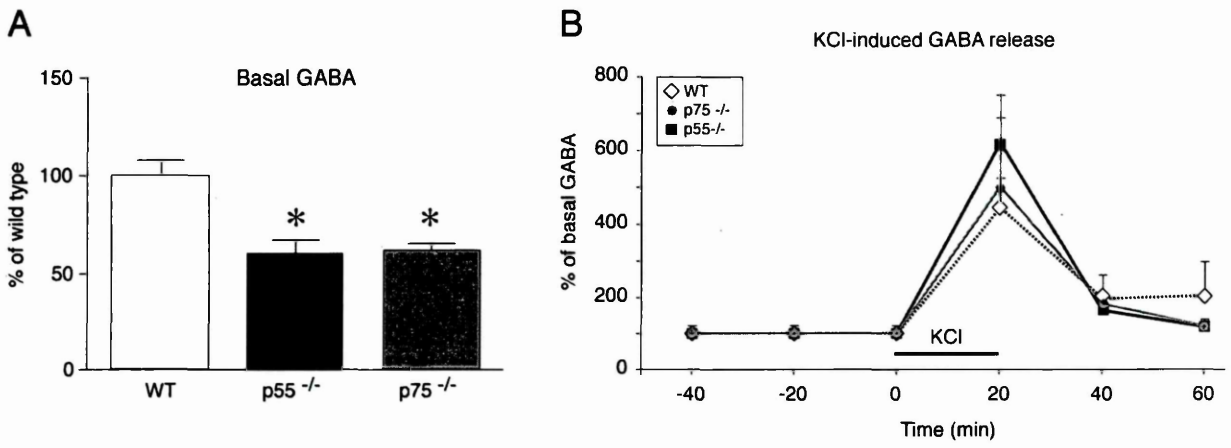


Figure 8.2. Spontaneous and depolarization-induced GABA release
Panel A: data represent the means ± SE (n=7-8) of the basal GABA in the ventral hippocampus of wild type (WT), p55$^{-/-}$ and p75$^{-/-}$ mice and are expressed as percentage of wild type.
Panel B: data represent the effect of 60 mM KCl on extracellular GABA concentrations in the ventral hippocampus of WT, p55$^{-/-}$ and p75$^{-/-}$ mice and are expressed as percentage of basal levels.
**p< 0.05 versus WT by one-way ANOVA followed by Dunnet's test.*

These data demonstrate that TNF-α functionally interacts with GABA neurotransmission in physiological condition.

8.3.3 AMPA, KA and NMDA receptor subunits in the hippocampus of p55$^{-/-}$ or p75$^{-/-}$ mice

Figure 8.3 shows the levels of AMPA, KA and NMDA receptor subunits in the hippocampus of mice lacking p55 or p75 receptors. p55$^{-/-}$ mice showed ~20% decrease of GluR3 ($p<0.05$) and ~30% decrease of NR1 ($p<0.05$) receptor subunits compared to wild type mice, while no differences were observed in the other glutamate receptor subunits (Fig. 8.3). Conversely, p75$^{-/-}$ mice had an up-regulation of glutamate receptor subunit levels; GluR2 and GluR3 subunits were enhanced by ~40% ($p<0.05$; $p<0.01$), GluR6/7 by ~50% ($p<0.01$) and NR2A/B subunits by 38% ($p<0.05$) compared to wild type mice (Fig. 8.3).

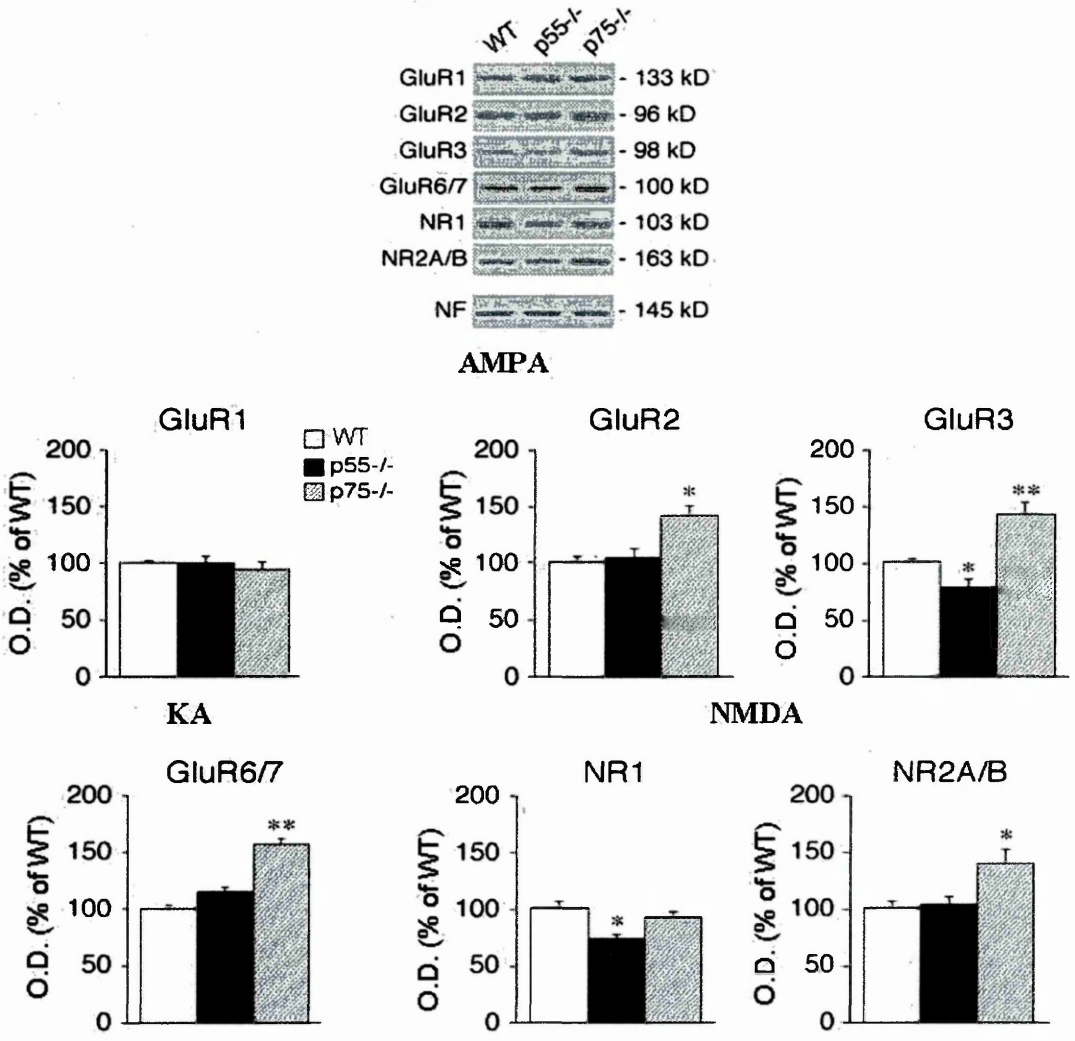


Figure 8.3. AMPA, KA and NMDA receptor subunits in total hippocampus of $p55^{-/-}$ or $p75^{-/-}$ mice

*Bargrams show densitometry analysis of the bands corresponding to the AMPA, KA and NMDA receptor subunits as assessed by western blot analysis of hippocampal homogenates of wild type (WT), $p55^{-/-}$, $p75^{-/-}$ mice (n=5). The optical density (O.D.) values of the relevant bands (as depicted in the representative western blot), are divided by the corresponding neurofilament (NF) value, used as internal standard; they are expressed as % of corresponding values measured in WT mice. *p< 0.05; **p< 0.01 versus WT by one-way ANOVA followed by Fisher's test.*

The changes in glutamate receptor subunit composition in mice lacking p55 or p75 receptors occur in the hippocampal membrane fraction. In particular, $p55^{-/-}$ mice showed a ~40% decrease of GluR3 ($p<0.05$) and NR1 ($p<0.01$) receptor subunits compared to wild type mice (Fig. 8.4A). In $p75^{-/-}$ mice, GluR2 subunit was enhanced by ~80% ($p<0.01$), GluR3 by ~40% ($p<0.05$), GluR6/7 by ~50% ($p<0.05$) and NR2A/B subunits by 40% ($p<0.05$) compared to wild type mice (Fig. 8.4B).

These findings suggest that TNF-α, by activating its receptors, regulate the trafficking of ionotropic glutamate receptor subunits from the cytoplasm to the membrane.

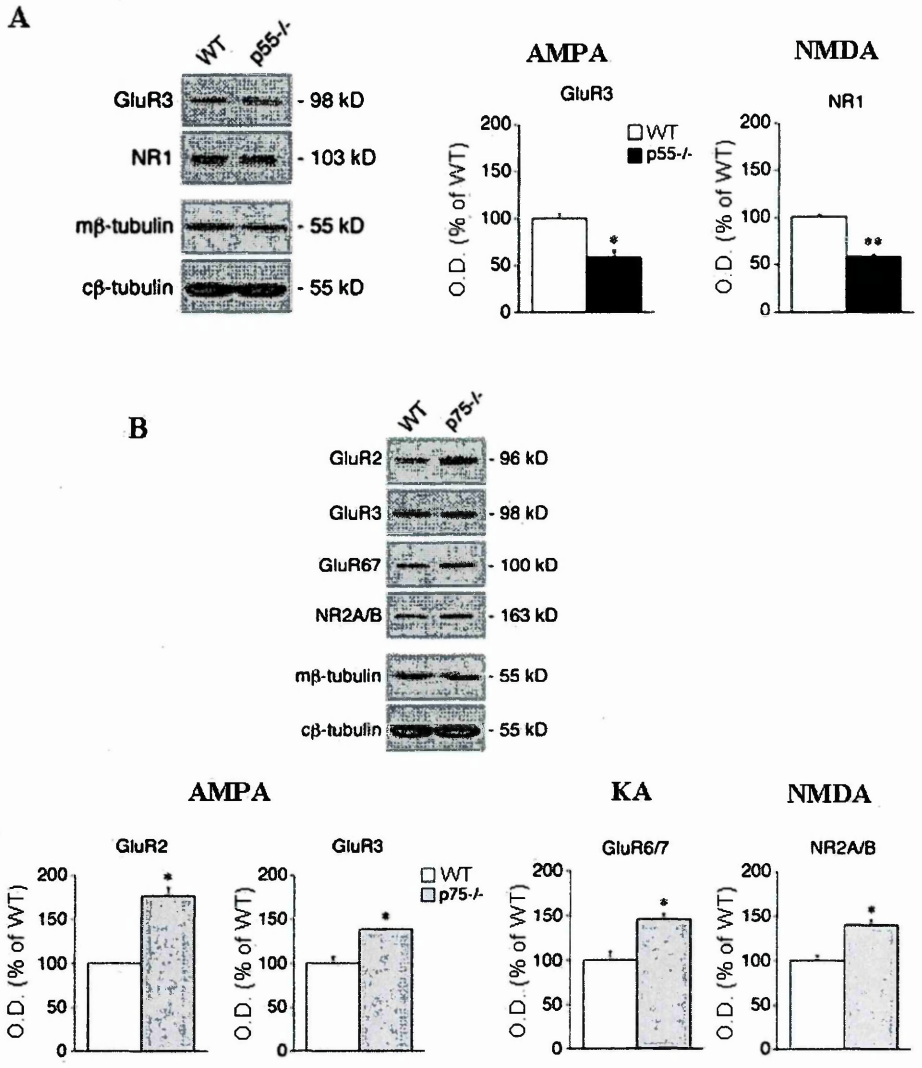


Figure 8.4. AMPA, KA and NMDA receptor subunits in the membrane fraction of $p55^{-/-}$ or $p75^{-/-}$ mice

Bargrams show densitometry analysis of the bands corresponding to AMPA, KA and NMDA receptor subunits as assessed by western blot analysis of hippocampal membrane fraction homogenates of wild type (WT), $p55^{-/-}$ (panel A) and $p75^{-/-}$ (panel B) (n=5).

*The optical density (O.D.) values of the relevant bands (as depicted in the representative western blots), are divided by the corresponding β-tubulin value, used as internal standard; they are expressed as % of corresponding values measured in WT mice. *p< 0.05; **p< 0.01 versus WT by one-way ANOVA followed by Fisher's test.*

mβ-tubulin: β-tubulin present in the membrane fraction; cβ-tubulin: β-tubulin present in the cytosol fraction.

In summary, this study has shown that:

1. $p55^{-/-}$ mice, that are less susceptible to seizures, show a reduction of spontaneous glutamate and GABA release and a decrease of GluR3 and NR1 receptor subunits.
2. $p75^{-/-}$ mice, that are more susceptible to seizures, show a reduction of spontaneous GABA release and an increase of GluR2, GluR3, GluR6/7 and NR2A/B receptor subunits.

It appears therefore that TNF-α receptor-mediated signaling controls the expression of glutamate ionotropic receptors bound on the membrane.

8.4 Discussion

The main finding of this study is that TNF-α interacts with glutamate and GABA neurotransmission in the mouse hippocampus. In particular, using mice lacking p55 or p75 receptors, we have found that, in physiological conditions, ambient concentrations of TNF-α can affect the release of glutamate and GABA and regulate the trafficking of ionotropic glutamate receptor subunits from the cytoplasm to the membrane. The final effect of TNF-α on glutamate and GABA neurotransmission depend on which TNF-α receptor is activated. The use of TNF-alpha receptors knock-out mice is a valuable tool to study the role of p55 and p75 receptor signalling in the physiopathological effects of this cytokine in CNS; thus, pharmacological tools that could distinguish unequivocally between these two receptors are lacking. Mice deficient in p55 and p75 are overtly normal indicates that neither p55 nor p75 are grossly required for normal mouse development and homeostasis under basal conditions (Peschon et al., 1998). These evidences are also supported by the fact that mice specifically

lacking TNF-α are viable and fertile, develop lymphnodes and show no apparent phenotypic abnormalities, indicating that TNF alpha is not required for normal mouse development (Pasparakis et al., 1996).

A reduced release of spontaneous GABA was observed in mice lacking p75 or p55 receptors; the latter strain displayed also a decreased spontaneous glutamate release, suggesting that the activation of both receptors by physiological concentration of TNF-α plays a role in regulating excitatory and inhibitory transmission. This may contribute to affect several forms of synaptic plasticity in hippocampus such as long-term potentiation (Cunningham et al., 1996; Tancredi et al., 1992) and long-term depression (Albensi and Mattson, 2000), that have already been shown to involve the interaction between TNF-α and glutamate receptor. We speculate that the decrease in basal extracellular glutamate levels in $p55^{-/-}$ mice is due to a reduction in astrocytic glutamate release for the following reasons: (1) the basal extracellular glutamate level measured by microdialysis likely reflects glutamate release from astrocytes since it is insensitive to TTX and has a low dependence on calcium (Del Arco et al., 2003; van der Zeyden et al., 2008); (2) TNF-alpha induces the astrocytic release of glutamate acting on p55 receptors (Bezzi et al., 2001), thus supporting our finding of a reduced glutamate release in the absence of p55 receptors.

In p75-/- mice, we found no significant changes in basal glutamate levels suggesting that this receptor subtype is not involved in the astrocytic glutamate release (Bezzi et al., 2001). Moreover, the lack of changes in high KCl-induced glutamate release in both receptor knock-out mice supports the view that TNF-alpha specifically affects astrocytic, but not neuronal, glutamate release (Bezzi et al., 2001; Del Arco et al., 2003). However, we can not exclude that the lack of changes in high KCl-induced glutamate release may be due to

microdialysis that is an invasive technique since it attempts to monitor the release of neurotransmitter from nerve terminals by inserting into the brain a probe several orders of magnitude larger than the biological structure under study. The injury caused by the insertion of the probe is limited to a short period of time and after 24 h animals exhibited only slight alteration in glucose metabolism and blood-brain barrier permeability. Typically after an initial period during which neurotransmitter levels are very high, they decrease and remain stable for several hours or days. Moreover, the development of reactive gliosis, occurring few days after probe implantation, limits the use of microdialysis probe. Another important aspect to consider is that after release, neurotransmitters can be involved into processes of uptake and metabolism, which can limit the amount of neurotransmitters recovered in the dialysate.

Significant alterations in the membrane levels of specific ionotropic glutamate receptor subunits in the hippocampus of naive mice lacking either p55 or p75 TNF-alpha receptors were observed. The absence of TNF-alpha receptors determines modifications in different glutamate receptor subunits, namely a decrease in NR1 and GluR3 in $p55^{-/-}$ mice and an upregulation of GluR2, GluR3, GLUR6/7 and NR2AB in $p75^{-/-}$ mice.

The glutamate receptor changes we have measured in naive p55 and p75 knock-out mice are compatible with their altered susceptibility to seizures, as reported in previous studies where these mice were intrahippocampally injected with kainic acid (Balosso et al., 2005). At the light of our present results, the increased seizure susceptibility in $p75^{-/-}$ mice (Balosso et al., 2005) could be explained by at least two non-mutually exclusive mechanisms: (1) an enhanced response of mice to kainate upon its intrahippocampal injection, due to the intrinsic upregulation of kainate GluR6/7 subunit. Accordingly, the

hippocampal overexpression of GluR6 predisposes to seizures (Telfeian et al., 2000) while GluR6 knock-out mice are resistant to kainate seizures (Mulle et al., 1998; Vincent and Mulle, 2008); (2) the intrinsic upregulation of NR2A/B and GluR3 subunits may amplify the neuronal responses to glutamate that is released during kainate-induced epileptic activity. In particular, the upregulation of NR2A/B protein level in $p75^{-/-}$ mice suggests the presence of an NMDA heteromeric receptor favoring both neuronal Ca2+ influx in response to glutamate (Viviani et al., 2003) and neuronal synchronization because of the lowest deactivation kinetics of NR2A/B containing receptors (Cull-Candy and Leszkiewicz, 2004). GluR3 receptor subunit also appears to be prominently involved in ictogenesis since rodents developing GluR3 receptor-activating antibodies develop seizures (Rogers et al., 1994; Levite and Ganor, 2008). The upregulation of AMPA GluR2 subunit, that confers low calcium permeability (Isaac et al., 2007), in $p75^{-/-}$ mice may represent a compensatory change to counteract the increased neuronal excitability.

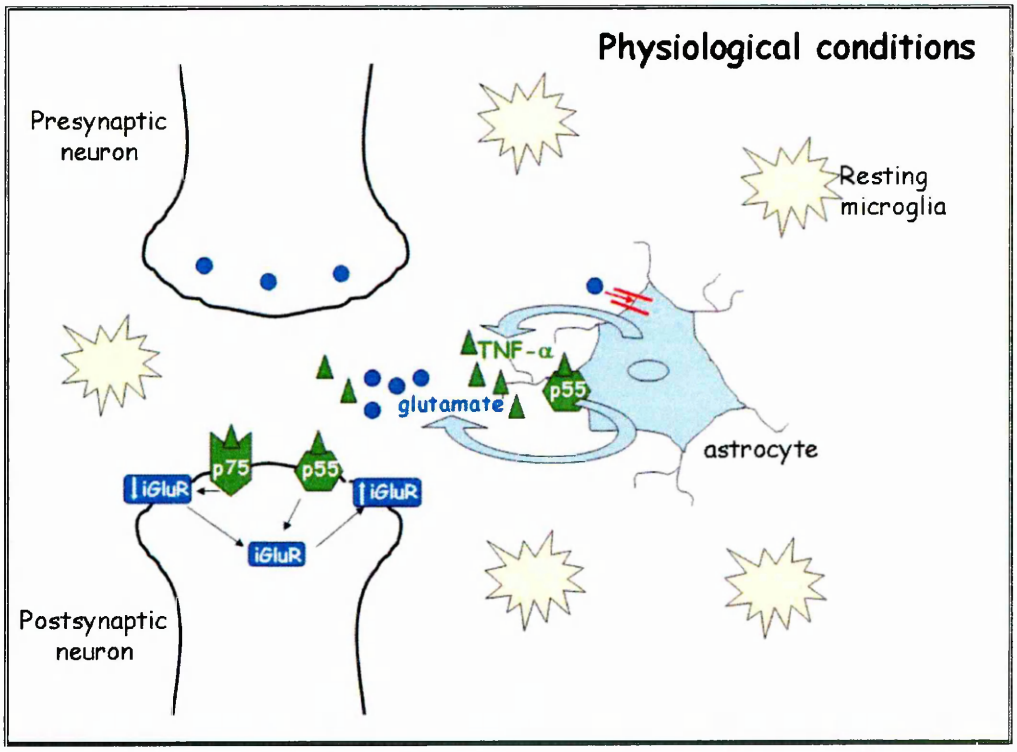
We did not detect p55 receptor using immunohistochemistry and Western blot analysis in normal mouse hippocampus, although measurable levels of p55 transcript and protein have been reported previously (Bette et al., 2003). The apparent absence of p55 protein implies a limited transcription of its messenger RNA in physiological conditions, which may represent a mechanism for limiting the potential harmful effects of TNF-α on neuronal survival that appear to be mediated by this receptor (MacEwan, 2002a).

In $p55^{-/-}$ naive mice, both GluR3 and NR1 receptor subunits are reduced and these modifications may contribute to decrease $p55^{-/-}$ mice susceptibility to kainate seizures. Indeed, as mentioned for GluR3 receptor subunit, NR1 subunit that is considered essential to form functional ion channels (Hollmann and Heinemann, 1994; Zukin and Bennett,

1995) is important for seizure generation and propagation. Indeed, it has been shown that a down-regulation of NR1 subunit using an antisense probe protects against sound-induced seizures in audiogenic mice (Chapman et al., 1996) and increases the latency to NMDA-induced seizures in mice (Zapata et al., 1997).

Although our biochemical evidence suggests only that significant alterations in the membrane levels of specific ionotropic glutamate receptor subunits in the hippocampus are present in mice lacking p55 or p75 receptors, we can speculate that these changes reflect modifications in hippocampal AMPA, KA and NMDA receptor subunits composition which may alter the functional properties of assembled ionotropic glutamate receptors modifying the balance of excitation and inhibition.

These novel functional glio-neuronal interactions may be relevant for controlling neuronal excitability in physiological and in pathological conditions (see Fig. 8.5).


Physiological conditions
Presynaptic neuron
Resting microglia
TNF-α
p55
glutamate
astrocyte
p75
p55
iGluR
iGluR
iGluR
Postsynaptic neuron

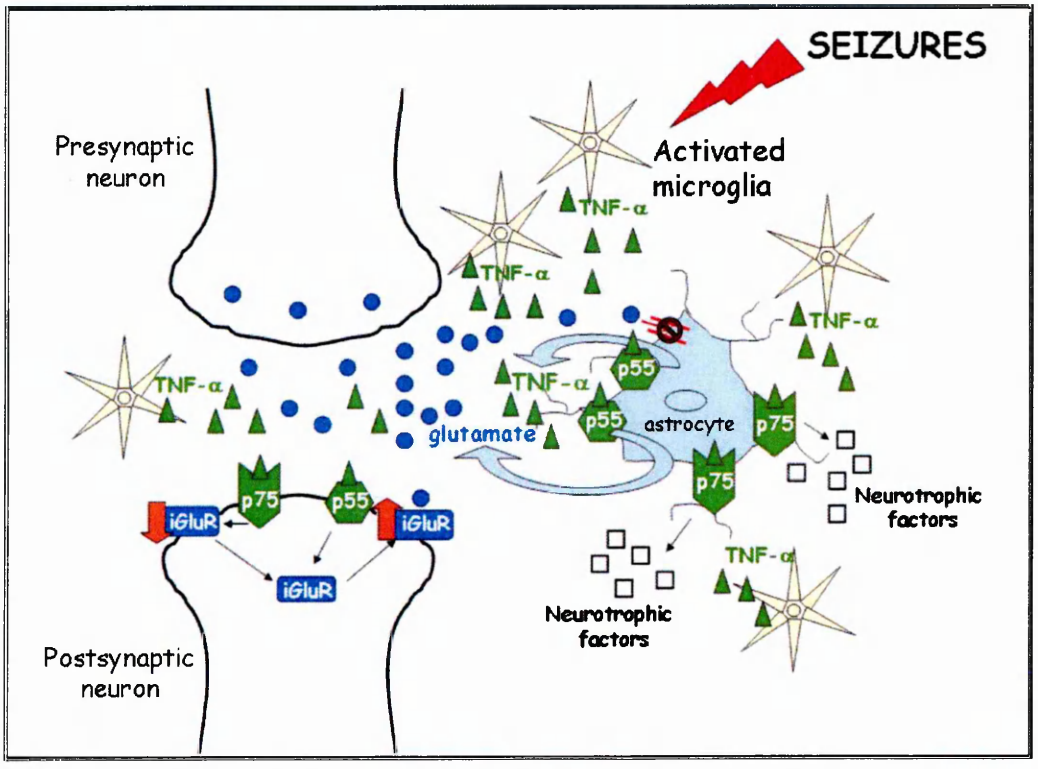

SEIZURES
Presynaptic neuron
Activated microglia
TNF-α
TNF-α
TNF-α
TNF-α
TNF-α
p55
p55
astrocyte
p75
p75
glutamate
Neurotrophic factors
p75
p55
iGluR
iGluR
iGluR
TNF-α
Neurotrophic factors
Postsynaptic neuron

Figure 8.5. Simplified representation of functional glia-neuronal interactions in physiological and pathological conditions

In physiological conditions, we detected p75 receptor expression in neurons by immunohistochemistry. Functional evidence provided by Stellwagen (Stellwagen et al., 2005) and Bezzi (Bezzi et al., 2001a) showed p55 receptors on neurons and astrocytes in in vitro preparation, although they were not detectable in vivo in our experimental setting. In physiological conditions, TNF-α is released by astrocytes (Beattie et al., 2002) and modulates astrocytic glutamate release (Bezzi et al., 2001a).Our microdialysis study in mice lacking p55 receptors suggests that p55 receptors are involved in regulating astrocytic glutamate release. Our western blot data indicate that TNF-α, possibly released by astrocytes, regulates glutamate ionotropic receptor (iGluR) expression on neuronal membrane acting on its receptor subtypes.

In pathological conditions, such as during seizures, we detected increased expression of p55 and p75 receptors on astrocytes. Microglia is activated and release high amount of TNF-α. This effect may enhance glutamate release from astrocytes (Bezzi et al., 2001a) and inhibit its re-uptake (Hu et al., 2000; Zou and Crews, 2005), leading to increase glutamate extracellular concentrations. Enhanced TNF-α concentrations in tissue may also exacerbate the effects of glutamate on its receptor subunits.

Thus, the activation of p55 receptors may increase the extracellular glutamate concentrations and enhance neuronal excitability; the activation of neuronal p75 mediates neuroprotective effects, increasing inhibition. The role of p75 receptors on astrocytes needs further investigations. We speculate they may mediate the production of neuroprotective agents (Kamiguchi et al., 1995; Kuno et al., 2006) to compensate for the detrimental actions due to p55-mediated signaling.

9.1 General discussion

The observation that brain inflammatory reactions occur in various epileptic disorders with different etiologies raises the possibility that inflammation may be a common factor contributing to the onset of epilepsy. Among the inflammatory mediators identified in epileptogenic tissue, IL-1β and TNF-α have been the ones best characterized for their expression profile in seizure models and in human epileptic tissue. In this thesis, the role of these proinflammatory cytokines in experimental models of acute and chronic seizures has been investigated using different experimental strategies: 1- elevated brain levels of the cytokine were attained by their intracerebral application; 2- their brain production or their receptor-mediated signaling was blocked using selective inhibitors 3. seizure susceptibility was assessed in genetically-modified mice with a perturbed cytokine system. We also studied the role of IL-1β in the excitotoxic neuronal damage, often associated with seizures, using in vitro approaches.

We provide evidence that:

1. pharmacological intervention aimed at inhibiting the IL-1β system reduces acute and spontaneous seizures induced in experimental models.

2. the proconvulsant effects of IL-1β are mediated by a novel transcription-independent pathway characterized by the activation of neutral sphingomyelinase and Src-family of kinases in the hippocampus which leads to the phosphorylation of the NR2B subunit of the NMDA receptor.

3. transient exposure of brain tissue to specific pro-inflammatory stimuli exacerbates neuronal susceptibility to a subsequent excitotoxic insult via induction of IL-1β release from activated microglia.

4. TNF-α reduces kainic acid-induced seizures by activating neuronal p75 receptors. The molecular and functional interactions between TNF-α receptors and ionotropic glutamate receptors contribute to the changes in seizure susceptibility mediated by this cytokine.

9.1.1 Inactivation of caspase-1 in rodent brain: a novel anticonvulsant strategy

Epilepsy in humans is associated with inflammatory processes in the brain involving the activation of the IL-1β/IL-1RI signaling (Ravizza et al., 2006a; Ravizza et al., 2008a). Experimental findings show that the activation of this signaling in neurons exacerbates acutely induced seizures (Vezzani et al., 1999) and lowers the threshold for seizure induction (Dubè et al., 2005). In contrast, IL-1ra, the naturally occurring receptor antagonist (Dinarello, 1996), mediates powerful anticonvulsant effects (De Simoni et al., 2000; Vezzani et al., 2000; Vezzani et al., 2002), and in mice overexpressing IL-1ra in astrocytes, seizure onset is delayed and seizure spread is strongly attenuated (Vezzani et al, 2000). These data indicate that a pre-existing inflammatory state in the brain associated with elevated levels of IL-1β may significantly contribute to the mechanisms of ictogenesis.

This evidence prompted us to study whether seizures can be inhibited by blocking the brain production of IL-1β by using pralnacasan and VX-765, two selective inhibitors of caspase-1, the key enzyme specifically involved in the production of the releasable and biologically active form of IL-1β. Intracerebral or systemic administration of these drugs significantly reduced the number and the duration of acute and spontaneous seizures that are refractory to

the treatment with various antiepileptic drugs (Riban et al., 2002). This powerful anticonvulsant effect was associated with the ability of these drugs to prevent seizure-induced increase of the biologically active form of IL-1β, as demonstrated by our biochemical data.

Thus, inhibition of caspase-1 may represent an effective and novel pharmacological strategy to control seizures which are refractory to classical anticonvulsant treatments.

9.1.2 A novel non-transcriptional pathway mediates the proconvulsant effects of IL-1β

The proconvulant effects of IL-1β in experimental models occur within minutes from the application of this cytokine; therefore, they cannot be explained by the classic signaling cascade involving activation of genomic transcriptional events. Using a detailed pharmacological approach, we demonstrated that the proconvulsant activity of this cytokine is mediated by the activation of the N-Smase-ceramide pathway since 3-O-MS, a selective N-Smase inhibitor, blocks the increase in the frequency and duration of seizures induced by IL-1β, and C2-ceramide faithfully mimics the IL-1β effect on seizures. Moreover, the proconvulsant effects of either IL-1β or C2-ceramide were dependent on activation of the Src family of tyrosine kinases since they were prevented by CGP76030, an inhibitor of this enzyme family. These results indicate that Src kinase activation is a downstream event subsequent to IL-1β activation of N-Smase. Finally, the proconvulsant effect of IL-1β was blocked by ifenprodil, a selective NR2B receptor antagonist, suggesting that this subunit is critically involved in IL-1β on seizures. These pharmacological results are supported by biochemical data showing that IL-1β or seizures enhanced the levels of the phosphorylated forms of Src-family of tyrosine kinases and the NR2B subunit in the hippocampus and

these effects were additive when seizures were increased by IL-1β. The phosphorylation of the NR2B subunit were prevented by 3-O-MS and CGP76030, thus supporting the involvement of N-Smase and Src-family kinases in NMDA activation. Phosphorylation of the NR2B subunit promotes Ca^{2+} influx into neurons (Viviani et al., 2003; Yu et al., 1997), thus resulting in potentiation of NMDA function which contributes to neuronal hyperexcitability (Meador, 2007). A similar pathway has been recently described in hypothalamic warm sensitive neurons (Sanchez et al., 2006) and appears to mediate the fast actions of IL-1β underlying the rapid phase of the febrile response to this cytokine.

These results highlights a novel, non-transcriptional mechanism underlying seizure exacerbation in inflammatory conditions and may allow the development of innovative strategies to block the activation of IL-1β signaling in diseased conditions.

9.1.3 The consequence of transient inflammation and microglia activation on neuronal susceptibility to an excitotoxic insult

One major hallmark of brain inflammation is the activation of microglial cells. These resident brain immune cells migrate and proliferate at sites of neuronal injury where they can support regenerative functions in neurons and help functional recovery from CNS injuries (Schwartz *et al*, 2006) or contribute to neuronal damage *via* the release of neurotoxic agents and proinflammatory cytokines such as IL-1β. Therefore, we investigated the consequences of inflammatory processes characterized by microglia activation and release of IL-1β on the AMPA-mediated excitotoxicity in organotypic hippocampal slice cultures. We observed that transient exposure of hippocampal slices to LPS plus ATP induced microglia activation and apoptosis-like cell death, accompanied with a sustained

IL-1β release. This effect was dependent on specific activation of $P2X_7$ receptors. Moreover, exposure of hippocampal slices to LPS plus ATP, but not either one or the other alone, induced an exacerbation of neuronal death mediated by AMPA in CA3 subfield. This toxic effect requires a critical IL-1β extracellular concentration and the activation of microglia-expressing $P2X_7$ receptors, but not microglia apoptosis-like cell death. Indeed, inhibitors of IL-1β production or its receptor signalling, prevented the potentiation of AMPA toxicity without altering microglial apoptosis.

This mechanism may contribute to determine neuronal cell death in acute and chronic neurodegenerative conditions associated with inflammatory events, such as in epilepsy.

9.1.4 Role of TNF-α and its receptors in seizures: interactions with glutamate and GABA neurotransmission

TNF-α is a pleiotropic cytokine rapidly upregulated in the CNS by seizures, but little is known about its role in epileptic activity. The biological actions of TNF-α are mediated through two distinct receptors, p55 and p75, that are present both in neurons and glia (Kuno et al., 2006; Sairanen et al., 2001). Each TNF-α receptor mediates different cellular responses, although partial overlap of their signaling has been reported (McEwan, 2002). We investigated the effect of TNF-α on seizures by using a dual approach: we produced increased cytokine levels in brain by applying the recombinant cytokine in mouse hippocampus immediately before the induction of seizures; we also studied seizure susceptibility in transgenic mice chronically overexpressing TNF-α in astrocytes or in mice lacking either p55 or p75 receptors. These experiments address whether acute or chronic

brain expression of a proinflammatory molecule changes the brain susceptibility to the induction or maintenance of seizures and which role TNF-α receptor subtypes play in these phenomena.

Differently from IL-1β, TNF-α has predominant anticonvulsant effect specifically mediated by p75 receptor subtypes.

In particular, we found that increased brain levels of murine TNF-α in hippocampus, due to exogenous application or gene overexpression, have a powerful inhibitory action on EEG seizures reducing their frequency and duration. Human TNF-α, which selectively activates p55 receptor subtype in mice, did not affect seizures in wild-type mice suggesting that p55 receptors are not involved in the anticonvulsant effect of mTNF-α.

Moreover, mice lacking p75 receptors exhibited prolonged seizures while mice deficient in p55 receptor, displayed reduced seizure activity. $p55^{-/-}$ showed also a marked upregulation of p75 receptors and in the related signaling pathway, suggesting that reduced seizure activity in $p55^{-/-}$ mice is a consequence of the adaptive increase in p75 receptors. p75 receptor is also the only receptor subtype detected by immunocytochemistry in normal mouse hippocampus supporting that the anticonvulsant effect of murine TNF-α in wild-type mice is mediated by neuronal p75 receptors and not p55 receptors.

This evidence shows a novel TNF-α function linked to fast changes in neuronal excitability and highlights the possibility that TNF-α functionally interacts with glutamatergic neurotransmission. It has been reported that TNF-α released by glial cells induces an increase in the surface expression of GluR2-lacking AMPA receptors leading to increased neuronal calcium influx and enhanced synaptic efficacy (Beattie et al., 2002; Stellwagen et al., 2005). TNF-α can also affect the GABAergic synaptic transmission by inducing

endocytosis of $GABA_A$ receptors, thus reducing the inhibitory synaptic strength (Stellwagen et al., 2005). We investigated the molecular interactions between TNF-α system and ionotropic glutamate receptor subunits and the receptor-mediated effects of TNF-α on glutamate and GABA release using mice lacking p75 or p55 receptors. We found that TNF-α receptor-mediated signaling controls the expression of glutamate ionotropic receptors. In particular, $p55^{-/-}$ mice that are less susceptible to seizures, showed a reduction of spontaneous glutamate (and GABA release) and a decrease of GluR3 and NR1 receptor subunits. On the other hand, $p75^{-/-}$ mice that are more susceptible to seizures, showed an increase of GluR2, GluR3, GluR6/7 and NR2A/B receptor subunits (and a reduction of spontaneous GABA release). The changes in hippocampal AMPA, KA and NMDA receptor subunit composition observed in the membrane fraction may alter the functional properties of ionotropic glutamate receptors modifying the balance between excitation and inhibition and contributing to the actions of TNF-α on neuronal excitability.

10.1 Summary and conclusions

The first part of this thesis has demonstrated that IL-1β and its receptor IL-1R1 system are chronically overexpressed in mice epileptic tissue. The activation of IL-1β system highlights the possibility that this phenomenon may have an active role in the pathophysiology of seizures, altering neuronal excitability and cell survival. Our findings from *in vivo* experiments support a role for IL-1β signalling in the mechanisms of ictogenesis because IL-1β exacerbates seizures in mice. In addition, seizures, that are refractory to the classical antiepileptic drugs, are drastically reduced by blocking the formation of the biologically active form of IL-1β, by selective inhibition of caspase-1, indicating that this treatment represent an effective and novel anticonvulsive strategy.

The mechanism underlying the proconvulsant actions of IL-1β appears to occur via a novel non-transcriptional *IL-1β-N-Smase-Src kinase* pathway that results in the potentiation of NMDA function which is responsible for the increased neuronal excitability. This fast post-translational effect of IL-1β represents a novel and non-conventional pathway by which inflammatory molecules produced in epileptic tissue can affect neurotransmission.

We have also demonstrated that a brief proinflammatory stimulus, characterized by the activation of microglia and subsequent lasting release of critical concentrations of IL-1β, primes neuronal vulnerability to a subsequent excitotoxic insult. These findings shed light on the mechanisms by which a pre-existent proinflammatory state increases hippocampal neuronal susceptibility to the excitotoxic damage associated with seizures (Auvin et al., 2007; Somera-Molina et al., 2007).

Because IL-1β exacerbates seizures (Vezzani et al., 1999), promotes neuronal loss (Bernardino et al., 2005; Viviani et al., 2003), inhibits neurogensis (Ekdahl et al., 2003; Monje et al., 2003), alters BBB permeability (Allan et al., 2005; Naldini and Carraro, 2005), pharmacological approaches specifically targeted to block the overproduction of IL-1β and its functions in diseased conditions may represent new strategies for the treatment of seizure disorders, which are refractory to classical anticonvulsant treatments.

The second part of this thesis has shown that TNF-α can significantly decrease epileptic activity by acting on neuronal p75 receptors. Activation of p55 receptors by TNF-α or chronically elevated levels of this cytokine (Akassoglou et al., 1997; Stalder et al., 1998) may trigger neuropathology and promote seizures.

Thus, the actions of TNF-α on neuronal excitability strictly depend on whether p55 or p75 receptors are preferentially involved and appear to be mediated also by changes in the assembly of glutamate receptor subunits.

These novel functional glia-neuronal interactions add important insights into the mechanism of ictogenesis and seizure-associated neuronal cell death, highlighting innovative pharmacological strategies to block the activation of cytokine-mediated signalling in diseased conditions.

11.1 Future work

1- Studies underlying the role of IL-1β system in the epileptogenesis

A relevant but still unresolved issue in epileptic disorders is whether inflammation plays a role in epileptogenesis, the process that transforms a non-epileptic neuronal circuit into a seizure-generating one. We have recently demonstrated that IL-1β system is upregulated in the epileptogenesis phase which follows induction of status epilepticus, and is prodromic to the onset of epilepsy (Ravizza et al., 2008a). Moreover, the selective inhibition of caspase-1 by VX-765 arrests kindling and impairs the development of generalized motor seizures (Ravizza et al., 2008b). These findings highlight the possibility that the activation of IL-1β system contributes to epileptogenesis by increasing neuronal susceptibility to spontaneous seizure induction (Vezzani and Baram, 2007). To address this issue we will use a mouse model of TLE where spontaneous seizures occur after a latency period (Riban et al, 2002). After status epilepticus, mice will be treated by VX-765 for 2 weeks and the time to onset of the first seizure and the duration of the spontaneous seizures will be evaluated by EEG recordings. If IL-1β plays a role in epileptogenesis, a significant delay in the time to onset of the first spontaneous seizure and a decrease in the time spent in epileptic activity will be observed.

Elucidation of the role of IL-1β in epileptogenesis may be very important to prevent epilepsy following brain injuries, such as neurotrauma and status epilepticus, which are two clinical conditions characterized by brain inflammation, elevated levels of IL-1β and associated with a high risk to developing epilepsy (Pitkanen and Sutula, 2002; Pitkanen et al., 2007).

2- Studies underlying the mechanisms of ictogenesis and epileptogenesis in inflammatory conditions

Inflammatory pathways activated by seizures share several common features with those activated by systemic or CNS infection. In this respect, the injection of LPS in rodents chronically lowers seizure threshold in immature and mature brain rodents and enhances seizure-induced cell loss (Sayyah et al., 2003; Auvin et al., 2007; Galic et al., 2008). LPS activates TLR4, which play a key role in the innate immune system during pathogen recognition; these receptors are up-regulated in neurons and glia by acute seizures and chronic epileptic tissue (our preliminary data). These results prompt us to explore whether TLR-signaling, activated by endogenous ligands released during tissue injury, contributes to neuronal hyperexcitability and to enduring alterations in gene expression programs that underlie the epileptogenic process.

The study of mechanistic aspects related to the pathways activated during brain inflammation, and their role in epileptogenesis, will be instrumental for envisaging novel therapeutic approaches.

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