Role of selected aspects of inflammation in age-dependent seizure susceptibility and neuronal injury in rats

Thesis

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Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.21954/ou.ro.0000fa7f

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Role of selected aspects of inflammation in age-dependent seizure susceptibility and neuronal injury in rats

by

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Thesis submitted for the degree of Doctor of Philosophy at the Open University

Discipline of Life Sciences

September, 2006
To Mara
Acknowledgements

I am grateful to Prof. J. W. Sander who kindly and promptly gave me helpful support during all stages of this work. A special thank you to Dr. Russell Monhemius, who kindly reviewed the thesis giving me helpful scientific and editing advice.

I would also like to thank Dr. A. Vezzani and Prof. Silvio Garattini for giving me the opportunity to attend the Open University PhD program.

Last, but not least, I do wish to thank the Open University for being as it is.
List of publications related to this thesis

Collaborations

The experiments exploiting seizure induction by flurothyl inhalation were kindly performed by Dr. Jana Veliskova at the Laboratory of Pediatric Epilepsy, Albert Einstein College of Medicine, New York, USA.
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<td>adrenocorticotropic hormone</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressing</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<tr>
<td>CA</td>
<td>Ammon’s horn</td>
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<td>CAMKII</td>
<td>Ca(^{2+})/calmodulin-dependent kinase II</td>
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<td>cAMP</td>
<td>cyclic-AMP</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
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<td>COX-2</td>
<td>cyclooxigenase-2</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding</td>
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<tr>
<td>CRF-R1</td>
<td>corticotropin releasing factor – receptor 1</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone/factor</td>
</tr>
<tr>
<td>CS</td>
<td>corticosterone</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalitis</td>
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<td>EEG</td>
<td>electroencephalogram</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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</tr>
<tr>
<td>EP4</td>
<td>prostaglandin E2 receptor 4</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fra</td>
<td>fos related antigens</td>
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<tr>
<td>GABA</td>
<td>γ-amino butirric acid</td>
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<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glia fibrillary acidic protein</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid responsive element</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>interleukin-1 receptor antagonist</td>
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<td>IL-1β</td>
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<td>IL-4</td>
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<td>IL-10</td>
<td>interleukin-10</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>I-κB</td>
<td>inhibitor of NF-κB</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>MTLE</td>
<td>mesial temporal lobe epilepsy</td>
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<tr>
<td>MTS</td>
<td>mesial temporal sclerosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>OX-42</td>
<td>complement receptor type 3</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PC-PLC</td>
<td>phosphocholine-specific phospholipase C</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PGE₂</td>
<td>prostaglandin E2</td>
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<td>PKA</td>
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<td>PN</td>
<td>postnatal</td>
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<td>POMC</td>
<td>proopiomelanocorticotropin</td>
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<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RIIβ</td>
<td>PKA-regulatory subunit II β</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SE</td>
<td>status epilepticus</td>
</tr>
<tr>
<td>SHRP</td>
<td>stress hyporesponsive period</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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Abstract

Besides systemic infection, proinflammatory signals in the CNS are also recruited by epileptic activity. Chronic stimulation of proinflammatory signals by seizures, or a persistent proinflammatory situation in brain 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.

Interestingly, in humans and in experimental models of epilepsy, seizure susceptibility and associated neuronal damage are age-dependent. Therefore, we wondered whether a hypothetical age-dependent relationship between proinflammatory molecules and seizure-susceptibility and related neurodegeneration there might exist and to what extent.

A first group of experiments investigated the hypothesis that activation of glia and subsequent production of proinflammatory molecules such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), as well as the naturally occurring anti-inflammatory molecule interleukin-1 receptor antagonist (IL-1Ra), are age-dependently involved in seizure-induced neuronal damage.

A second group of experiments investigated whether a pre-existing LPS-induced inflammatory state in the CNS may enhance the predisposition of rat pups to develop seizures.

We concluded that the induction of proinflammatory cytokines, per se, cannot be regarded as a general mechanism which underlies the appearance of neurodegeneration. In addition, there were no clear indications emerging from our data concerning the role of proinflammatory cytokines in modulating
seizure-susceptibility in rat pups. Nonetheless, we showed that *prostaglandin E*₂ *(PGE*₂*) production and the activation of the Hypothalamic-Pituitary-Adrenal axis play a relevant role in the modulation of seizure-susceptibility in rat pups. Interestingly, our experiments seem to support the hypothesis that production of proinflammatory molecules such as IL-1β and TNF-α in rat pups might be down-regulated upon elevation of parenchymal PGE₂ levels.
“...make everything as easy as possible, but not simpler”

A. Einstein
1. Introduction
1.1 Foreword

Tiergartenstrasse 4 in Berlin still represents the address of the building that used to be the headquarter of the authority responsible for the accomplishment of the so-called *Euthanasia Operation T4* (T4 stood just for Tiergartenstrasse 4), a ruler promulgated in Germany, 1940, according to the Third-Reich theories on *Racial Hygiene*. All people not conforming to Aryan standards were to be definitely eliminated thus making the society free from racial imperfections. Among them, people affected by the so-called *hereditary falling sickness*. 'Hereditary falling sickness' was the generic terminology by which many symptoms of epilepsy used to be classified at that time. After mass sterilization imposed by the *Law for the Prevention of Offspring with Hereditary Disease* (promulgated on 15th July 1933, fig. 1) the final step of building gassing chambers was undertaken, as it was probably intended from the start.
Gesetz zur Verhütung erbkranken Nachwuchses

§ 1
Wer erbkrank ist, kann durch chirurgischen Eingriff unfruchtbar gemacht (sterilisiert) werden, wenn nach den Erfahrungen der ärztlichen Wissenschaft mit großer Wahrscheinlichkeit zu erwarten ist, daß seine Nachkommen an schweren körperlichen oder geistigen Erbschäden leiden werden. Erbkrank im Sinne dieses Gesetzes ist, wer an einer der folgenden Krankheiten leidet:

1. angeborenem Schwachsinn,
2. Schizophrenie,
3. zirkulärem (manisch-depressivem) Irresein,
4. erblicher Fallsucht,
5. erblichem Veits-Tanz (Huntingtonsche Chorea)
6. erblicher Blindheit,
7. erblicher Taubheit,
8. schwerer erblicher körperlicher Mißbildung.
Ferner kann unfruchtbar gemacht werden, wer an schwerem Alkoholismus leidet.

Law for the Prevention of Offspring with Hereditary Diseases

§ 1
People who suffer from a hereditary disease can be made infertile surgically (sterilised), if the knowledge gained from medical science points to the probability that the offspring will suffer from severe physical or mental hereditary damage.

The law views people as suffering from a hereditary disease if they have the following:

1. congenital mental deficiency,
2. schizophrenia,
3. circular (manic depressive) insanity,
4. hereditary falling sickness,
5. hereditary Huntington's Disease,
6. hereditary blindness,
7. hereditary deafness,
8. severe hereditary physical deformity.

People suffering from severe alcoholism can also be sterilised.

Source: German Epilepsymuseum Kork - Museum for epilepsy and the history of epilepsy
Thus, History simply shows that even the modern and rational-based XX century witnessed a strong discrimination against people suffering from epilepsy. Indeed, what joins the majority of people suffering from epilepsy throughout ages and civilizations is a strong social discrimination which likely originates from the feelings of fear and horror linked to the image with which epilepsy is usually associated, the grand mal attack.

Epilepsy is an illness as old as human kind and it is one of the most documented pathologies in the history of medicine, and can be traced back to the dawn of the most ancient civilizations.

As may be expected, the multitude of descriptive terms by which epilepsy used to be referred to throughout the ages directly reflected the medical, cultural and social history surrounding this disease at those times. A significant example is shown in fig. 2 but also in more recent times, terms such as lunatism (a disorder caused by the phases of the moon), daemonic suffering (brought about by evil spirits) or the scourge of Christ, were used, among many others, to define epilepsy.
Fig. 2. The origin of the ancient Egyptian name for epilepsy, *nesejet*

Sources: H. Schneble, Krankheit der ungezählten Namen, Huber-Verlag Bern, 1987, p. 9-11; German Epilepsymuseum Kork - Museum for epilepsy and the history of epilepsy
In the last two centuries, scientific progress and dissociation between Science and the dominant culture has yielded fundamental insights towards a better, though still limited, understanding of the intrinsic nature of this neurological disorder. Although the medical treatments of many patients affected by epilepsy have improved in the last half-century, from the point of view of medical sciences epilepsy still remains an unresolved matter and still represents one of the most common neurological disorder affecting about 0.5 to 0.8% of worldwide population.

The Greek word from which the term epilepsy comes from, epilambanein, (to seize upon – to attack unexpectedly) describes the main feature of such a neurological disorder well. Thus, epilepsy is a disease that causes seizures to occur. Often, aetiologies are well-determined, such as stroke or head injury, but in a high percentage of cases they can be unknown.

The singular term epilepsy is not appropriate to describe what actually is a cluster of disorders rather than a single disease, hence the plural term epilepsies is the most correct, (see tab. 1 and 2 for official classifications of epilepsies and seizures). Nonetheless, the singular term is still in use among neurologists and so it will be in this work as well, unless differently specified.
Table 1: Classification of epileptic seizures

Partial (focal, local) seizures

1- Simple partial seizures (consciousness not impaired)
   - With motor signs (ex: facial motor, vocalisation, arrest of speech..)
   - With autonomic symptoms (ex: epigastric sensation, piloerection..)
   - With somatosensory symptoms (ex: visual, vertiginous..)
   - With psychic symptoms (ex: hallucinations, affective-fear, anger..)

2- Complex partial seizures (with impairment of consciousness)
   - Simple partial onset followed by impairment of consciousness
   - With impairment of consciousness at onset
   - With simple partial features (AI-4) followed by impairment of
     consciousness
   - With automatisms

Generalised seizures (convulsive or nonconvulsive)

1. Absence seizures
   - Impairment of consciousness only
   - With mild clonic components
   - With atonic components
   - With tonic components
   - With automatisms

2. Atypical absence
   - Myoclonic seizures
   - Clonic seizures
• Tonic seizures
• Tonic-clonic seizures
• Atonic seizures

3. Unclassified epileptic seizures

Correspond to all seizures that cannot be classified according to the descriptions above, due to incomplete or inadequate data.

Table 2: Classification of epileptic syndromes

Localisation-related (focal, local, partial) epilepsies and syndromes

- Idiopathic with age-related onset
- Benign childhood epilepsy with centrotemporal spike
- Childhood epilepsy with occipital paroxysms
- Symptomatic

- Syndromes are of great individual variability, mainly based on the anatomical localisation. Examples are:
  - Frontal lobe epilepsies (ex: motor cortex)
  - Temporal lobe epilepsies (ex: Hippocampus – mesiobasal limbic)
  - Parietal lobe epilepsies
  - Occipital lobe epilepsies

Generalized epilepsies and syndromes

A. Idiopathic with age-related onset

- Benign neonatal familial convulsions
- Benign neonatal convulsions
- Benign myoclonic epilepsy in infancy
- Childhood absence epilepsy
- Juvenile absence epilepsy.
- Epilepsy with "grand mal" seizure on awakening

Other generalized idiopathic epilepsies, if they do not belong to the above syndromes, can still be classified as generalized idiopathic epilepsies.
Idiopathic and/or symptomatic, in order of age of appearance

- West syndrome
- Lennox-Gastaut syndrome
- Epilepsy with myoclonic-astatic seizures
- Epilepsy with myoclonic absences

Symptomatic

- Non-specific aetiology (early myoclonic encephalopathy)
- Specific syndromes (several aetiologies can be included here)

Epilepsies and syndromes undetermined as to whether they are focal or generalized

with both generalized and focal seizures

- Neonatal seizures
- Severe myoclonic epilepsy in infancy
- Epilepsy with continuous; spike waves during slow-wave sleep
- Acquired epileptic aphasia (Landau-Kleffner syndrome)
- Without unequivocal generalized seizures (many cases of sleep grand-mal)

Special syndromes

- Situation-related seizures (ex: febrile convulsions, sleep deprivation, drugs..)
- Isolated unprovoked epileptic events

According to the classification and Terminology of the International League Against Epilepsy. Proposal for revised clinical and electroencephalographic classification of epilepsies and epileptic syndromes (Epilepsia, 1989, 30:389-399)
Epilepsy can arise at any age and the mechanisms leading to epileptogenesis are still unknown. At the cellular level, epileptic tissue comprises neurons whose pattern of discharge depends on intrinsic and/or environmental alterations, giving rise to a peculiar neuronal firing named as burst. Burst is a functional consequence of what is called the paroxysmal depolarizing shift (PDS), this feature being considered as the hallmark of epilepsy from the electrophysiological point of view. Since neurons within epileptic tissue are particularly prone to fire in bursts, and as such a pattern of discharge requires a prolonged state of depolarization of the membrane potential (PDS), nerve cells involved in seizure generation have been classified as hyperexcitable neurons.

Hence, an old common definition of epilepsy stated that such neurological disorder is characterized by excessive discharge of cerebral neurons due to their intrinsic hyperexcitability. This definition has been somewhat misleading since it focuses attention mainly, if not exclusively, on the abnormal behaviour of the individual neuron. Indeed, speaking of a single epileptic neuron is meaningless since a seizure is the macroscopic manifestation of a synchronizing mechanism involving broad neuronal populations. Thus, nowadays, although research on epilepsy has been focused on the properties of neuronal networks with greater attention than that it used to be, significant knowledge is still missing.

From a clinical perspective, seizures are associated with a variety of manifestations, spanning from auras to behavioural convulsions, often accompanied by a loss of consciousness, depending on which cerebral areas (and to what extent) are actively involved in seizures.
A simplified classification of clinical epilepsies is depicted in Fig. 3, according to the International Classification of Epilepsies and Epileptic Syndromes (tab. 2) which, despite current advancements in knowledge, has not been amended yet, although suggestions for more clinically practical and updated classification (and related glossary) have already been proposed. In Fig. 3 terms as cryptogenic and localization-related are preserved despite, nowadays, their inappropriateness. Nonetheless, these terms and others are still common among neurologists, and are therefore adopted in this discussion.

Generally, two main categories of epilepsies can be sketched out (Fig. 3), idiopathic and symptomatic, being cryptogenic a synonym of “symptomatic with unknown aetiology due to technical limitations of available diagnostic tools”.

Both categories can be subdivided in generalized and localization-related clusters, depending on the origin and the extent of the cerebral areas involved in seizure propagation. Symptomatic localization-related epilepsies constitute a prominent group which comprises epileptic syndromes originating from cerebral lesions as brain tumours, traumatic scars, vascular malformations, dysplasia, etc...(often affecting neocortex and named lesional partial epilepsies) and cryptogenic partial epilepsies as well.

Of particular relevance among symptomatic localization-related epilepsies are those of temporal-lobe origin, usually referred to as Temporal Lobe Epilepsy (TLE) which represents the most common form of human epilepsy. In fact, in the majority of patients affected by epilepsy seizures have a localized onset and, among these patients, about one third actually present seizures originating from temporal lobe.
Fig. 3. Schematic classification of epilepsies.

- Idiopathic (primary)
  - age-related
  - familial
  - often benign

- Symptomatic (secondary)
  - pathological substrate

- Cryptogenic
  - no idiopathic
  - no symptomatic

- Generalized seizures
  - Localization-related
    - Partial seizures with or without secondarily generalized seizures

- Localization-related
  - Lesional Partial Epilepsy
    - Tumors, scars, etc..., Usually neocortical

- Mesial Temporal Lobe Epilepsy

- Symptomatic Epilepsies of Temporal-lobe origin

- Presumed to be symptomatic but technical limitations of available diagnostic tools are not able to identify the aetiology yet

- Unknown aetiology
TLE can be subdivided into three groups as well, within which definitions as *lesional TLE* and *cryptogenic TLE* preserve the same meaning as before.

Indeed, the most relevant and frequent epileptic syndrome of temporal-lobe origin is named *Mesial Temporal-Lobe Epilepsy* (MTLE) and it constitutes one of the major challenges that epileptologists have been facing as it represents one of the most diffuse and problematic forms of human epilepsies.

The clinical relevance of MTLE stems from the observation that patients affected by MTLE often experience at least one of the two main problems characterizing this form of epilepsy, i.e. refractoriness to antiepileptic drug (AED) treatment and structural abnormalities of specific brain areas leading to cognitive dysfunctions.

Refractoriness to AEDs affects about 25% of epileptic patients and, among them, 70% is diagnosed with MTLE. Antiepileptic drug resistance is commonly defined as lacking of seizure control when at least three appropriate drugs have been used at the maximum therapeutic doses. Drug resistance to AEDs is probably a multifactorial phenomenon (Sisodiya, 2003) and among several prognostic factors particularly relevant are structural abnormalities such as hippocampal and amygdaloid sclerosis, the former being a very common feature of MTLE which will be discussed in detail below. It should be stressed that, however, structural abnormalities, together with the totality of known prognostic factors, cannot account for drug resistance in clear terms of a cause-effect relationship.

Conversely, the relationship between structural abnormalities and cognitive impairments affecting people with MTLE is undoubtedly much clearer since patients often exhibit memory impairments as deficits in acquiring facts
and events related to their personal past (declarative memory) and strongly reduced performance in tasks related to spatial memory (Helmstaedter et al., 2001; Abrahams et al., 1999).

Also, these cognitive dysfunctions are strongly related to the damage of neuroanatomical structures involved in memory processes such as the hippocampus. The nature of this damage is well established since early studies in the XIX century and essentially relies on a pronounced cell death affecting (to varying degrees) principal and non-principal neuronal populations in the hippocampus. This peculiar pattern of cell loss represents the hallmark of the so-called hippocampal sclerosis which is the major histopathological finding frequently associated to MTLE.

Thus, neuronal cell loss (also referred to as neurodegeneration) often associated with MTLE appears to be involved in both resistance to AEDs treatment and cognitive impairments affecting patients. Indeed, 50% to 70% of patients with drug refractory MTLE present hippocampal damage and 30% to 60% are found with amygdaloid damage (Pitkänen, 2002; Engel, 1996). Hence, neuronal damage can be well regarded as a hallmark of MTLE, in particular hippocampal sclerosis where neurodegeneration is most prominent. As mentioned before, such structural abnormality was already known since XIX century from studies conducted by visual and microscopic inspection of brains post-mortem. At that time it was also observed that neurodegeneration often involves other cerebral areas as well as enthorinal cortex, amygdala and midline thalamic nuclei.

The aetiology of neurodegeneration still remains unknown. A basic question still largely unanswered concerning epileptogenesis is whether
structural abnormalities are already present at the time of the first spontaneous seizure or they appear later as a consequence of recurrent epileptic symptoms, or both. This applies in particular to hippocampal sclerosis. This topic has long been debated and, substantially, still remains controversial. On the one hand, surgical resection of sclerotic hippocampus is usually associated with a good outcome for seizure control/remission (thus pointing hippocampal structural alteration as a leading cause of seizure generation) while, on the other hand, recent high-resolution magnetic resonance imaging has repeatedly revealed that seizures can induce hippocampal atrophy per se, thus suggesting hippocampal sclerosis is a consequence of recurrent seizures. Nowadays, a growing amount of evidence has been accumulating in support of the latter hypothesis. Many studies have repeatedly shown that intense and prolonged seizures involving the limbic temporal-lobe can induce neuronal damage, in particular of some neuronal populations in the hippocampus.

A partial explanation, although still hypothetical, conciliating seizures, hippocampal atrophy and epileptogenesis involves an initial insult to hippocampus, as it is known to occur following prolonged febrile seizures and/or status epilepticus. Then, after a silent period while structural alterations appear to take place, spontaneous and recurrent seizures occur, that is, the patient becomes affected by epilepsy with all its possible consequences, such as refractoriness to AEDs treatment and cognitive dysfunctions. Usually, the origin of seizures is the hippocampus.

Whether the risks/benefits ratio is considered acceptable, surgery is undertaken. However, it should be stressed that in the majority of cases (80%), operated patients do not become really seizure-free, but they reach an
acceptable level of responsiveness to common AEDs, thus keeping seizures under a pharmacological control which was previously impossible to achieve before surgery. The number of real seizure-free patients is variable and often depends on the underlining pathology associated with epilepsy. However, it never reaches 100%. For instance, 60% of patients affected by some forms of cortical dysplasia become seizure-free after surgery and this is considered a good outcome. Moreover, total or partial resection of hippocampus, while not improving memory dysfunction, is always associated with an increased risk of post-surgery anterograde amnesia which can be severe and permanent.

Hence, understanding the causes leading to neurodegeneration is an absolute priority since neuronal cell loss appears to be the substrate of a series of severe clinical problems associated to MTLE, which are difficult, often impossible, to manage.

Among the possible, hypothetical, causes of neurodegeneration associated with seizure occurrence, the involvement of pro-inflammatory cytokines and related molecules has been gaining increasingly greater attention. In particular, clinical evidence exists of inflammatory markers in the brains of epilepsy sufferers and experimental work (using animal models of seizures and human MTLE) demonstrates that tissue expression of inflammatory markers increases during seizures, following a time-course that can be correlated to the onset of neuronal damage.

The subsequent chapters will deal in greater detail with the many aspects of this topic, adding novel information from experiments undertaken using animal models of seizures.
Before entering the core of this work, it is of interest to see fig. 4 which shows famous people who had (or were presumed to have) epileptic seizures during their life or who suffered of a chronic form of epilepsy for many years. Thus, from fig. 4, it may be inferred that the social and political decline of some famous people could be plausibly explained in terms of neurological impairment arisen as a consequence of what, nowadays, it is known as epilepsy-related neurodegeneration.
Fig. 4. Famous people who had epileptic seizures at a certain stage of their life or who suffered of a chronic form of epilepsy for many years (German Epilepsymuseum Kork - Museum for epilepsy and the history of epilepsy)

- Vincent van Gogh: Dutch painter
- G. Julius Caesar: Roman Statesman
- Gustave Flaubert: French Writer
- F.M. Dostoyevsky: Russian Writer
- Saint Paul: Apostle
- Alexander the Great: Macedonian King
- Socrates: Greek Philosopher
- Napoleon Bonaparte: French Emperor
- Pius IX.: Pope
- Vladimir Ilyich Lenin: Russian Revolutionist
- Alfred Nobel: Swedish Chemist
- Lord Byron: English Poet
Cardinal Richelieu
French Statesman

Hermann von Helmholtz
German Physicist

Joan of Arc
French Saint

Molière
French Playwright
1.2 Mesial temporal lobe epilepsy with hippocampal sclerosis

1.2.1 Clinical signs, symptoms and therapeutic intervention in human

Key clinical features of mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS) are characteristic aura, arrest, alteration of consciousness (and amnesia) and automatisms (Weiser, 2004).

The most common aura, usually occurring in isolation, is in the form of epigastric sensation (40 to 70% of patients, depending on the diagnostic tools such as MRI or histopathology by which HS has been proven) often evolving into ictal oral or manual automatisms, while, to a lesser extent, olfactory-gustatory auras may occur. Up to 50% of patients experience emotional auras which trigger fear and anxiety and autonomous-vegetative auras such as widened pupils, palpitations, arrhythmia, etc may occur as well. In children, abdominal pain associated with fear is more frequent. It is interesting to note that about 20% of patients who were rendered seizure-free after resection of sclerotic hippocampus still have persistent auras (Fried et al., 1995), this finding suggesting that in patients with HS an anatomic dissociation may occur between seizures and auras.

Alteration of consciousness is frequently observed to varying degrees, ranging from preservation to loss of consciousness and, generally, it is assumed...
that the more severe the alteration of consciousness, the larger the ictally perturbed brain volume will be.

Although non-specific for MTLE with HS, aphasia/anomia may occur during the ictal as well as the postictal period. Also automatisms can happen during ictal and postictal periods as well; usually, oroalimentary ictal automatisms prevail over gestural or manual automatisms.

On seizure spread to suprasylvian brain areas, positive motor symptoms may occur ranging from contralateral, predominantly clonic (or clonic/tonic) facial-brachial motor symptoms to secondary generalized motor symptoms with a clonic-tonic-clonic pattern.

In the postictal period, some degree of dysfunction is always present. The deficits most frequently observed are cognitive impairments, memory deficits, mood changes and language deficits.

In general, neuropsychological examination of patients affected by MTLE with HS reveals specific memory impairment and, often, additional general intellectual impairment, although the latter is a non-specific symptom for this pathology. The impairment of long-term memory consolidation or retrieval of newly acquired information (episodic memory) are the most typical types of cognitive dysfunction. In this contest, material-specific memory impairments develop, depending on hemispheric language dominance and the affected hemisphere.

Memory impairments found in patients affected by MTLE with HS have been shown to be progressive and significant memory changes can be expected after an interval of about 30 years (Helmstaedter and Elger, 1999). This time interval has also been suggested for hippocampal neuron loss to
occur as a consequence of repetitive seizures (Helmstaedt and Elger, 1999; Mathern et al., 2002). However, it should be noted that HS can account for memory impairments in no more than 30% of patients affected by MTLE and additional determinants such as age at the onset of the lesions, medication frequency, severity of seizures, IQ, chronical age, etc should always be kept in consideration.

Among partial epilepsies, MTLE with HS is the most resistant to AED treatment for the majority of patients. However, a small percentage of subjects achieve good control over seizures and it is likely that prevalence/incidence of good responders is indeed higher than it used to be thought in the past. Interestingly, Özkara et al. (Özkara et al., 2004) reported that although both responder and non-responders to AEDs had significantly lower memory scores than control patients, no relevant differences were detected between the two groups of subjects thus showing that memory impairment in subjects affected by MTLE with HS was permanent and might be related to the direct effect of HS itself, independently from the rate of response to AED treatment.

Indeed, for AED resistant subjects, surgery still represents the most effective therapeutic intervention with highest rates of seizure-free patients, being generally graded as patient-group of outcome class I, according to Engel (Engel et al., 1993; Paglioli et al., 2004, TAB 3). However, a relevant percentage of operated patients (15-20%) still have convulsions after surgery and this percentage increases over time from surgical intervention (Paglioli et al., 2004). Seizures occurring after surgery can still be more or less disabling at varying degrees although, in the majority of cases, their frequency is reduced. However,
no worthwhile improvement or even seizure worsening can occur in a small percentage of subjects after surgery (outcome class III and IV, TAB 3).

Generally, the majority of operated patients are still administered with AEDs which usually have their efficacy increased due to total or partial resection of presumed-to-be-epileptogenic sclerotic hippocampus, alone or together with other limbic structures such as amygdala and temporal cortex as well.
Table 3: Engel’s classification of the surgical outcome

- **Class I: Free of disabling seizures**
  
  IA, completely seizure-free since surgery;  
  IB, non disabling simple partial seizures only since surgery;  
  IC, some disabling seizures after surgery, but free from disabling seizures for ≥ 2 years;  
  ID, generalized convulsions with antiepileptic drugs withdrawal only.

- **Class II: Rare disabling seizures (“almost seizure-free”).**
  
  IIA, initially free of disabling seizures but has rare seizures now;  
  IIB, rare disabling seizures since surgery;  
  IIC, more than rare disabling seizures after surgery, but rare seizures for ≥ 2 years;  
  IID, nocturnal seizures only.

- **Class III: Worthwhile improvement.**
  
  IIIA, worthwhile seizures reduction;  
  IIIB, prolonged seizure-free intervals amounting to more than half the follow-up period, but < 2 years.

- **Class IV: No worthwhile improvement.**
  
  IVA, significant seizures reduction;  
  IVB, no appreciable change;  
  IVC, seizures worse.
Neuropsychological evaluations of post operated patients have long established that surgery always increases the risk of additional memory impairment, with verbal memory most often affected, in particular in left-side operated patients (~25%), usually being this cerebral area the language-dominant hemisphere, whereas a worsening in visual memory often occur in right-side operated patients (~20%) (Paglioli et al., 2004; Gleissner et al., 2002; Lee et al., 2002).
1.2.2 Histopathology of hippocampal sclerosis in adult humans

Hippocampal sclerosis (HS), which often occurs unilaterally, is the most common lesional pathology identified in patients with TLE and, although it has been intensively studied over the last decades, its aetiology still remains elusive.

A general histological evaluation of resected tissue reveals the classical pattern of HS affecting about 70% of patients, with severe neuronal loss in CA1 area and the bordering, proximal, part of the subiculum, accompanied by more or less severe cell loss in the hilus. CA3 sector is also affected, whereas pyramidal cells of CA2 subfield are usually relatively preserved (Babb and Brown, 1987; Sloviter, 1994; Proper et al., 2000). The less frequent patterns of hippocampal neurodegeneration affecting the remaining patients comprise the so-called end-folium sclerosis (cell loss essentially limited to the hilus only), mild neurodegeneration involving about 30% of neurons in CA1 area with general sparing of hilar neuronal populations and, for a small percentage of patients, severe cell populations loss affecting all hippocampal subfields, CA2 area included. All patterns of neurodegeneration manifest, however, a prominent astrocytic gliosis (Thom et al., 2004).

Specific to HS is the architecture of the granule cell (GC) population. With the exception of end-folium sclerosis and generalized severe neurodegeneration, where a depletion of GCs occurs as well, the most evident pattern affecting this cell population is dispersion (Houser et al., 1990). This pattern of granule cell architecture is evident in ~40% of HS specimens and consists of the broadening of granule cell layer, in particular toward the
molecular layer of the dentate gyrus. In the majority of cases, the degree of
dispersion ranges from mild to severe, the latter being also detected together
with nests of GCs toward the hilus and, also, in ectopic locations within the hilus
or CA3 area (Thom et al. 2002). Less frequently, an extensive or localized
bilaminar pattern of the granule cell layer also occurs, this too with clusters of
GCs in the molecular layer.
1.2.3 Major morphological and immunohistochemical findings in adult human HS

Beyond stereotypical loss of pyramidal cells in CA1 and CA3 fields, and relative sparing of CA2 neurons and GCs, several non-principal cell populations are shown to be selectively involved in the neurodegeneration characterizing HS.

Thus, compared to autopsy controls and lesion-associated TLE specimens, hippocampal tissue from patients with HS presents a significant decrease of hilar mossy cells, a morphologically peculiar cell population resident in the polymorphic cell layer, that are known to be glutamatergic thus excitatory (Blümccke et al., 2000).

Generally, fascia dentata of HS specimens shows the highest number of cell populations involved at varying degrees in neurodegeneration and this has attract the attention of investigators since this hippocampal subfield is considered the "gatekeeper" to whole hippocampal circuitries. Thus neuropeptide-targeted immunocytochemical studies have shown a loss of hilar somatostatin (SOM) and neuropeptide Y (NPY) immunoreactive interneurons, usually subgranular-located, with a preferential involvement of SOM positive cells (Robbins et al., 1991; Mathern et al., 1995). About 30% of hilar cells present a double immunostaining for both SOM and NPY and, moreover, a subpopulation of these peptide-immunoreactive hilar cells have been shown to co-localized with glutamic-acid-decarbossylase (GAD), the enzyme responsible for γ-aminobutyric-acid (GABA) synthesis.
As far as GABAergic neurons are concerned, one of the most abundant hippocampal GAD-positive cell population is represented by basket-like interneurons which are located in all hippocampal subfields. These cells have been shown to be relatively preserved in specimens affected by HS as well as immunoreactivity reported from fibres and synaptic terminals, suggesting a GABA hyperinnervation on surviving cells. This finding, in a scenario of diffuse loss of cellular populations, make GABAergic interneurons to become proportionally abundant as compared to normal hippocampal tissue, thus suggesting an apparent increase in inhibitory neurotransmission (Babb et al., 1989; De Lanerolle et al., 1989; Mathern et al., 1995).

A relevant phenomena occurring in HS, also involving the same cellular populations directly involved in neurodegeneration, is reactive synaptogenesis also known as sprouting. This terms equally refers to arborisation of both axonal projections of surviving interneurons and axonal projections of GCs.

Sprouting of GCs axons (mossy fibres) is one of the most striking aberrant finding related to HS. The richness in Zn$^{2+}$ of mossy fibres synaptic terminals have made these axonal projections amenable to be easily marked by Timm’s staining (Danscher, 1981). Thus, whereas a normal hippocampus shows a dense staining in the hilus and CA3 area, corresponding to ordinary synaptic contacts of GCs axonal projections, a sclerotic hippocampus directly involved in seizures generation clearly shows a strong decrease of staining in fascia dentata and CA3 subfield, with a strong, peculiar, Timm-positive region located in the inner molecular layer of the dentate gyrus. These terminals were actually shown to make new synaptic contacts onto proximal apical dendrites of GCs and their immunoreactivity to synaptophysin (a calcium-binding
glycoprotein located in the membrane of neurotransmitter-containing small vesicles at presynaptic terminals) and presynaptic growth-associated protein (GAP-43, usually overexpressed in newly forming presynaptic terminals) suggests intrinsic synaptic activity of sprouted mossy fibres (Proper et al., 2000).

However, although GC axons sprouting appears to be one of the most prominent synaptic reorganization related to HS, other cellular populations show similar patterns of reactive synaptogenesis, here again almost exclusively located in the fascia dentata. Thus, enhanced immunoreactivity to SOM, NPY and substance-P (SP) have been particularly shown in dentate gyrus where electron microscope studies revealed new synapses deriving from sprouted axon terminals in the molecular layer (De Lanerolle et al., 1992), with SOM- and NPY-positive synaptic terminals mostly localized in the region of proximal apical dendrites of GCs (inner molecular layer, de Lanerolle et al., 2003).

Particular attention was paid to sprouting of GAD-positive fibres which were shown to hyperinnervate GC bodies and the full length of their apical dendrites, i.e. involving inner and outer molecular layer in GABAergic reactive synaptogenesis (Mathern et al., 1995; Babb et al., 1989). Also CA1 and CA3 areas of sclerotic hippocampus were shown to be involved in hyperinnervation of surviving pyramidal neurons by GAD-positive fibres which preferentially make significant new synaptic contacts on cell bodies and proximal apical dendrites, that is, GABAergic hyperinnervation is oriented toward stratum pyramidale and stratum oriens of these Ammon’s horn subfields which are affected by neurodegeneration as particularly reported to occur in CA1 (Babb et al., 1989).
The importance of intracellular Ca\(^{2+}\) concentration with respect to intracellular signalling and potential excitotoxic damage, has raised the question as to whether patterns of cell death and survival in HS could be explained in terms of alterations of buffering capabilities of intracellular calcium-binding proteins.

Thus, the expression of calbindin-D28k, normally detected in principal and non-principal cell populations of all hippocampal subfields with the exception of CA1 pyramidal cells, was shown to significantly decrease in GCs whereas interneurons appear generally preserved although morphological alterations make them actually different from comparative control specimens (Maglóczky et al., 1997; Sloviter et al., 1991).

A different pattern of degeneration affects cells expressing parvalbumin (PV), a calcium-binding protein exclusively localized in non-principal neurons. In control tissue, PV is usually detected in interneuronal populations throughout all layers of hippocampal areas, whereas in HS specimens PV immunoreactivity shows a generalized decrease in Ammon's horn subfields and a strongly pronounced loss of PV-positive cells close to pyramidal neurons in fascia dentata (Arellano et al., 2004; Zhu et al., 1997; Sloviter et al., 1991).

Another calcium-binding protein, calretinin (CR), is frequently used as a marker for a cell population morphologically identifiable as Cajal-Retzius like, although CR is not specific for these cells since it is also present in interneurons where was shown to be co-localized with GABA. Cajal-Retzius cells, neurochemically characterized by p73-immunoreactivity (Abraham et al., 2003), have attracted the attention of investigators since they represent the major releasing source of the extracellular matrix protein reelin, which is known to play
a fundamental role in neuronal migration and targeting during development (Mienville, 1999). This cell population, commonly resident in all hippocampal subfields and in particular in the molecular layer of dentate gyrus and in stratum granulosum-moleculare, is known to strongly decrease in number as development approaches maturation. In HS specimens it was shown a higher persistent number of these cells as compared to control tissue as well as tissue resected from patients affected by TLE with extrahippocampal origin. This finding has been suggested to be related to GC dispersion as a sign of a pregressed hippocampal maldevelopment as a potential factor contributing to the establishment of HS as a malformation per se not directly related to seizures occurring (Thom et al., 2002; Blumcke et al., 1996).

Considering the morphology of surviving cell populations, it has been shown that, in up to half of patients, cytoskeletal abnormalities are generally prominent, for instance, in residual hilar neuronal populations. Morphology is altered, showing enlargement and/or ballooning of cell bodies and processes at varying degrees, accompanied by extensive dendritic ramifications. The number of mitochondria is usually increased. Morphological alterations are actually confirmed by specific immunohistochemistry for structural proteins. Thus, immunostaining for neurofilament proteins reveals accumulation of both phosphorilated and non-phosphorilated neurofilaments, both in cell bodies and dendritic processes (Blümcke et al., 1999). Microtubule-associated protein 2 (MAP2) immunostaining which appears prominently in molecular layer projecting apical dendrites of granule cells is also increased (Thom et al., 2002).

Glial cells are always involved in HS mainly in terms of activated microglia and reactive astrocytic gliosis which are particularly prominent in the
hippocampal subfields mostly involved in neurodegeneration (CA1 and DG), whereas in CA3 and CA2 they appear to a lesser extent. Indeed, an increased number and size of astrocytes can be shown by immunoreactivity to GFAP, thus confirming a strong proliferation and replacement of dead principal and non-principal cells by astrocytes, with frequently observed radial infiltrates of glial fibres among surviving pyramidal and granular cell populations (Thom, 2004).
1.3 Seizures and epilepsy in infants and children

The incidence of epilepsy is approximately 5-7 cases per 10,000 children from birth to age 15 years, and in any given year, about 5 of every 1,000 children. Increasing risk factors include congenital malformations of the central nervous system (CNS), moderate and severe head trauma, CNS infections, certain inherited metabolic conditions and genetic factors. However, these factors are not able to account for more than 45% of occurring epilepsies thus leaving the remnant cases without any identifiable aetiology (Cowan, 2002).

Although epilepsies in infants and children can be still classified according to the official classification, i.e. idiopathic, symptomatic and cryptogenic, a re-clustering of ictal disorders in the following four groups probably better characterizes the occurrence of seizure disorders in infants and children, that is i) symptomatic epilepsies, ii) epileptic encephalopathies, iii) febrile seizures and iv) seizures of temporal lobe origin.

As well as in adults, symptomatic epilepsies originate from injuries and/or acute insults induced by underlying neuropathologies as cerebral malformations, tuberous sclerosis or acute insults as hypoxia. Differently from adults, as a general peculiarity, status epilepticus (SE) is more likely to occur in infants and children in response to neuropathologies and/or acute insults and it is known to often anticipate epilepsy in 30-75% of children, in particular when afebrile and drug refractory (Maytal et al., 1989; Sahin et al., 2001).

Epileptic encephalopathies represents a cluster of disorders in which cognitive, sensorial and/or motor functions deteriorate as a consequence of
epileptic activity which consists of frequent seizures and/or so-called interictal paroxysmal activity (Nabbout et al., 2003). Electroclinical features yield to a subdivision of these disorders in i) epileptic encephalopathies produced by frequent and/or severe seizures such as Dravet syndrome and Rasmussen's encephalitis (both occur in infants within the first year of life) and ii) epileptic encephalopathies with continuous or nearly continuous spike and slow wave activity such as West syndrome in infants and Lennox-Gastaut syndrome in age-school children. Generally, epileptic encephalopathies are age-related syndromes whose pathophysiology is largely unknown, although sometimes they can be partially accounted for by structural and genetic alterations.

Febrile seizures are common ictal events affecting infants and children older than 1 month, originating from febrile illness (body temperature > 38.5°C) without CNS infections and/or metabolic imbalances. Usually, they represent the very first ictal event thus without any afebrile seizures occurring beforehand. They can be short lasting seizures or febrile SE, although the latter being more common when neurological dysfunctions are present.

The clinical seizure semiology in infants and children affected by epilepsy of temporal lobe origin is different as compared to adults and adolescents.

Generally, with regard to the aspects of seizures of temporal lobe origin in infancy and childhood, a distinction can be made between young children aged less than 3 years and older children. The main differences characterizing seizures in infants and young children can be summarized as i) a predominance of behavioural arrest, ii) no identifiable auras before the age of 5 years, iii) the automatisms are predominantly oroalimentary and less gestural and/or purposeful, iv) seizure duration is substantially longer and more convulsive with
tonic, clonic and myoclonic activity, v) from the perspective of electrophysiology, interictal EEG discharges are less frequent and ictal discharges may be less focal (Yamamoto et al., 1987; Duchowny, 1987). The impairment of consciousness often observed in adults as a feature of complex partial seizures is difficult to evaluate in infants and young children (aged < 3 years). An attempt at measuring the degree of altered consciousness was made by Duchowny (Duchowny, 1987) who observed that infants did not react to visual threat or loud noise during seizures, thus concluding that also in infants consciousness was impaired at least in part. Interestingly, histopathology reveals that structural abnormalities associated with seizures of temporal lobe origin mostly consist of dysplasias, migrational disorders, hamartomas and low-grade tumors such as gangliogliomas. Differently from what is encountered in adult and adolescent epilepsy sufferers, a neurodegenerative pattern of mesial temporal sclerosis has a reduced prevalence, ranging from 15 to 40%, often detected together with aforementioned structural alterations thus ascribing a dual-pathology pattern which occurs more frequently in children than in adults (Murakami et al., 1996; Duchowny et al., 1992; Wyllie et al., 1993). Generally, several studies have shown that the older the children the higher the prevalence of mesial temporal sclerosis among neuropathological findings, as assessed both by histopathology of surgical specimens (Mizrahi et al., 1990; Duchowny et al., 1992; Wyllie et al., 1993; Kuzniecky et al., 1993; Bourgeois, 1998; Bocti et al., 2003) and by MRI studies which show that although HS can also be detected in children as aged as 2 years, the incidence of this neuropathological finding remains very low as far as the age is less than 5 years (Cross et al., 1993; Harvey et al., 1995; Harvey et al., 1997; Murakami et al., 1996).
1.3.1 Ictal events in infants and children as predisposing risk factors for MTLE development in adulthood

Studies in humans have not yet provided any clear relationship between MTLE in adulthood and any epileptic-like activity occurring in infancy and early childhood, the latter evaluated in terms of being either an epiphenomenon or a causative factor. Generally, investigations on epilepsy in the field of pediatric neurology are affected by the question of whether ictal events occurring in infancy and early childhood are a cause of structural abnormalities such as HS, or the consequence of an already damaged cerebral structure that should be ascribed as the pathological substrate for seizures. Information available from human studies on the evaluation of febrile seizures as a predisposing risk factor for MTLE to develop in adulthood ranges from data showing the high proportion of patients with HS who had experienced prolonged febrile seizures in early childhood (Abou-Khalil et al., 1993; Cendes et al., 1993 (b); Kuks et al., 1993) as well as data showing a lack of any relationship in long-term follow-up (Tarkka et al., 2003). The association between febrile SE and the severity of hippocampal atrophy in patients with MTLE is better defined. Thus a history of febrile SE correlates positively with the degree of HS (Cendes et al., 1993 (a)) and children with long lasting febrile seizures, such as SE, were shown to have a higher incidence of MTLE than siblings (even twins) with short lasting febrile seizures. This supports the hypothesis that seizure duration may be directly related to the development of epilepsy in patients with common genetic background (Jackson et al., 1998).
Generally, the diversity of early childhood as compared to adults is also manifested in the different prognostic significance of the occurrence of status epilepticus (SE), both in association with febrile illnesses (see above) and as a consequence of more or less manifested neurological alterations. In adult patients, SE usually occurs following a neurological insult resulting in a lesion or underlying pathology, have a relatively high mortality rate (Wu et al., 2002) and it was shown to be the first manifestation of epilepsy in about 10% of adult patients (Janz, 1983). Conversely, afebrile SE in childhood, often precedes the onset of epilepsy (30-75% of cases), although a first seizure rarely presents as SE in the absence of a neurological insult (Maytal et al., 1989; Aicardi et al., 1970). Mortality induced by SE is lower in children compared to adult patients although children are more prone to manifest SE, in particular when the brain is compromised such as in cerebral malformations (Sisodiya, 2004) and cerebral palsy (Gururaj et al., 2003). Although common in infants (the incidence of both febrile and afebrile SE is higher in the first year of life) as well as in people aged over 60 years there are no studies at present that definitively prove SE as a source of permanent injury to the brain, since prospective data in human beings is still missing. Thus, SE in children may be an ictal event triggering HS that will boost MTLE in adulthood as well as a consequence of a pre-existing pathological substrate that will progress towards hippocampal atrophy the more the age of children approaches adolescence (Haut et al., 2004; Sutula et al., 2003; Berg and Shinnar, 1997).

It should be emphasized, however, that data so far collected from human beings are retrospective, thus making separation of seizure-induced pathology from the cause of the seizure difficult.
1.3.2 Experimental studies on the susceptibility to seizure-induced damage of immature as compared to adult brain

The major limitation of human studies is the lack of prospective data as patients undergoing diagnosis for the very first time have usually suffered a seizure already. Hence, brain condition before the ictal event is actually unknown. Experimental models of SE as well as epileptogenesis, resembling human pathology, are therefore important for elucidating basic mechanisms. In particular, animal models of SE are well known to be associated with an age-dependent onset of neuronal injury, hence making prospective studies possible.

As in humans, investigations exploiting experimental models have focused on evaluating structural and functional alterations.

Anatomical consequences of SE

A pattern of neurodegeneration resembling human MTS occurs in adult rats treated with kainic acid (KA) and pilocarpine or undergoing continuous electrical stimulation of the hippocampus or the amygdala in order to trigger a SE originating from limbic structures. Thus, neuronal loss appears in CA1, CA3 and hilus of DG, with neurodegeneration involving extrahippocampal areas as well (Ben-Ari, 1985; Turski et al., 1986; Sloviter, 1994; Nadler, 1981). The extent of damage is positively correlated with SE duration and cell death can be detected as early as 2 h, lasting up to 2 months (Ben-Ari, 1985; Covolan et al.,
2000), and accompanied by synaptic reorganization (Nadler, 1981; Yang et al., 1998 (a); Haas et al., 2001).

Conversely, although immature rats are more prone to seizures and show a decreased threshold to epileptogenic stimuli as compared to adults (Pitkanen et al., 2002 (b); Cavalheiro et al., 1987), the neuronal damage affecting limbic structures, as well as synaptic reorganization, are absent or little expressed. They are usually undetectable until rats are aged ≥ 3 weeks (Haas et al., 2001; Cavalheiro et al., 1987; Nehlig et al., 1996; Sperber et al., 1999; Wasterlain et al., 2002). However, SE early in life may prime the brain to the harmful effects of subsequent insults as seizure-induced brain injury in adulthood (Koh et al., 1999).

**Association of SE with development of epilepsy**

In adult rats, induction of SE by administration of KA leads to spontaneous seizures in 2-3 months, as it also occurs after SE induced by electrical stimulation (Wasterlain et al., 2002; Stafstrom et al., 1992; Gorter et al., 2003).

In immature rats, spontaneous seizures subsequent to KA-induced SE do not occur until animals are aged ≥ 3 weeks, and seldom occur in lithium-pilocarpine model of SE (Wasterlain et al., 2002). At postnatal (PN) 21 it was shown that spontaneous seizures can occur in 10-73% of immature animals as compared to 100% of adults (Wasterlain et al., 2002; Stafstrom et al., 1992; Roch et al., 2002).
Anatomical consequences of febrile SE and the development of epilepsy

A peculiar pattern of neuronal damage affects immature rats undergoing hyperthermia-induced seizures, a model of febrile seizures in humans. Hippocampus and amygdala show acute neuronal damage which appears to be transient as no long-term decrease of neuronal number or neurogenesis is detectable. However, the density of mossy fibres increases in adulthood as well as the likelihood of seizures (Baram et al., 2002; Bender et al., 2003).

Brief seizures and seizure-induced damage

In the kindling model of epileptogenesis in adult rats, it was shown that a single electrical stimulation of the hippocampus (resulting in a long-lasting electrographical afterdischarge without behavioural seizures) slightly increases apoptosis in the DG (Bengzon et al., 1997). Repeated seizures cause cumulative damage in adult affecting the hippocampus and accompanied by synaptic reorganization, shown exploiting experimental models in which brief single seizures where induced by pentylenetetrazole, electroshock and kindling (Haas et al., 2001; Pitkanen et al., 2002 (a); Bengzon et al., 1997; McCabe et al., 2001; Lukoyanov et al., 2004).

In immature brains, the general trend confirms that despite having more severe seizures, neuronal damage is often absent or mild compare to that observed in adult animals. This has been shown for kindling and tetanus-toxin induced recurrent seizures as well. It is interesting to note that in both these models susceptibility to seizures persists in adulthood despite the lack of any
detectable neuronal loss in immature brains (Haas et al., 2001; Lee et al., 2001).

Similar observations were made also for recurrent neonatal seizures (Holmes et al., 2002) while brief hyperthermic seizures in early life may or may not increase seizure susceptibility in adulthood (Chang et al., 2003; McCaughran et al., 1982).

Overall, the available data converge to point out that seizure-induced injury is age-dependent and the most convincing evidence comes from animals studies as data collected from human beings are retrospective, thus making the discerning of seizure-induced pathology from the cause of the seizure difficult. Experimental studies strongly suggest that although immature brains are highly prone to develop seizures and SE, they are more resistant to seizure and SE-induced damage than adult brains, in particular as concerns the development of a neurodegenerative pattern as MTS.
1.3.3 Current hypothesis for the resistance of the immature brain to SE-induced changes

The mechanisms underlying the decreased vulnerability of immature brain to seizure-induced neuronal death remain unknown. Some hypothesis have been proposed, supported by experimental evidence.

Overall, the leading hypothesis towards all experimental investigations have been related to a reduced sensitivity of the immature brain to excitotoxic damage. Indeed, both immature and adult animals release equivalent amount of glutamate following SE (Liu et al., 1997), therefore, any plausible hypothesis should contemplate a different ability of immature brain to manage the toxic load carried by excessive glutamate release (excitotoxicity) such as the huge increase of Ca\(^{+2}\) influx.

An early investigation (Friedman et al., 1997) was aimed to evaluate the expression, in terms of both mRNAs and protein levels, of three subunits involved as assembling monomers in the ionotropic receptors for AMPA, namely GLUR1, GLUR2 in particular and GLUR3.

The presence of subunit GLUR2 confers the heteromeric AMPA receptor with impermeability to Ca\(^{+2}\) influx and it is known that most of the principal neurons of the adult hippocampus contain this subunit in their AMPA receptors, thus making them Ca\(^{+2}\) impermeable. In adult rats, SE has been shown to reduce the expression of GLUR2 mRNA in CA3 pyramidal neurons at times preceding significant cell loss (Pollard et al., 1993; Friedman et al., 1994 (b)), thus suggesting that a decrease of GLUR2 expression yields to an increased formation of Ca\(^{+2}\) permeable AMPA receptors in CA3 neurons and possibly
contribute to the delayed hippocampal neuronal cell loss following kainate-induced seizures.

The hypothetical age-dependent effect of SE on GLUR2 as well as GLUR1 and GLUR3 was tested by triggering SE in PN5 and PN14 rats, then comparing results with those obtained in adult rats following KA administration (Friedman et al., 1997). In the same experimental session the authors also investigated the expression of mRNAs of NMDA receptor (NMDAR1) and α1-subunit of GABA<sub>A</sub> receptor.

In PN5 animals, no significant changes in glutamate or GABA<sub>A</sub> receptor mRNA was detected in CA1, CA3 and DG at 24 h after the induction of SE, whereas a marked increase of GLUR1 and GLUR2 mRNAs were shown to occur in DG, but not in the other hippocampal subfields, in PN 14 rats. However, these changes in mRNA expression were transient, then approaching the respective basal level in about 1 week. Protein levels of AMPA receptor subunit where measured by immunohistochemistry in PN 14 rats, i.e. in the age-group where mRNA expressions were shown to be increased after SE. No detectable changes for any subunit studied were shown in CA3 subfield at 24 and 48 h after induction of seizures. Also the DG did not show any increased level for all subunits studied. Therefore, despite the transient higher level of expression of GLUR1 and GLUR2 mRNAs induced by SE, corresponding protein levels were not affected. Thus, conversely to reports from adult animals where KA-induced SE was shown to significantly decrease both mRNA and protein level of GLUR2 in the vulnerable CA3 hippocampal subfield, in young rats aged PN 5 and PN 14 levels of expression of both mRNA and respective GLUR2 protein are still sustained and preserved after SE in CA3, hence giving support to the
hypothesis that CA3 pyramidal cell resistance to neurodegeneration in young animals might be due to a diffuse presence of GLUR2 subunit among the monomers making up AMPA receptors. Also in DG, the unaltered expression of protein GLUR2 is in accordance with the resistance of this hippocampal subfield to neuronal cell loss in pups and, also, in adults since it was shown that SE induces a prolonged enhancement of GLUR2 mRNA expression in DG thus supporting the potential protective role of this subunit to neurodegeneration in this age-group as well (Friedman et al., 1994 (a)). The authors also paid attention to age-dependent effect of SE on GABAergic system since GABA represents the major inhibitory neurotransmitter in the CNS, hence, it is of particular relevance to counteract the excess of excitation occurring during seizures. Thus, the observation of unchanged levels of mRNA expression of α1-subunit of GABA<sub>A</sub> receptor in PN 5 and PN 14 rats after KA-induced SE (Friedman et al., 1997), has prompted the authors to hypothesize that the sustained expression of this subunit may contribute to reduce the vulnerability of developing CA3 neurons to seizure-induced damage. To support this, in adult animals SE was shown to decrease the mRNA expression in the same subfield at times preceding the occurrence of neuronal cell loss (Friedman et al., 1994 (a)). However, immunohistochemistry for α1-subunit was not performed.

More recently, the electrophysiological properties and the subunit composition of GABA<sub>A</sub> receptors localized on GCs in PN 10 rats that underwent lithium-pilocarpine administration have been investigated (Zhang et al., 2004) thus attempting to integrate the early results reported by Friedman et al, described above. In this study it was shown that unlike rats experiencing SE as adults, PN 10 rats present alterations affecting GABA receptors that are much
less pronounced and in the opposite direction compared to those seen in the adult rats. In particular, the mRNA expression of the α1-subunit was shown to be increased two-fold compared to control. This was the only change detected in pups by this group of investigators concerning the effect of SE on subunit mRNA expression. This result is somewhat different from that reported by Friedman and colleagues, where no change in mRNA expression of α1-subunit was detected. In contrast, this study confirmed that in adult animals SE decreases α1-subunit expression, whereas significantly increases subunits α4 and δ. Electrophysiological investigation revealed that the application of type I benzodiazepine agonist, such as zolpidem, elicited a higher level of GABA-induced current. As GABA receptors made of at least one α1-subunit are known to be more sensitive to type I benzodiazepine agonist and since α1 was the only subunit to show a significant increase in relative expression after SE induced in PN 10 rats, it appears that the greater zolpidem augmentation of GABA currents in GCs in young animals most likely reflects an increased number of α1 containing GABA_A receptors on the membrane of these cells, hence a subsequent increase of inhibitory current on GCs following SE.

The effect of SE on GABAergic system was also investigated by determining the rate of synthesis of GABA at different ages. The rate of synthesis of GABA in immature rat hippocampus increases during normal development. During SE induced by lithium-pilocarpine administration, the rate of GABA-synthesis was shown to be significantly lower as compared to control rats, in all age-groups ranging from 1 to 4 weeks (Sankar et al., 1997). This trend was also observed in adult rats (Wasterlain et al., 1993), together with a consistent reduction (40-45%) in GABA release from slices collected from rats.
undergoing KA-induced SE (Arias et al., 1990). The ratio expressed as a percentage of GABA synthesis during SE to that under basal conditions was taken as a measure of the capability of the hippocampus to maintain GABA synthesis under conditions of metabolic stress. Thus, in the hippocampus of 1-week-old rats, the GABA synthesis rate was shown to have declined from 74.5\% to 44.1\% in 4-week-old animals (Sankar et al., 1997). Since the basal rate of GABA synthesis increases with maturation, it appears that only a progressively smaller fraction of the basal rate can be maintained in the face of SE, suggesting a relative collapse in inhibition. The authors speculated that the resistance of immature hippocampus to SE-induced neuronal cell death as well as to mossy fibre sprouting may be related to the capability of the developing hippocampus to maintain high levels of GABA in the face of SE compared to basal levels.

Thus, although with slightly different results, all data reviewed so far converge onto a persistent functionality of the GABAergic system in pups with no corresponding system in adults.

Other hypothetical mechanisms that might account for the resistance of immature brain to SE-induced neurodegeneration focus on mitochondrial functionality.

Linked to intense neuronal excitation is the production of reactive oxygen species (ROS) in mitochondria that may lead to insurgence of oxidative stress. Oxidative stress is defined as an imbalance into which production of ROS overwhelm endogenous antioxidant defences and repair. An important mediator of oxidative stress is the superoxide radical $\text{O}_2^-$ which is known to both directly oxidise minor cellular targets and, most importantly, constitute the precursor of
much more powerful reactive species such as hydroxyl radicals OH$^-$ and peroxynitrite ONOO$^-$. The intracellular concentration of O$_2^{-}$ is usually kept at a constant level by superoxide dismutases (SODs) which are located in the cytoplasm, mitochondria and extracellular compartment. These enzymes catalyze the conversion of O$_2^{-}$ into H$_2$O$_2$ and O$_2$ at a rate which approaches the diffusion limit.

It has been shown that in adult rats the production of mitochondrial superoxide radical increases following KA-induced seizures, at times preceding the death of vulnerable neurons affected in the subsequent hippocampal damage. Moreover, scavenging of O$_2^{-}$ by endogenous or synthetic antioxidants was shown to protect against seizure-induced neuronal damage (Liang et al., 2000).

The hypothesis of age-dependent oxidative mechanisms as a possible explanation for the resistance of immature brain to seizure-induced neuronal damage was tested in rats at different ages (PN 12, 21, 30, 45 and 60) by assays aimed at evaluating the degree of aconitase inactivation and 8-hydroxy-2-deoxyguanosine (8-OHdG) concentration as compared to 2-deoxyguanine, as markers of mitochondrial oxidative stress and oxidative DNA damage respectively. Results obtained are in support of age-dependent oxidative damage induced by seizures triggered by KA administration. Thus, immature animals (PN 12-21) did not show an increase of mitochondrial aconitase inactivation or 8-OHdG, suggesting that both levels of O$_2^{-}$ and the amount of oxidative DNA damage did not increase following SE. These animals did not show hippocampal cell loss. Conversely, adult rats (PN 30-60) were shown with a significantly increase of aconitase inactivation and a significantly higher
concentration of 8-OHdG at times preceding hippocampal neuronal loss (Patel et al., 2003). The investigators also showed that the lack of oxidative stress in immature brains was not due to increased levels of either mitochondrial and cytoplasmatic SOD, which were shown to be comparable to those detected in control animals in all age-groups. Among hypothesised mechanisms that could account for a reduced oxidative stress in immature rats as compared to adults, an intrinsically lower production of $O_2^-$ by itself or as a consequence of immature neurotransmitter/receptor systems in the developing brain have been proposed.

The lack of a significant increase of ROS production and mitochondrial dysfunctions following KA-induced seizures in neonatal rat brains compared to adults was also confirmed by another group of investigators (Sullivan et al., 2003) and associated to mitochondrial uncoupling protein-2 (UCP-2) expression that was shown to be significantly higher in neuronal mitochondria of immature hippocampus. Uncoupling proteins (UCPs) are members of the superfamily of mitochondrial carriers which transport small molecules across the inner mitochondrial membrane. In particular, these proteins were shown to dissipate the mitochondrial proton gradient by transporting protons across the inner membrane, thereby uncoupling electron transport from ATP production that become dissociated from oxygen consumption. One of the main consequences of UCPs activity is a reduced production of ROS. Hence, it is conceivable that resistance of limbic neurons of immature rat to seizure-induced cell death may due, at least, to partial uncoupling of mitochondria in these neurons as a result of the high levels of UCP-2 expression. Researchers showed the UCP-2 function and uncoupling capacity of neuronal mitochondria from immature rat brain (PN 10) are significantly greater than in mitochondria from limbic neurons.
of the adult. In particular, UCP-2 immunoreactivity in immature brain appears to be more pronounced in neurons located in hippocampal areas which are known to be sensitive to seizure-induced neuronal injury in adults, such as CA3. As a possible explanation for the role of UCP-2 in counteracting excitotoxicity, an overall picture depicts UCPs to dissipate mitochondrial membrane potential with subsequent decrease of ROS production as well as decrease of mitochondrial uptake of Ca\(^{2+}\) that was shown to be associated with glutamate-induced neuron death (Stout et al., 1998). Indeed, these two steps are interlinked since, usually, mitochondrial dysfunction due to Ca\(^{2+}\) overload is known to lead to ROS production, hence UCP-2 may reduce ROS formation also by limiting mitochondrial Ca\(^{2+}\) concentration.

Finally, interesting results come from the evaluation of brain-derived neurotrophic factor (BDNF) as a possible candidate involved in neuroprotection of immature brain with respect to SE-induced neuronal damage. BDNF is the most abundant and widely expressed neurotrophin in the brain, involved in neuronal growth, differentiation and survival of neurons in both the developing and the adult brain (Schmidt-Kastner et al., 1996). BDNF has been shown to be protective against hypoxic-ischemic injury in neonatal brain (Cheng et al., 1997) and seizures are known to increase BDNF mRNA in both the adult and the immature brain (Dugich-Djordjevic et al., 1992; Kornblum et al., 1997). Moreover, regulation of BDNF expression and release from hippocampal neurons is known to be mediated by non-NMDA glutamate receptors (Wetmore et al., 1994) thus further showing that seizures not only lead to excitotoxicity and neuronal loss but also elicit trophic responses in neurons. Using PN 20 rats injected with KA as an animal model of SE, it was shown that blockade of the
increased expression of BDNF elicited by KA-induced seizures resulted in a long-lasting SE and loss of protection from neuronal damage. Control animals, whose BDNF production was not impaired, did not show any evidence of cell loss and alteration of SE duration (Tandon et al., 1999), thus supporting the hypothesis that BDNF may protect neurons of immature brain from seizure-induced excitotoxicity.

In summary, although all proposed mechanisms seem to be fairly robust, evidence so far collected cannot be allowed to infer any general mechanism accounting for resistance of immature brain to seizure-induced neurodegeneration, which actually appear to be a multifactorial phenomenon.
1.4 Inflammation and epilepsy

As has been shown in other neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, Hungtington’s disease, multiple sclerosis and amyotrophic lateral sclerosis, specific histopathological evaluations of brain specimens of epilepsy sufferers have made it possible to observe that also some epileptic encephalopathies and febrile seizures in children as well as epilepsy in adults share common features directly related to immune/inflammatory reactions, in particular mediated by proinflammatory cytokines and related molecules which have been described in brain after seizures in clinical cases and in experimental models of epilepsy.

Little is known about the role of inflammation in epilepsy. It has been hypothesized that activation of the innate immune system, and associated inflammatory reactions in brain, may mediate some of the molecular and structural changes occurring during and after seizure activity. Indeed, as for other mechanisms, two issues have been raised as to i) whether these proinflammatory signals represent a mere epiphenomenon or ii) if they are significantly involved in the etiopathogenesis of seizures and possibly contribute to epileptogenesis.
1.4.1 Evaluation of proinflammatory cytokines in association with epilepsy in humans

In adults, levels of immunoreactive IL-1α were found to be elevated in surgically resected human temporal lobe tissue from patients with intractable epilepsy as compared to post-mortem tissue from neurologically unaffected subjects (Sheng et al., 1994). Quantitative evaluation of IL-1α-immunoreactive cells revealed a threefold increase of these cells in epileptic than in control tissue, also showing that these cells had the morphological features of activated microglia.

In cortical specimens from patients affected by focal cortical dysplasia and glioneuronal tumors (both pathologies being recognized as causes of chronic intractable epilepsy) IL-1β and its signalling receptor IL-1RI were shown to be highly expressed by more than 30% of neurons and glia whereas the decoy receptor IL-1RII and IL-1Ra were expressed to a lesser extent by approximately 10% and 20% of cells, respectively. Moreover, the number of IL-1β- and IL-1RI-positive neurons was positively correlated with the frequency of seizures, whereas the number of IL-1Ra-positive neurons and astroglial cells was negatively correlated with the duration of epilepsy (Ravizza et al., 2006).

Inflammatory reactions in MTLE with HS have also been reported by measuring increased expression of NF-κB both in reactive astrocytes and in surviving hippocampal neurons (Crespel et al., 2002).

The aforementioned results actually suggest that in HS inflammatory processes that might be chronically active or transiently re-induced by recurrent seizures, or both, can take place.
Recent genetic studies have shown that a polymorphism in the promoter region at position -511 of the IL-1β gene is associated with therapy resistant epilepsy in subjects affected by MTLE with HS and in children with febrile seizures (Kanemoto et al., 2000; Virta et al., 2001). This polymorphism when present in homozygotes appears to be associated with an overexpression of cytokine production upon stimulation. Thus, minor events during development such as febrile convulsions could set up in these subjects a cascade of proinflammatory events leading to HS. However, several studies did not confirm the evidence reported by Kanemoto and Virta (Heils et al., 2000; Buono et al., 2001; Jin et al., 2003; Tilgen et al., 2002). This apparent discrepancy may be due to specific ethnicity (Heils et al., 2000) or the existence of methodological bias (Tan et al., 2004).

Interesting results were reported by studies aimed to evaluate proinflammatory molecules in the CSF and serum of epileptic patients.

IL-6 is the cytokine consistently found to be significantly enhanced in plasma and CSF of epilepsy sufferers with recent tonic-clonic seizures. Contrasting results are reported for IL-1β in CSF where either no increase or significant elevation was measured. Analysis of peripheral blood mononuclear cells highlighted in some but not all (Hulkkonen et al., 2004) instances an enhanced ability of these cells to produce inflammatory molecules and express markers of inflammation when harvested from epileptic patients, or children with recent febrile seizures (Pacifici et al., 1995; Helminen et al., 1990). Therefore, these data are compatible with an inflammatory state in the epileptic brain and perhaps in peripheral monocytes from epileptic patients.
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). In particular, expression of molecules involved in cytokine signalling (ICAM-1 and NF-κB) and TNF-α, have been found in dysplastic neurons and giant cells in tubers. This observation leads to the hypothesis that initiation of an inflammatory response in tubers may be directly related to epileptogenesis in these lesions.

Finally, one of the best indication 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 affected brain tissue revealed both perivascular lymphocytes and scattered microglia nodules in close proximity with neurons. In addition, a huge increase in the expression of several inflammatory-related genes (i.e. IL-1β and TNF-α) was described in brain specimens from a patient affected by Rasmussen's encephalitis and active seizures (Baranzini et al., 2002).

Table 4 describes Inflammatory markers in human epilepsies and convulsive disorders. Pharmacological treatment in clinical practice is also indicated.
Table 4. Inflammation in human epilepsies and convulsive disorders

<table>
<thead>
<tr>
<th>Epileptic syndrome</th>
<th>Inflammatory markers</th>
<th>Brain tissue</th>
<th>Antiinflammatory treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convulsive disorder</td>
<td>Plasma or CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rasmussen encephalitis</td>
<td>GluR3 Ab, Munc-18 Ab</td>
<td>GluR3 Ab</td>
<td>ACTH, steroids, IV Ig</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD-8+ lymphocytes</td>
<td>PEX, PAI, immunosuppressant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GrB; MAC; cytokines</td>
<td></td>
</tr>
<tr>
<td>West syndrome</td>
<td>IFN-alpha, TNF-alpha, IL-2</td>
<td>n.d.</td>
<td>ACTH, steroids, IV Ig</td>
</tr>
<tr>
<td>Lennox–Gastaut syndrome</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ACTH, steroids, IV Ig</td>
</tr>
<tr>
<td>Landau Kleffner syndrome</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ACTH, steroids, IV Ig</td>
</tr>
<tr>
<td>Febrile seizures</td>
<td>IL-1beta, IL-1Ra, IL-6</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>IL-10, TNF-alpha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLE</td>
<td>IL-6, IL-1beta, IL-1Ra</td>
<td>IL-1, NFKB*</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tonic-clonic seizures</td>
<td>IL-6, IL-1alpha, IL-1beta</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tuberculosis Sclerosis</td>
<td>n.d.</td>
<td>CD-68 macrophages</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICAM-1, TNF-alpha, NFKB, MAPK</td>
<td></td>
</tr>
</tbody>
</table>

*Only in patients with MTLE and HS. n.d., not determined; PEX; plasma exchange; PAI; protein A immunoabsorption; IV Ig, intravenous immunoglobulin; MAPK, mitogen activated protein kinase.

Adapted from Vezzani and Granata, 2005
1.4.2 Evaluation of proinflammatory cytokines in experimental models of epilepsy

Seizure activity induced in experimental models of SE has been reported to rapidly increase the production of proinflammatory cytokines, as well as various markers of the innate immunity (i.e. NF-κB system, prostaglandins and their pathway enzymes, Toll-like receptors, monocytes chemoattractant protein-1, complement system) both in glia and in neurons (Jankowsky and Patterson, 2001; Peltola et al., 2001). The increase is rapid (≤ 30 min) and reversible after SE in rats developing spontaneous seizures, with the exception of IL-1β which is still upregulated in brain 60 days after induction of SE (De Simoni et al., 2000). Cytokines were shown to specifically increase in brain regions involved in seizure onset and spread (De Simoni et al., 2000; Minami et al., 1991; Oprica et al., 2003). Evidence of increased production of inflammatory molecules in brain has also been reported in genetic models of audiogenic seizures and in kindling (Gahring et al., 1997; Plata-Salaman et al., 1992).

Microglia and astrocytes are the first cells producing cytokines during seizures and, in general, they represent the main sources of proinflammatory molecules in brain. However, cytokine receptors are localized both in glia and neurons in normal brain. This suggests that proinflammatory cytokines are soluble mediators which may establish functional communication among microglia, astrocytes and neurons (Ban et al., 1991; Benveniste, 1992; De Simoni et al., 1998). In this respect, electrophysiological findings support a neuromodulatory role of glia-born cytokines showing that these molecules affect
ionic conductances in neurons, and synaptic plasticity (i.e. LTP) (Zeise et al., 1997; Wang et al., 2000; Kelly et al., 2003).

So far, it appears that proinflammatory molecules and their signalling pathways are similarly activated by systemic infections and seizures. Indeed, there are clear differences in the duration of these events and in the cell specific distribution of these changes.

The inflammatory response observed following pilocarpine-induced seizures in mice is different in many aspects from that described after systemic injections of bacterial LPS (Rivest, 2003; Turrin and Rivest, 2004 (a)). Thus, proinflammatory molecules are predominantly and firstly expressed after endotoxemia in circumventricular organs, the choroid plexus, the leptomeninges and along brain microvessels; the involvement of parenchymal microglia is delayed and more restricted. Neurons do not typically express inflammatory markers after endotoxemia. On the other hand, seizures induce a massive inflammatory response in parenchymal cells involving both microglia and neurons (i.e. NF-κB and COX-2 are significantly expressed by neurons after seizures but not after LPS). Moreover, changes induced by endotoxemia are relatively short-lasting when compared to those observed after seizures. These observations suggest that inflammation induced by seizures results from complex neurophysiological events specific to brain tissue which differ in their duration, and in the cell populations involved, from classical immune reactions triggered by bacterial/viral infections.

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. Although there is indication of a late wave of CD-45 positive monocyte penetration into the brain parenchyma after seizures, Turrin and Rivest (Turrin and Rivest, 2004 (a)) recently 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. Accordingly, immunostaining for T-cells, B-cells and NK-cells was negative in the brain of KA treated rats 8-72h after seizure induction, although granulocytes, macrophages/monocytes and microglia cells were all detected (Dinkel et al., 2003). Thus, the innate immune response in experimental models of seizures does not appear to be associated with adaptive immune and B- or T-cells infiltration and differentiation with a restricted time window after induction of seizures.

The evidence that seizures can trigger lasting inflammatory reactions opened the additional question as to whether a protracted inflammatory state in CNS may enhance the predisposition of brain tissue to develop seizures and brain damage.

Using models of hippocampal seizures or SE in rodents, it has been shown that the IL-1β system significantly exacerbates seizure activity. Thus, pre-administration of this cytokine using concentrations in the range of those endogenously produced during seizures, prolongs the duration of electrographic and behavioural kainate-induced seizures (Vezzani et al., 1999). Subsequent studies have shown that the intracerebral administration of IL-1ra, a naturally occurring molecule which antagonizes the effect of endogenous IL-1β, has powerful anticonvulsant activity (Vezzani et al., 2002). Accordingly, IL-1ra overexpressing mice display a reduced susceptibility to seizures (Vezzani et al.,
2000). These findings suggest that an endogenous rise in IL-1β levels has proconvulsant effects. It appears that IL-1β prolongs seizures by increasing glutamatergic neurotransmission (Vezzani et al., 1999). In this respect, functional interactions at the molecular level have been reported between IL-1β and N-Methyl-D-Aspartate (NMDA) receptors which are co-expressed by hippocampal neurons (Viviani et al., 2003). Other actions may account for the excitatory effect of this cytokine: IL-1β can inhibit glutamate uptake by astrocytes (Ye et al., 1996) and decreases the peak magnitude of GABA-mediated currents in cultured hippocampal neurons (Zeise et al., 1997; Wang et al., 2000).

On the other hand, the effect of TNF-α on seizures is still controversial. Data obtained using in vivo pharmacological approaches in wild-type and TNF-α knock-out mice, showed that TNF-α reduces seizure activity by interacting with TNF-α type II (p75) receptors (Balosso et al., 2005). Mice overexpressing TNF-α in glia show a decreased sensitivity to kainate-induced seizures (Balosso et al., 2005). However, seizures and brain damage in a rat model of bacterial meningitis were attenuated by large spectrum inhibitors of both matrix metalloproteinases and TNF-α converting enzyme, which reduce the soluble form of TNF-α (Meli et al., 2004). These apparent discrepancies can be reconciled by taking into account either differences in TNF-α concentrations or the receptor subtypes predominantly involved in the various experimental models. In accordance with this view, relatively high concentrations of TNF-α exert suppressive effects on Shigella dysenteriae-induced seizures while lower concentrations were proconvulsive (Yuhas et al., 2003).
Table 5 summarizes the inflammatory markers occurring in experimental models of seizures and the effect of anti-inflammatory pharmacological treatment on seizure susceptibility.
Table 5. Inflammation in experimental models of seizures

<table>
<thead>
<tr>
<th>Exp Models</th>
<th>Inflammatory markers</th>
<th>Pharmacological Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Kainic acid</td>
<td>Cytokine and their receptors</td>
<td>Low corticosterone</td>
</tr>
<tr>
<td>*Bicuculline</td>
<td>Signaling pathways (NFkB; F38MAPK)</td>
<td>TNF-alpha/p75 receptor</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>COX-2, mPGES</td>
<td>IL-1Ra</td>
</tr>
<tr>
<td></td>
<td>Adhesion molecules</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td></td>
<td>Chemokines</td>
<td>COX-2, mPGES</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspirin (10 mg/kg)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hyperthermia</th>
<th>IL-1beta</th>
<th>IL-1beta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>IL-1beta, TNF-alpha</td>
<td>IL-1beta, TNF-alpha</td>
</tr>
<tr>
<td>Infection</td>
<td>n.d.</td>
<td>Aspirin (100-500 mg/kg)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>n.d.</td>
<td>Panacetamol</td>
</tr>
<tr>
<td>MES</td>
<td>n.d.</td>
<td>COX-2 inhibitors</td>
</tr>
<tr>
<td>PTZ</td>
<td>n.d.</td>
<td>COX-1/2 inhibitors</td>
</tr>
<tr>
<td>Audiogenic seizures</td>
<td>IL-1alpha</td>
<td>IL-1beta</td>
</tr>
<tr>
<td>Rodent Kindling</td>
<td>Cytokine and their Receptors</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Cat Kindling</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

*Refers to pharmacological experiments carried out in rats intracerebrally or systemically injected with kainic acid, or intracerebrally injected with bicuculline methiodide (for IL-1beta and IL-1Ra only).

MES, maximal electroshock test; PTZ, pentylenetetrazol; mPGES, membrane/microsomal prostaglandin E synthase.

After hyperthermia or infection the cytokines were measured in plasma only; n.d., not determined.

Adapted from Vezzani and Granata, 2005
Seizure susceptibility was also assessed in transgenic mice overexpressing IL-6 in glia, showing that they have a profound increase in sensitivity to glutamatergic (but not cholinergic) agonists (Samland et al., 2003). Interestingly, IL-6 overexpressing mice showed neuronal loss of GABA- and parvalbumin-positive neurons in the hippocampus.

In different studies, TNF-α and IL-6 transgenic mice develop neurodegenerative changes and behavioural seizures in an age-dependent manner when overexpressing these cytokines in glia, but not in neurons (Campbell et al., 1993; Akassoglou et al., 1997). These studies with transgenic mice suggest that a pre-existing chronic proinflammatory state in the brain can predispose to seizures and promote neurological dysfunctions. Accordingly, systemic administration of LPS to mice was reported to decrease their threshold to pentylenetetrazole-induced seizures. This effect was blocked by anti-inflammatory drugs (Sayyah et al., 2003). Finally, sequential infusion of individual proteins of the membrane attack pathway (MAC) of the complement system into the rat hippocampus induced both behavioural and electrographic seizures as well as cytotoxicity (Zhi-Qi et al., 2003). This implies that deposition of the complement MAC in brain tissue may contribute to epileptic seizures and cell death in diverse CNS diseases (i.e. Rasmussen's encephalitis).

Experimental models have also been exploited in order to evaluate the effects of anti-inflammatory drugs on seizures.

Various pharmacological studies reported inhibition of seizures using nonsteroidal anti-inflammatory drugs. An early report shows attenuation of penicillin induced electrocorticographic and motor seizures in rats using ibuprofen, paracetamol and indomethacin (Wallenstein, 1987). Aspirin was also
reported to protect mice from MES and pentylenetetrazole-induced seizures and to potentiate the anticonvulsant action of diazepam and sodium valproate (Srivastava et al., 2001).

Interestingly, both sodium valproate and carbamazepine inhibit the production of inflammatory mediators in vitro. In particular, among the commonly used AEDs, sodium valproate inhibits LPS-induced activation of NF-κB as well as the production of TNF-α and IL-6 in monocytes and glioma cells; carbamazepine was shown to decrease LPS-induced production of prostaglandins and activity of phospholipase A in rat glial cells (Ichiyama et al., 2000; Matoh et al., 2000). Conflicting data are available on the effect of prostaglandins on seizures. Several studies have demonstrated increased synthesis of cerebral prostaglandins following convulsions; however, it appears that they can either reduce or promote seizures depending on the type of prostaglandins produced. For instance, it was shown that tacrine-induced seizures in rats, and the consequent hippocampal damage, can be abolished by inhibiting PGE2 production using COX-2 inhibitors (Paoletti et al., 1998). Similarly, a cyclooxygenase/lipoxigenase inhibitor protected from irreversible brain lesions and reduced the severity of seizures induced by KA in rats (Baran et al., 1994).

The possible relationship between inflammation and seizure-induced long-term sequelae has also been investigated. Inflammatory processes have been implicated in both acute and chronic neurodegenerative conditions. Accumulating evidence has shown that brain injury per se in the absence of seizure activity is accompanied by a marked inflammatory reaction characterized by increased expression of various proinflammatory molecules,
activation of microglia and infiltration of monocytes/macrophages (Allan and Rothwell, 2001). However, in the presence of seizures triggered by damage to brain structures, it is difficult to distinguish between the cytokine response to injury and seizures that might contribute to cell death.

When administered individually, cytokines tend not to induce cell death directly, but rather to have synergistic effects with other molecules that can result in neurotoxicity (e.g. IL-1 plus TNF-α). Intracerebral injections of TNF-α or IL-1 exacerbate excitotoxic injury in vivo while IL-1ra reduces excitotoxic, traumatic and ischemic brain damage (Allan et al., 2005). An involvement of cytokines in apoptotic mechanisms has also been shown (Allan and Rothwell, 2001). A study by Dinkel (Dinkel et al., 2003), shows that the extent of damage in the hippocampus after an excitotoxic insult correlates significantly with the antecedent inflammatory cell infiltration (granulocytes and macrophages) and activation of microglia. This study supports the hypothesis that neuronal damage is, at least in part, caused by a preceding state of inflammation. Cytokines can also have neurotrophic actions on neurons, possibly mediated by an increase in the production of neurotrophins (Jankowsky and Patterson, 2001; Spranger et al., 1990). Glia, in particular astrocytes, are a principal source of neurotrophins and growth factors which are induced by cytokines and exert neuroprotective actions.

Other actions of cytokines which may be relevant for epilepsy are their reported effects on brain microvasculature (IL-1 can induce neovascularization) and damage to the blood-brain barrier (BBB) leading to its increased permeability to normally excluded substances and cells (i.e. up regulation of cell
adhesion molecules such as ICAM-1 and e-selectin in endothelial cells are involved in leucocytes infiltration).

As regards neurogenesis, an inhibitory action of inflammatory molecules such as IL-6 has been reported (Monje et al., 2003). In particular, neuroinflammation induced by radiation injury or by LPS inhibits neurogenesis while inflammation blockade with indomethacin or inhibition of microglia activation by minocycline, restore neurogenesis (Monje et al., 2003; Ekdahl et al., 2003).
1.4.3 Involvement of proinflammatory cytokines in CNS injury and neurodegenerative diseases

Cytokines are a heterogeneous and complex group of polypeptide mediators which act as communication signals among cells of the immune system as well as among other cells and tissues in the body. Cytokines include interleukins, interferons, tumor necrosis factor and chemokines, and their production as well as that of related receptors are well documented among neuronal and glial cells within the CNS. Generally, glial and endothelial cells produce TNF-α, IL-1 and IL-6 (Riccardi-Castagnoli et al., 1990; Fabry et al., 1993; Sébire et al., 1993), whereas in neurons the expression of these cytokines is hardly detectable in normal brain.

However, astrocytes and microglia represent the main source of cytokine production and each of them express receptors for almost any cytokine. Proinflammatory cytokines are known to exert central effects such as fever, sleepiness, anorexia, activation of the hypothalamic-pituitary-adrenal axis, and to trigger and sustain the machinery of innate immunity. It is important to note that the inflammatory response is not confined to pathogenic infection but also occurs as a consequence of brain injuries, and in both acute and chronic neurodegenerative conditions. Indeed, among proinflammatory cytokines, IL-1β and TNF-α appear to be particularly relevant to several CNS disorder, since their levels of mRNA, protein or both, are increased in these conditions in both animals and humans. Thus, experimental models of global and focal ischemia have shown an increase of both mRNA transcription and level of protein for both IL-1β and TNF-α in cerebral areas particularly vulnerable to this kind of insult.
such as hippocampus, striatum and cortex (Minami et al., 1992; Orzyłowska et al., 1999; Buttini et al., 1994; Saito et al., 1996; Liu et al., 1994). These changes occur in both the core and the penumbral regions within a few minutes of the insult, can peak in a few hours, and the overexpression may last for several days.

Proinflammatory cytokines are elevated in terms of both mRNA and protein expression in experimental models of traumatic CNS injury. For instance, IL-1β protein levels are increased in the rat hippocampus following transection of the perforant pathway (Fagan and Gage, 1990), and in the CNS parenchyma after a stab wound injury (Fan et al., 1995; Herx et al., 2000; Rostworowski et al., 1997). Similarly TNF-α mRNA and protein levels increase rapidly in the rat brain after traumatic brain injury. (Fan et al., 1996; Shohami et al., 1997; Taupin et al., 1993). TNF-α mRNA and protein levels also increase in several brain structures in mice receiving a stab or surgical injury (Tchelingerian et al., 1993; Tchelingerian et al., 1994).

Considerable data is available concerning proinflammatory cytokine levels in patients affected by neurological diseases. Inflammatory processes have been shown to occur in the CNS of sufferers of Alzheimer’s disease, Parkinson’s disease, Huntington’s chorea, multiple sclerosis and amyotrophic lateral sclerosis, as confirmed by elevated levels of IL-1β, TNF-α and IL-6 in the serum and cerebrospinal fluid of these patients (Pasinetti, 1998; Gonzalez-Scarano et al., 1999; Julien, 2001). Increased levels of TNF-α were observed in plasms serum of humans after severe head injury (Goodman et al., 1990) and increased levels of IL-1β are seen following neurotrauma (Fassbender et al., 2000).
The observation that inflammatory processes occur in association with several neurodegenerative disorders as well as together with traumatic brain injuries has raised the question as to whether neuroinflammation plays a beneficial or detrimental role, maybe directly or indirectly contributing to the pathology itself.

In the context of the aforementioned findings about increased levels of IL-1β and TNF-α in CNS injuries, it has been demonstrated that injections of IL-1β in the lateral ventricle during ischemia produces an increase in the infarction size, whereas injections of anti-IL-1β monoclonal antibody produces a decrease in post-ischemic infarction size (Stroemer and Rothwell, 1998; Yamasaki et al., 1995). Analogous results were obtained by administration of IL-1ra in a model of focal ischemia thus showing that neuronal death was decreased in the penumbral region and infarction size was reduced as well (Garcia et al., 1995; Relton and Rothwell, 1992). Similar experiments have also been done with TNF-α. Thus, rats undergoing focal cerebral ischemia exhibit neuronal loss when TNF-α is injected into the CNS (Barone et al., 1997), whereas inactivation of TNF-α activity by administration of either the soluble TNF-α type I receptor or antibody anti-TNF-α yields to a reduced ischemic brain injury (Barone et al., 1997; Nawashiro et al., 1997; Yang et al., 1998 (b)).

Indeed, increasing evidence shows that proinflammatory cytokines can provoke an increase in extracellular glutamate by acting on the mechanisms of its release and re-uptake, as well as potentiating the function of ionotropic glutamate receptors and enhancing the production of mediators of oxidative stress (i.e. arachidonic acid and nitric oxide) (Xu et al., 2003).
However, despite several observations showing a detrimental role of IL-1β and TNF-α in CNS injury, a number of observations support the hypothesis that these cytokines may also act as relevant neurotrophic mediators in the CNS. For example, microglia-derived IL-1β can induce the synthesis of neurotrophic factors from astrocytes, such as nerve growth factor, ciliary neurotrophic factor, insulin-like growth factor which can promote repair of the CNS (Juric and Carman-Krzan, 2001; Mason et al., 2001). Interestingly, IL-1β was shown to induce neuronal sprouting in residual dopaminergic neurons in the ventral tegmental area in an animal model of Parkinson's disease yielding to a functional recovery (Wang et al., 1994) as well as in rat hippocampal cholinergic neurons following transection of perforant pathway (Fagan and Gage, 1990). TNF-α was shown to be protective in rat embryonic cultures of hippocampal, septal and cortical neurons with respect to injury induced by glucose deprivation and excitatory amino acid toxicity (Cheng et al., 1994). Moreover, in animal models of seizure, TNF-α was shown to act as a neurotrophic factor thus improving neuronal survival (Jankowsky and Patterson, 2001). In vivo application of TNF-α to the injured adult rabbit optic nerve has been reported to produce regeneration of axons that traverse the site of injury (Schwartz et al., 1991).

IL-1β and TNF-α have been shown to exert a synergistic stimulation of nerve growth factor release from cultures of rat astrocytes (Gadient et al., 1990). Conversely, whereas individual low concentrations of either IL-1β or TNF-α were shown to stimulate insulin release, their combination was suppressive (Mehta et al., 1994).
Thus, the available evidence show that proinflammatory cytokines such as IL-1β and TNF-α can either exacerbate excitotoxic cell death or be neuroprotective depending on the brain microenvironment. In particular, it is increasingly clear that the action of proinflammatory cytokines on cell function and survival is highly dependent on the extent to which these molecules are produced, the length of time the tissue is exposed to their action, and the balance between the neurotrophic and inflammatory effects mediated by these molecules.

Finally, it is worth remembering that the regional and cellular patterns of induction of inflammatory molecules, and their time-course of activation and persistence in brain tissue, appear to depend on the nature of the CNS injury. For instance, IL-1 and TNF-α producing cells in the CNS may differ depending on stimuli as well as on diseases involved. Thus, TNF-α appears to be mainly produced by glial cells in experimental autoimmune encephalomyelitis (Renno et al., 1995), whereas in excitotoxic and ischemic brain injury a TNF-α production of neuronal origin has been reported (Liu et al., 1994; Bruce et al., 1996).
1.4.4 Proinflammatory molecules and the Hypothalamic Pituitary-Adrenal axis

A system linked to the inflammatory response and that deserves attention is the Hypothalamic-Pituitary-Adrenal (HPA) axis. Indeed, any modulation or induction of an inflammatory state in the periphery as well as in the CNS cannot be dissociated from the activation of the HPA axis. In particular, proinflammatory molecules such as LPS and proinflammatory cytokines such as IL-1β are known to increase the corticosterone levels in rodents when administered both systemically and centrally. However, proinflammatory cytokines and the HPA axis reciprocally affect their levels of expression and functionality. The main feature of the HPA axis activation is the synthesis and secretion of glucocorticoids (GCs, cortisol in humans and corticosterone in rodents) from the adrenal cortex. This facilitates the mobilization of substrates which are used as an energy source, potentiates the release of catecholamines, increases the cardiovascular tone and suppresses biological systems which are not considered essential to the organism's response to stressors. Among these suppressed systems are, growth, reproduction and immunity. In relation to the immune response, the classical anti-inflammatory action of corticosteroids, the inhibition of transcriptional activation of proinflammatory molecules such as IL-1β and TNF-α has been well documented. Nonetheless, the mechanism by which proinflammatory cytokines activate the HPA axis which turns to inhibit proinflammatory cytokines production at genomic level may not hold true for the immature organism. As it
will be discussed in the following paragraphs, the ability to respond to stressful events at this stage of development is remarkably different from that encountered in adults. From this perspective, a massive release of proinflammatory cytokines in the developing brain may lead to long-term consequences. For instance, the induction of proinflammatory cytokines in the fetal environment following intraperitoneal injection of LPS to pregnant rats has been shown to increase the mRNA levels of CRH in the whole fetal brain, thus possibly influencing the degree of neuronal excitability of immature brain and possibly affecting the response of the immature brain to stressful stimuli by abnormal activation of hypothalamic-pituitary-adrenal (HPA) axis (Gayle et al., 2004). This latter phenomenon has been shown to prime this system to inadequate responses to stressors in adulthood, as it was shown to occur following immune challenge during prenatal stages of life (Reul et al., 1994). Generally, the exposure to high levels of proinflammatory cytokines during perinatal life, together with documented long-term alterations of relevant biological systems such as HPA axis could induce significant changes in immature brain (e.g. elevation of brain excitability, neuronal circuitries hypomyelination, excessive induction of innate immune cell response, abnormal activation of HPA axis) such to account, in principle, for an increased susceptibility to seizures and neurodegeneration in later stages of life. It should be mentioned, however, that corticosterone and, in particular, agonists to mineralocorticoid receptors have been shown to be anticonvulsant at young ages, as it will be discussed in the following paragraphs. Thus, in principle, the activation of the HPA axis may positively affect seizure susceptibility as long as it occurs at young ages.
The following paragraphs will briefly outline the ontogeny of the HPA axis in rodents, with particular emphasis on the limbic-HPA axis system.
1.4.4.1 The adult limbic-HPA axis

Interactions between the endocrine system and the CNS result in a diurnal rhythm of GCs secretion, with a peak occurring at the time of awaking and a nadir during the first few hours of sleep. Blood levels of circulating GC increase in response to virtually any type of stimulus that is perceived as a real or potential threat to body homeostasis. The regulation of blood levels of GC is subject to diverse sensory inputs, and this information is integrated at the level of the hypothalamus. Relevant within this brain structure are the parvocellular neurons of the paraventricular nucleus (PVN) which constitute the main source of the 41-amino acid peptide corticotrophin releasing hormone (CRH), which is the major physiological regulator of pituitary adrenocorticotropin hormone (ACTH) secretion. Indeed, parvocellular neurons also express, to a lesser extent, the peptide arginine vasopressing (AVP) which together with CRH is a secretagogue of the HPA axis. Neuron producing CRH and AVP in the PVN project to the external zone of the median eminence (ME) where, in response to physiological and psychological stressors, CRH and AVP are released into a specialized capillary network from which hypophysial portal vessels arise and vascularize the anterior pituitary. Within the anterior pituitary, CRH and AVP synergistically stimulate the synthesis of the ACTH precursor peptide proopiomelanocortin (POMC) and the secretion of ACTH and other POMC-derived peptides. ACTH, transported in the circulation, interacts with adrenal cortex receptors causing steroidogenesis and elevation of plasma GCs.

Generally, the stress response of the HPA axis is meant to be acute or limited in duration. The time-limited nature of this process makes the
accompanying antireproductive, antigrowth, catabolic and immunosuppressive effects not detrimental to the organism. Thus, inhibition of steroid secretion is also an important feature of the system. The termination of the response to stressful stimuli appears to be mediated mainly by two feedback mechanisms which seem to be independent from each other, i.e., a rate sensitive fast feedback and a delayed feedback (Vázquez, 1998).

By the fast feedback mechanism, which operates on a time scale of minutes, CRH and ACTH are inhibited by the direct action of GC in a rate sensitive manner, that is, the quicker the rise of GC levels the more effective the inhibition. It has been shown that fast feedback inhibition is influenced by GC binding to specific receptors in limbic structures, mostly in the hippocampus. Indeed, specific lesions to neural connections from the hippocampus to the hypothalamic CRH neurons and/or a decrease of GC receptors as occurs in aging, result in a selective decrease in the termination of the stress response (Jacobson et al., 1991; Sapolsky, 1984).

Delayed feedback operates on a time scale of hours and its inhibitory action is accomplished by the translocation of corticoid-receptor complex to the nucleus where it acts at transcriptional level by suppressing gene expression, hence decreasing the ACTH stores in the pituitary and all key molecules in the limbic-HPA structures such as hypothalamic CRH and hippocampal glucocorticoids receptors (Fremeau et al., 1986).

Several lines of evidence support the hippocampus as a key structure in the limbic-HPA interaction. For instance, hippocampectomy results in an upregulation of hypothalamic CRH mRNA expression even with high levels of circulating GCs (Herman et al., 1989; Jacobson et al., 1991). Moreover,
genomic inhibitory feedback exerted by GCs on hypothalamic parvocellular neurons fails in animals that underwent hippocampectomy (Jacobson et al., 1991).

As far as corticoid receptors are concerned, two types have been described in the brain. At the cellular level, they both are located in the cytoplasm. The type I receptor, also known as the mineralocorticoid receptor (MR), selectively binds corticosterone (CS) in rodents and is usually described as a high affinity corticoid receptor. In the adult rodent brain, MRs are mostly localized in hippocampal and septal neurons. Conversely, the type II receptor, also known as the glucocorticoid receptor (GR), is usually defined as a low affinity corticoid receptor. These receptors are widely distributed in brain neurons (including hippocampus and hypothalamus), glial cells and pituitary cells.

GRs bind corticosterone with a lower affinity compared to MRs. It is largely accepted that MRs appears to be operative at low CS concentrations thus playing a tonic inhibitory role on HPA axis during the nadir of circadian rhythm. When high concentration of CS occurs, MRs saturate and the return to homeostasis appears to be mediated by GRs (Vázquez, 1998).
1.4.4.2 The limbic-HPA axis in the immature brain

The first two postnatal weeks are characterized by a hyporesponsiveness to stressors (a time-window also known as stress hyporesponsive period, SHRP), in that the rat pups respond weakly to a variety of acute stressful stimuli such as ether inhalation, surgery or handling, whereas the same stimuli would considerably increase the levels of plasma CS in older animals.

Indeed, several studies have shown how this hyporesponsiveness appears at all levels of the HPA axis at this developmental stage. Measures of anterior pituitary peptide content and POMC mRNA levels have shown a clear age-dependency. The anterior pituitary content of peptides derived from cleavage of POMC (ACTH, β-lipotropin and β-endorphin, the latter from cleavage of β-lipotropin) increases specifically with age for each individual peptide (Vázquez and Akil, 1992), showing that ACTH and β-endorphin increase 4-8 fold from newborn to PN 21. Interestingly, by comparing the amount of each processed peptide to the amount of its immediate precursor, it is evident that in the developing anterior pituitary there is a greater proportion of cleaved and acetylated end products as compared to adults, thus showing that corticotroph appears to process the POMC precursor at a faster rate than the adult corticotroph, in particular during the first two postnatal weeks, reaching adult rate at PN 21. In this contest, POMC mRNA was shown to parallel the age-dependent increase of peptides in the anterior pituitary (Vázquez and Akil, 1992).

The plasma levels of ACTH released by the developing pituitary have been shown to maintain a low steady state until PN 21 after which time a
significantly increase occurs (Vázquez and Akil, 1992). The CS levels show an initial decrease after birth, than increase on PN 14 and 21 (Sapolsky et al., 1986; Vázquez, 1998). Two interesting observations arise by considering the pattern of plasma ACTH and CS levels in PN 14 and 21 rats (fig. 5) i) at PN 14 the adrenal cortex appears more sensitive to ACTH since a tendency to increase CS is evident despite plasma levels of ACTH being unchanged at this age, ii) at PN 21, although the levels of circulating ACTH are significantly increased, there is not a corresponding rise in CS levels (fig. 5). This latter observation appears to be consistent with the decrease in adrenal sensitivity to circulating ACTH and limited adrenal steroidogenic capacity observed at this age (De Kloet et al., 1988; Henning et al., 1985).
Plasma CS and ACTH levels from unhandled pups. Adapted from Vazquez 1998 Psychoneuroendocrinology 23:663-700
The response of the immature HPA axis to stressful stimuli is worth a comment. Repeated maternal isolation, used as a paradigm of chronic intermittent stressor (Vázquez and Akil, 1992), causes activation of the limbic-HPA axis in the pups. Under this stressful challenge (isolation from the respective mother for 1 hour, on 3 consecutive days), pups aged at the second and third week of life do not exhibit changes in stored anterior pituitary peptides with the exception of the PN 21 rats that showed a depleted content of all peptides descending from the POMC precursor (Vázquez and Akil, 1992). Indeed, it was shown that during the SHRP the developing corticotroph, under stressful conditions, processes the POMC precursor at a faster rate compared to age-matched unstressed animals (Vázquez and Akil, 1992). This leads to a minimal (if not absent) increase of anterior pituitary peptide content and a limited increase of circulating POMC related peptides.

The pattern of peptide content at different ages are paralleled by similar changes in POMC mRNA levels. Thus, at PN 21 a decrease of POMC mRNA occurs whereas no changes appear in younger animals.

Animals challenged with repeated maternal isolation show an increase of CS as early as PN 7 although ACTH levels were unaltered by stress, suggesting a greater adrenal sensitivity to circulating ACTH levels at this developmental stage. The response to stress by increasing CS levels becomes gradually more pronounced in PN 14 and PN 21 rats despite ACTH levels appearing unaffected by stress at all ages, with the exception of PN 21 where a decrease in plasma levels of circulating ACTH occurs.

Therefore, in general, animals which undergo stressful stimuli during the first three weeks of life maintain a greater level of CS as compared to
unstressed controls. It is interesting to note that at PN 21 there is i) an increase in circulating CS, ii) a decrease in POMC mRNA levels, iii) a decrease in pituitary peptide stores and iv) a decrease in pituitary peptide plasma levels. Taken together, this suggests the emergence of negative CS feedback acting on the pituitary at the level of genomic transcription by PN 21 (Vázquez, 1998). Nonetheless, the emerging CS negative feedback by PN 21 has been shown to be a consequence of the inability of weanling rats to quickly terminate the adrenocortical response following exposure to stressful stimuli, due to a combination of adrenal developmental factors leading to a slow CS rise and pituitary and/or brain factors which result in decreased responsiveness to negative feedback, even when the rate of CS increase is high (Vázquez and Akil, 1993). In particular, it is of interest to note that, compared with adults, the exposure of weanling animals (aged about PN 22, on average) to a stressor like inhalation of ether vapor induces a robust CS peak but a slower return to resting CS levels. In this situation glucocorticoid concentrations are still significantly elevated 2 hours after the exposure to the stressor (Goldman, 1973; Vázquez and Akil, 1993).

Thus, to summarize what has been reported so far, it is clear that during development the HPA axis is different from the adult in both structure and function. The immediate postnatal period is characterized by a time-window (SHRP) in which the young animals show a relative insensitivity to environmental perturbations, followed by a peculiar pattern of stress responsiveness in weanling animals, which show an inability to quickly terminate glucocorticoid secretion. Indeed, experimental evidence has been accumulated
in support of the notion that the mechanisms underlying normal HPA development and the mechanisms of adaptation are not necessarily those which the mature system would employ under those same challenges. Thus, during the first 2 weeks of life, pups respond to chronic intermittent stressors by increasing anterior pituitary POMC post-translational events, while adults are known to increase genomic events. At this developmental stage, although the increment of circulating ACTH concentration is minimal, the adrenocortical response to stress is already observable at PN 7, due to the augmented sensitivity of adrenal gland to circulating ACTH levels, and this responsiveness progressively increases during the following 2 weeks of life. It is relevant to note that in both the mature and PN 21 rat, the adrenocortical response to chronic intermittent stressful stimuli leads, eventually, to an increase of CS levels. However, the adult rat sustains this response by increasing the releasable pool of ACTH and reducing steroid feedback at the level of the pituitary. Conversely, the developing animal increases POMC molecule processing which leads to a minimal increase in circulating ACTH levels. Finally, levels of circulating CS in the immature rats in response to stressors have been shown to be long lasting as compared to adults.
1.4.4.3 Changes in mineralocorticoid and glucocorticoid receptors in the developing hippocampus

Similar to other receptor systems, cytoplasmic receptors for CS also undergo a developmental change. Studies exploiting the in situ hybridization technique have shown that in almost every hippocampal subfield the mRNA for mineralocorticoid receptors increases with age, whereas the levels of transcript for glucocorticoid receptors decreases (Vázquez et al., 1996). Nonetheless, GR and MR protein expression generally increases from low levels on day 6 to adult levels by day 22, and between PN 22 and PN 45 both receptors reach the greatest absolute increase of protein levels, which then gradually decrease to adult levels (Vázquez et al., 1993). Binding studies have shown that the affinity of both corticoid receptors does not change with age. These findings suggest that the failure to terminate the stress response in young animals is neither due to a decrease in corticoid receptor numbers nor to a change in their affinity.

It is of interest to observe the sensitivity of both corticoid receptors in the early developmental stages of rat pups following an increase of circulating CS induced by stimulation of the HPA axis. Animals that underwent 24 hours of maternal deprivation clearly show an increase in CS levels which are significantly higher at PN 9 and PN 12. Moreover, this paradigm of stress was shown to prime the organism to increase its responsiveness to novel stressors that could be as mild as isotonic saline injection or inhalation of ether vapor (Rosenfeld et al., 1992). This feature was evident not only at PN 9 and PN 12 but also at PN 6, which is during the SHRP time window.
The mineralocorticoid and glucocorticoid receptor pattern in the hippocampus was evaluated in animals aged PN 6, 9 and 12, challenged with 24 hours of maternal deprivation. The mineralocorticoid receptor (MR) gene expression was shown to undergo a 25-35% decrease in the CA1 area of the hippocampus at all ages, while significant changes in GRs mRNA levels appeared unaffected by maternal deprivation. Thus, the MR to GR ratio is influenced by the challenge of stressful stimuli. Unstressed animals usually showed a MR to GR ratio of about 1 at PN 6, 2 at PN 9 and 3 at PN 12, whereas rats which were challenged with 24 hours of maternal deprivation show a ratio of about 1 at PN 6 and PN 9, while the ratio increased to 2 at PN 12 (Vázquez et al., 1996).

It is largely accepted that MRs and GRs complement each other. The MRs appear to exert their tonic inhibition to the HPA axis as far as CS concentrations are low, whereas at high CS levels MRs saturate and the effects become mediated by GRs which are expected to restore the homeostasis. Thus, the dual action of these receptors in the hippocampus appears to be central for both basal modulation and stress regulation of the limbic-HPA axis. It is of interest to observe that the down-regulation of MRs in animals that underwent 24 hours of maternal deprivation appears to be associated to an alteration of the tonic inhibition of the system which is manifested by an elevation of basal CS levels.
1.4.4.4 Membrane mineralocorticoid receptors

Membrane mineralocorticoid receptors which mediate non-genomic actions of corticosteroids (Wehling et al., 1991; Schmidt et al., 2000) have been identified on several cell types, including synaptic membranes, and have been shown to be developmentally regulated (Sze et al., 1993). At present, little is known about their physiologic significance. In some cases, their function has been linked to alterations of electrolyte transport (intracellular Na\(^+\), K\(^+\), and Ca\(^{2+}\)) and cell volume (Mihailidou et al., 1998; Wehling et al., 1991). Inherent to modulation of neuronal activity are experimental findings from electrophysiological measurements made in amphibian synaptic membrane preparations, which have shown that agonists of mineralocorticoid receptors cause a decline or cessation of firing in spontaneously active and sensory-responsive neurons (Rose et al., 1993).
1.4.4.5 Mineralocorticoid receptors and modulation of seizure susceptibility

Indeed, a role of mineralocorticoid receptors in modulating seizure susceptibility has been established showing different effects in adult and infant rats. In adult rats, pre-treatment with a mineralocorticoid agonist enhances susceptibility to pentylenetetrazole and kainic acid seizures and these proconvulsant effects are blocked by antagonism of mineralocorticoid receptors (Roberts et al., 1994; Reddy et al., 2002). Glucocorticoid receptors appear to have little or no effect on seizure susceptibility in adult rats (Reddy et al., 2002). Conversely, in infant rats activation of mineralocorticoid receptors were shown to counteract seizure activity in a variety of seizure models and these anticonvulsant effects are blocked upon administration of an antagonist to mineralocorticoid receptors (Edwards et al., 2005; Edwards et al., 2002 (a, b)).

Generally, two conclusions have emerged from these studies. Firstly, although the great majority of experiments were accomplished using PN 15 rats, the anticonvulsant properties triggered by mineralocorticoid receptor activation are age-dependent since they appear effective within the first four-five weeks of postnatal life. Secondly, the effects mediated by agonists to mineralocorticoid receptor, including corticosterone, have a short latency of occurrence (within 15 min or less, depending on the agonist used) that is not compatible with the longer time required to elicit genomic effects by binding to nuclear receptors, hence suggesting an involvement of membrane mineralocorticoid receptors. However, available evidence about the mechanism of action of membrane corticosteroid receptors is too scarce to infer any reasonable explanation for
their age-dependent protective effect onto seizure susceptibility, although this property has been well documented.
1.4.5. Activation of the HPA axis and the modulation of proinflammatory cytokines expression by PGE$_2$

A molecule closely related to proinflammatory cytokines and the HPA axis activation and that may be relevant in the context of brain excitability and neurodegeneration at young ages is prostaglandin E$_2$ (PGE$_2$), the major enzymatic product of cyclooxygenase-2 (COX-2) activity (Brock et al., 1999).

Despite being formerly depicted as a strict proinflammatory molecules, PGE$_2$ has been shown to significantly modulate the level of proinflammatory cytokines expression and the HPA axis activation by proinflammatory stimuli. Indeed, in rodents, PGE$_2$ has anti-inflammatory effects in the CNS following brain injury and administration of LPS (Zhang and Rivest, 2001), while LPS-induced activation of the HPA axis has been shown to occur through the production of PGE$_2$ (Zhang and Rivest, 2003; Turrin and Rivest, 2004 (b); Blais et al., 2005). At present, it is largely accepted that PGE$_2$ may have a dual role in the CNS, being pro- or anti-inflammatory depending on the target cells and the type of insult. In particular, it has been shown that although PGE$_2$ increases LPS-induced NF-κB activity and COX-2 transcription in vascular-associated elements, it significantly reduces microglial activation and TNF-α expression in the brain parenchyma. Intracerebral PGE$_2$ infusion has been shown to largely prevent the expression of TNF-α and IL-1β mRNA elicited by tissue injury (Zhang and Rivest, 2001).
A reduction of microglial activation by PGE$_2$ is supported by in vivo evidence (Caggiano and Kraig, 1996) and in vitro (Théry et al., 1994; Caggiano and Kraig, 1996; Caggiano and Kraig, 1998; Minghetti et al., 1997), showing that PGE$_2$ can inhibit several microglial/macrophage functions, including the expression of major histocompatibility complex class II, NO production and synthesis of proinflammatory cytokines. The deactivating effect on microglial cells appears mediated by elevation of intracellular cAMP levels, and several studies suggest the involvement of the EP2 receptor subtype in this mechanism, although details of the cellular pathways involved are still lacking (Petrova et al., 1999; Caggiano and Kraig, 1998; Caggiano and Kraig, 1999).

The following paragraphs describe findings related to COX-2 and PGE$_2$ expression and modulation in glial and neuronal cells.
1.4.5.1 COX-2 and PGE$_2$ expression and regulation in glial cells

The COX-2 promoter contains binding sites for several transcription factors including NF-κB and CREB (Chun et al., 2004). However LPS-stimulated COX-2 expression in microglia occurs via several other pathways. Indeed, pathways related to p38 MAPK, p42/44 MAPKs and phosphocholine-specific phospholipase C (PC-PLC) appear to act in parallel without apparent cross-talk (Akundi et al., 2005), thus showing that overexpression of COX-2 might be a consequence of both increased transcription and enhanced mRNA stability. Of particular interest is the PC-PLC pathway which leads to activation of protein kinase C (PKC) by production of diacylglycerol (DAG). This kinase is able to activate NF-κB via phosphorylation of I-κB as well as p42/44 MAPKs through phosphorylation of Ras/Raf pathway (Sweatt, 2001). In microglia, PKC stimulates COX-2 transcription following LPS stimulation through a NF-κB as well as p42/44 MAPKs independent mechanism (Bauer et al., 1997; Akundi et al., 2005).

cAMP dependent modulation of LPS-induced COX-2 expression has been clearly shown in microglial cells (Minghetti et al., 1997). Interestingly, accumulation of cAMP by the adenylyl cyclase activator forskolin did not affect basal levels of COX-2 protein, but enhanced COX-2 protein levels following stimulation by LPS, or the cAMP analogue dibutyryl cAMP. This effect is mediated by protein kinase A (PKA) since its inactivation abolished the enhancing effect of LPS onto COX-2 protein expression. Importantly, PGE$_2$ release was shown to parallel the COX-2 pattern of expression following all
treatments reported above. Thus, these experiments suggest that cAMP accumulation \textit{per se} is not sufficient to induce COX-2 transcription and that another element is required to act in concert to modulate COX-2 expression. This element may be PKA, whose expression would be expected to be enhanced by LPS stimulation since basal levels of PKA are not enough to increase COX-2 transcription even in presence of an augmented cAMP availability (Minghetti et al., 1997).

Interestingly, exogenous PGE$_2$ enhances the LPS-induced expression of COX-2 without affecting its basal levels (Minghetti et al., 1997). This effect is mediated by EP$_2$ G$_s$-coupled receptors.

PKC regulates adenylyl cyclase activity differently in microglial cells and astrocytes. cAMP accumulation is quickly enhanced in astrocytes, peaking within a few minutes of exposure to PKC activators, but is unchanged in microglia, despite the phosphorilating activity of the kinase was increased (Patrizio et al., 1997). However, when microglia is stimulated with PGE$_2$, phosphorylation of EP$_2$ receptors by PKC results in a reduced accumulation of cAMP (Patrizio et al., 2000).
1.4.5.2 COX-2 and PGE\textsubscript{2} expression and regulation in neuronal cells

Although microglial cells account for the majority of COX-2 production (Minghetti and Levi, 1998), seizure-activity per se enhances COX-2 and PGE\textsubscript{2} expression in neurons (Cole-Edwards and Bazan, 2005).

In the mature hippocampus, COX-2 has been shown to play a significant role in neuronal excitability and physiological phenomena such as LTP. COX-2 is enriched in the dendritic spines of excitatory synapses (Kaufmann et al., 1996) and constitutive levels of expression of COX-2 are regulated by synaptic activity and up-regulated by high-frequency stimulation associated with long-term synaptic plasticity (Yamagata et al., 1993), a physiological feature which is particularly evident in brain structures such as the hippocampus.

A fundamental step in LTP induction is the spatiotemporal correlation of postsynaptic firing and excitatory postsynaptic potentials (Koester et al., 1998; Magee et al., 1997; Markram et al., 1997). A decrease in postsynaptic neuron membrane excitability, with a subsequent decrease of the number of postsynaptic action potentials during high-frequency stimulation of the presynaptic ending, diminishes the probability of LTP induction. Inhibition of constitutive COX-2 attenuates postsynaptic membrane excitability, thus decreasing the probability of LTP induction. Another important factor in LTP establishment is the active propagation of axonally generated action potentials back into the dendrites, where associated dendritic influx plays a significant role (Magee et al., 1997). Specifically, pharmacologic COX-2 blocking decreases
back-propagating dendritic action potential-associated Ca^{2+} influx (Chen et al., 2002).

Since PGE_{2} is the main product from the enzymatic conversion of arachidonic acid by COX-2 (Brock et al., 1999) and PGE_{2} receptors are expressed in the hippocampus (Zhu et al., 2005) then it is likely that PGE_{2} is the effector of COX-2 mediated activity-dependent synaptic plasticity (Chen et al., 2002). Consistently, DG granule cells specific inhibition of COX-2 (but not COX-1) was shown to decrease the induction of LTP, whereas administration of PGE_{2} (but not PGD_{2} or PGF_{2α}) reversed COX-2 inhibitor-induced suppression of LTP (Chen et al., 2002). Interestingly, it has been shown that PGE_{2} acts as a retrograde messenger in excitatory synaptic transmission through a presynaptic EP_{2} receptor that increases the probability of glutamate release at hippocampal synapse (Sang et al., 2005). In particular, the increased COX-2 expression (at postsynaptic dendritic spines) causes enhanced production of PGE_{2} which in turn augments synaptic transmission via a presynaptic EP_{2}-PKA pathway. Chen and Bazan (Chen and Bazan, 2005) showed that somatic and dendritic membrane excitability was significantly reduced when endogenous PGE_{2} was depleted using a selective COX-2 inhibitor (NS398). In addition, it was observed that exogenous application of PGE_{2} significantly increased the firing rate, excitatory postsynaptic potential amplitude, and temporal summation in slices treated with the COX-2 inhibitor. Interestingly, PGE_{2}-induced enhancement of excitatory postsynaptic potentials was blocked by inhibiting both PKA and PKC activities (Chen and Bazan, 2005). Accordingly, PKA and PKC are involved in the modulation of K^{+} currents in dendrites of hippocampal CA1 pyramidal neurons (Hoffman et al., 1998). Indeed, the activation of either PKA or PKC
significantly increase the amplitude of back-propagating action potentials in distal dendrites by down-regulation of transient $K^+$ channels, thus supporting a prolonged cell excitability as shown by an increased amplitude of back-propagating action potentials upon either PKA or PKC stimulation (Hoffman et al., 1998).

Thus, these data suggest that PGE$_2$ production and its interaction with its cognate receptors involve functional relationship among COX-2, PKA and PKC, possibly leading to relevant alteration of physiological phenomena such as LTP. In this regard, the developmental regulation of LTP induction is particularly relevant. In the adult hippocampus, one enzyme commonly implicated in LTP is Ca$^{2+}$/calmodulin-dependent kinase II (CAMKII) (Lisman et al., 2002). In rats aged PN 7-18 it was shown that parallel kinase cascades are involved in the induction of LTP at hippocampal CA1 synapses (Wikström et al., 2003). At this developmental stage, CAMKII participates in LTP induction, although its involvement is only manifest when either PKA or PKC is inhibited.

A stressful stimulus as mild neonatal isolation during the first postnatal week was shown to accelerate the developmental switch in the molecular mechanisms underlying hippocampal CA1 LTP from a PKA- to a CAMKII-dependent pattern (Huang et al., 2005). Importantly, the upregulation of CAMKII was mediated by CRH acting through CRF-R1, suggesting that activation of HPA axis could play an important role in modulation of neonatal LTP.

In the immature organism the role of PGE$_2$ is not well established since often the available evidence comes from adult rodents. On the one hand, the accumulation of free arachidonic acid and the formation of prostaglandins and
lipoxigenase reaction products in the brain during seizures is well established (Cole-Edwards and Bazan, 2005) and ibuprofen, a non-selective COX inhibitor, was shown to delay the onset of flurothyl-induced seizures in adult rats (Wallenstein and Mauss, 1984), suggesting a proconvulsant role of PGE$_2$. On the other hand, by activating the HPA axis, PGE$_2$ may exert an anticonvulsant action through the occupancy of membrane mineralocorticoid receptors by circulating corticosterone.
2. Aims
The available evidence shows that innate immune response, usually triggered in brain by systemic infection, involves proinflammatory signals which are also recruited by epileptic activity. The adaptive response to infection is rapid, reversible and aimed at eliminating pathogens from the host tissue. Conversely, chronic stimulation of proinflammatory signals by seizures, or a persistent proinflammatory situation in brain may contribute to the establishment of a pathological substrate (i.e. neurodegeneration, neuronal hyperexcitability, BBB damage, etc) playing a role in epileptogenesis and in the acute manifestation or reinforcement of seizures. In particular, it appears that the consequences of an increase in brain cytokines and related molecules on brain function depends on the extent and duration of these changes, on the subtype of cytokine receptors recruited and the pre-existing functional status of the cells exposed to cytokines.

In humans and in experimental models of epilepsy, seizure susceptibility and associated neuronal damage are age-dependent. The key determinants of this developmental occurrence are still unclear although evidence collected suggests that this is a multifactorial phenomenon.

Thus, in light of recent findings on inflammation and epilepsy, we hypothesized an age-dependent cause-effect relationship between proinflammatory molecules and seizure susceptibility and related neurodegeneration. Therefore, the goal of this thesis is to gain insights into this question. In particular, this work evaluates two hypothesis, i) the age-dependent changes in pro-inflammatory cytokines and their relation to
seizure induced neuronal damage and ii) the effect of inflammatory challenge and possible molecular mechanisms.

In relation to the age-dependent changes in pro-inflammatory cytokines and their relation to seizure induced neuronal damage, experiments will investigate the hypothesis that activation of glia and subsequent production of proinflammatory molecules such as IL-1β and TNF-α, as well as the naturally occurring anti-inflammatory molecule interleukin-1 receptor antagonist (IL-1Ra), are age-dependently involved in seizure-induced neuronal damage. In addition, as some proinflammatory molecules such as IL-1β have been shown to modulate susceptibility to seizures and ictal activity in experimental models of SE in adults, a possible IL-1β-induced age-dependent susceptibility to seizures will be also evaluated.

As it concerns the effect of inflammatory challenge at young ages and possible molecular mechanisms, experiments will investigate whether a pre-existing inflammatory state in the CNS may enhance the predisposition of rat pups to develop seizures. In this regard, these studies will use systemic administration of LPS in rat pups as a proinflammatory stimulus to investigate the relationship between IL-1β and TNF-α mRNA levels and what the role of the HPA axis activation and COX-2/PGE₂ might be in the modulation of seizure susceptibility in this experimental paradigm.

If a link between inflammatory reactions in brain and epileptogenesis/ictogenesis were found, this novel information may open new therapeutic perspectives for the treatment of seizures or their prevention.
3. Materials and methods

- general procedures -
In the following section, the general experimental procedures adopted in the experiments described in this thesis are provided. Further methodological details are given for each specific experiment before the presentation of the results.

*Please note that the experiments involving seizure induction by flurothyl ether inhalation were run in collaboration with Dr. Jana Veliskova at the Laboratory of Pediatric Epilepsy, Albert Einstein College of Medicine, New York, USA.*
3.1 Experimental animals

3.1.1 Choice of experimental animals

To validate experimental results in terms of a meaningful comparison with human pathology, we should consider how the developing rat brain compares with human brain (Moshè, 1987; Holmes, 1986). Rats are known to be born in a premature state relative to humans, thus several factors must be taken into account to ensure the rats used are representative of specific stages of human development. The evaluation of some developmental parameters such as the length of gestation, the rate of brain growth, synaptogenesis, protein synthesis, myelination and cerebral glucose utilization have led to defined age-intervals of rat development that match stages of human development (Moshè, 1987; Nehlig, 1997; Avishai-Eliner et al., 2002; Romijn et al., 1991), as summarized in the following table 6:
Table 6

Age-intervals of rat development that match stages of human development

<table>
<thead>
<tr>
<th>Rat (age-intervals)</th>
<th>Human (corresponding stages of development)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN8-10</td>
<td>Full-term newborn</td>
</tr>
<tr>
<td>PN12-18</td>
<td>Infant/toddler</td>
</tr>
<tr>
<td>PN25-32</td>
<td>Peripubertal child</td>
</tr>
<tr>
<td>PN32-38</td>
<td>Onset of puberty</td>
</tr>
<tr>
<td>&gt;PN60</td>
<td>Adult</td>
</tr>
</tbody>
</table>

Adapted from Haut et al., 2004
Accordingly, experiments were performed using rats aged postnatal day (PN) 9, 15 and 21 since these age-intervals match human brain development from birth to peripuberty, thus embracing human ages at which susceptibility to neuronal injury is known to progressively increase. Concomitantly, rats in these age groups are known to be susceptible to seizure-induced brain injury in an age-dependent manner (Albala et al., 1984; Ben-Ari et al., 1984; Holmes and Thompson, 1988 (b); Nitecka et al., 1984).
3.1.2 Animal care

Male Sprague-Dawley rats (Charles River, Calco, Italy) at PN 9, 15, and 21 (with PN 0 defined as the day of birth) were used. All animals were used before weaning. Pups were housed with their dams 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 conformity with the institutional guidelines that are in compliance with national (D.L.n.116, G.U, suppl. 40, Feb. 18, 1992) 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).
3.2 ‘In vivo’ models of seizures and status epilepticus

3.2.1 Induction of status epilepticus (SE) following systemic administration of kainic acid

Kainic-acid (KA) induced SE in rat is one of the widely accepted models of MTLE. KA acts specifically on the kainate receptor (one of the two ionotropic non-NMDA glutamate subtype receptors, the other being AMPA receptor). It is believed that induction of SE and the subsequent neurodegenerative pattern (see below) is triggered by activation of kainate receptors in the CA3 region of the hippocampus (Krnjevic et al., 1980), followed by release of the endogenous excitatory aminoacids glutamate and aspartate (Ferkany et al., 1982; Köhler et al., 1979) which in turn further activate all subtypes of glutamate receptors.

Typically, the progression to KA-induced SE consist of a sequence of stereotyped behaviours usually starting with staring and freezing within 5-15 min from administration. This is followed by mastication, head nodding, myoclonic twitches of the head and ‘wet dog' shakes in the subsequent 15-30 min, lasting for about 30 min. Eventually, within 1 h of administration of KA, recurrent generalized tonic-clonic seizures develop (Sperk, 1994). The EEG dynamics correlate well with behavioural changes, clearly defining ictal and interictal events. Injection of KA also induces biphasic changes in body temperature, with hypothermia in the first 30-60 min, followed by hyperthermia lasting for several hours (Ahlenius et al., 2002).
This experimental model of MTLE shows two relevant features. Firstly, after several hours SE ceases and a silent period follows during which epileptogenic mechanisms are believed to occur. This period lasts for weeks and, eventually, spontaneous seizures develop. Secondly, behavioural changes are followed by neurodegeneration which appears prominent in hippocampus, entorhinal and piriform cortex, thalamus and amygdala (Sperk et al., 1985). In particular, a typical pattern of neurodegeneration, similar to pathological findings in human MTLE, occurs in the hippocampus. Thus, cell loss mostly affects CA3 pyramidal cells and interneurons in the dentate gyrus (Ben-Ari, 1985) followed by CA1 neurons (Sperk et al., 1985; Phelps et al., 1991), inducing cell death both through necrotic and apoptotic mechanisms (Ferrer et al., 1995; Simonian et al., 1996; Popescu et al., 2002). CA2 pyramidal neurons as well as granule cells are the most resistant to neurodegeneration. Structural alterations are reminiscent of human pathology, thus mossy fibre sprouting, astrocytosis and remodelling of GABAergic connections are known to take place.

This reproducible pattern of neurodegeneration can be induced by systemic, intrahippocampal, intra-amygdaloid or intracerebroventricular administration of KA, provided the generalized motor seizures are not terminated within about 20-60 min (Ben-Ari, 1985; Tanaka et al., 1992). Importantly, the onset of neuronal injury induced by KA administration has been shown to be age-dependent (Albala et al., 1984; Ben-Ari et al., 1984; Holmes and Thompson, 1988 (b); Nitecka et al., 1984). KA administration triggers behavioural SE at all ages, each manifesting its own motor pattern. In PN9 and PN15 rats SE is characterized by bilateral clonic seizures of all the extremities,
whereas in PN21 rats, limbic motor seizures were characterized by continuous forelimb clonus (Albala et al., 1984; Friedman et al., 1997).

The preference of KA over pilocarpine for inducing SE in rats has also been driven by the consideration that the intensity of damage appears to be more severe in pilocarpine-treated rats (Covolan and Mello, 2000). In particular, pilocarpine administration can induce extensive damage to granule cells in the DG, whereas KA produces little damage (Covolan et al., 2000). Therefore, the neurodegenerative pattern induced by KA administration appears to be more similar to human MTLE-associated histopathological findings, since damage to granule cells in the DG accounts for no more than 5% of total neurodegenerative patterns occurring in MTLE sufferers. Finally, the human hippocampus is known to be vulnerable to excitotoxicity triggered by activation of kainate receptors. In 1987 several Canadian patients were affected by domoic acid poisoning following ingestion of mussels. Neuropathological studies revealed lesions predominantly in the hippocampus and amygdala, resembling the pattern of neurodegeneration induced by administration of KA. Indeed, domoic acid is a structural analogue of KA, thus acting on kainate receptors, but its excitotoxicity is much higher as compared to KA itself. It was determined that the mussels had accumulated domoic acid, synthesized by the phytoplankton *Nitzchia pungens* (Teitelbaum et al., 1990). A further study (Cendes et al., 1995) described a patient responding with complex partial SE following acute intoxication of domoic acid. After a latent period of 1 year after the acute episode, the patient developed MTLE. MRI and post-mortem examinations showed a MTS pattern of neurodegeneration.
3.2.2 Seizure induction by inhalation of flurothyl ether

Flurothyl (bis-2,2,2-trifluoroethyl ether, Flura Corp., USA) is a liquid convulsant which quickly evaporates, thus inducing seizures by inhalation. In our experimental setting, rat pups were placed in an air-tight chamber (9.38 l) and flurothyl was continuously delivered into this chamber via a precise microinfusion pump (WPI, Inc., USA).

The advantage of continuous flurothyl infusion is that seizures always occur. Two seizure types develop after flurothyl exposure: clonic and tonic-clonic seizures. Clonic seizures consist of clonic convulsion of head and forelimb muscles with preserved righting reflex. Tonic-clonic seizures are usually preceded by wild running followed by a loss of righting reflex. Then, there is a short tonic contraction of fore- and hindlimbs followed by long clonic convulsions of all four limbs. In rat pups, the first episode of clonic seizures is almost immediately followed by a tonic-clonic seizure.

In the case of SE induction, flurothyl was infused at 30 µl/min for the first 10 min and then at 15 µl/min for the remaining time, and rat pups were left exposed to SE for 1 h.

In the case of seizure threshold testing, flurothyl was infused at 40 µl/min. We recorded the latency to the onset (measured from the beginning of flurothyl delivery) of the first clonic and first tonic-clonic seizure, with a limit of 20 min flurothyl exposure. Since flurothyl was infused at a constant rate, the latency to the onset of seizures allowed us to calculate the amount of infused flurothyl necessary to elicit seizures, i.e., the flurothyl seizure threshold for clonic or tonic-clonic seizures for our chamber size. The seizure threshold inversely reflects
seizure susceptibility. Thus, the higher the seizure threshold, the lower the seizure susceptibility.
3.3 Drugs

3.3.1 Butaprost, SC19220 and SC58125

In the rat, Butaprost (9-oxo-11α, 16S-dihydroxy-17-cyclobutyl-prost-13E-en-1—oic acid) is a selective EP2 receptor agonist and SC19220 (8-chlorodibenz(b,f)(1,4) oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide) is a selective EP1 receptor antagonist (Boye et al., 1997; Coleman et al., 1997). Doses and time of administration were chosen on studies based on functional studies exploiting subcutaneous or intraperitoneal injection as drug delivery method (Takeuchi et al., 2003; Pons et al., 1994; Pons et al., 1989; Barbieri et al., 1977). Butaprost and SC19220 (Cayman Chemicals, USA) were dissolved in 25% Tween 80 and injected subcutaneously (3 mg/kg, each). Their functional effects were evaluated 30 min after the administration. SC58125 (1-((4-methylsulfonyl)phenyl)-3-tri-fluoromethyl-5-(4-fluorophenyl)-pyrazole) is a selective inhibitor of COX-2 (Seibert et al., 1994; Kurumbail et al., 1996). The doses and the time of administration of SC58125 were selected on the basis of studies indicating i) the time the molecule requires to penetrate the blood-brain barrier (McCarthy et al., 2002) and ii) dose-response data showing significant inhibition of PGE₂ levels in the brain parenchyma (Nakayama et al., 1998). SC58125 (Cayman chemicals, USA) was dissolved in 0.1 M PBS and injected intraperitoneally (3 mg/kg) and its functional effects were evaluated 30 min after the administration.
3.3.2 Canrenoic acid and RU486

Canrenoic acid (the main metabolite of Spironolactone) and RU486 are selective antagonists of mineralocorticoid receptor and glucocorticoid receptor respectively. The doses of antagonists and timing of injections were chosen based on functional studies and binding assays demonstrating the time span necessary to allow proper passage of the drugs through the blood-brain barrier and their selective antagonism of mineralocorticoid and glucocorticoid receptors (Grupp et al., 1985; Semler et al., 1989; Baron et al., 1991; Herman and Spencer, 1998; Smriga et al., 1998; Koenig and Olive, 2004). Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg). RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg). The functional effects of Canrenoic acid and RU486 were evaluated 30 min after drug delivery.
3.3.3 IL-1β

The doses and the time of administration of IL-1β were selected on the basis of the studies indicating excitatory effects of these doses on seizure activity and neuron viability (Vezzani et al., 2000; Vezzani et al., 2002; Rothwell, 1991; Morganti-Kossmann et al., 1992). Doses of IL-1β administered were 5-100-200-500 ng/5 µl PBS 0.1 M, icv (see protocol for icv injection below). The effect of cytokine on seizure threshold was tested 5 min after the icv administration.

3.3.4 Intracerebroventricular (icv) injections of IL-1β

Rats were surgically implanted with cannulas under stereotaxic guidance as previously described (Vezzani et al., 1999). Briefly, rats were deeply anesthetized by administration of Equithesin (1% Phenobarbital and 4% chloral hydrate, 3 ml/kg i.p.). Bilateral stainless-steel guide cannulas (5 mm length) were implanted 1 mm above the lateral ventricles (AP, 0.2-0.3 mm caudal from bregma; L, ± 1.6 mm; V, 3.3 mm from the skull surface). The cannulas were secured with fast-curing dental acrylic. Bilateral icv infusion of IL-1β was performed by using stainless-steel internal cannulas (6 mm length), each connected by a polyethylene tube to a 10-µl Hamilton syringe.
3.4 Immunohistochemistry, Western blotting and Fluoro-Jade staining

3.4.1 Immunohistochemical detection of microglia and astrocytes

For immunohistochemical analysis of microglia and astrocytes, experimental rats and their controls (n = 5-10 each group) were killed 4 and 24 h after SE onset. At these time points we previously found significant glial cell activation by SE in adult rat brain (Vezzani et al., 1999; De Simoni et al., 2000). Rats were deeply anaesthetized using Equithesin [1% pentobarbital/4% (v/v) chloral hydrate; 3.5 ml/kg, i.p.] and transcardially perfused with 0.05 M PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were postfixed in fixative for 4 h then cryoprotected in 20% sucrose in PBS overnight and rapidly frozen at —25°C.

Serial horizontal 40μm forebrain sections were cryosectioned at —20°C. The sections encompassed the whole septotemporal aspect of the hippocampus. Adjacent slices were used in each brain for detection of microglia and astrocytes using specific markers, complement receptor type 3 (OX-42) and glia fibrillary acidic protein (GFAP) respectively and for detection of neuronal injury using Fluoro-Jade (Schmued et al., 1997).

Slices were incubated at 4°C for 30 min in 0.4% Triton X-100 in PBS followed by a 15-min incubation in 3% fetal bovine serum (FBS) in 0.1% Triton X-100 in PBS. They were subsequently incubated overnight at 4°C with the
primary antibodies: mouse monoclonal antibody against OX-42 (Serotec Ltd, Oxford, UK, 1:100) or GFAP (Chemicon Int. Inc., Temecula, USA, 1:2500) in 3% FBS in 0.1% Triton X-100 in PBS. Immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Labs, USA). Sections were then reacted by incubation with 3',3'-diamino-benzidine (Sigma, Milan, Italy), mounted onto gelatine-coated slides, dehydrated in graded alcohols, and coverslipped.
3.4.2 Detection of degenerating neurons by Fluoro-Jade staining

Fluoro-Jade is an anionic tribasic fluorescein derivative with an emission peak at 550 nm and excitation peaks at 362 and 390 nm respectively. Fluoro-Jade staining was carried out as originally described by Schmued et al., (Schmued et al., 1997).

Brain sections were mounted with distilled water onto gelatine coated slides and dried on a slide warmer at 45° C. Dried tissue was then immersed in 100% ethanol for 3 min followed by a 1 min change in 70% ethanol and a 1 min change in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 15 min and were gently shaken on a rotating platform. Then, the slides were rinsed for 1 min in distilled water and transferred to the Fluoro-Jade staining solution (0.001% Fluoro-Jade and 0.1% acetic acid, in distilled water), where they were gently agitated for 30 min. After staining, the sections were rinsed with three 1 min changes of distilled water. Excess water was drained off, and the slides were air dried on a slide warmer. When dry, the slides were immersed in xylene and then coversliped.
3.4.3 Western blotting

Rats were decapitated 4 h after the SE onset. Both hippocampi were dissected out at 4°C, pooled, 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, and 2 µg/µl leupeptin (30 mg tissue/100 µl homogenization buffer).

Total proteins were measured in each sample using the Bio-Rad Protein Assay (Bio-Rad Labs, Munchen, Germany). Twenty-five micrograms of total proteins per lane were analyzed using SDS-PAGE, 10% acrylamide, and each sample was run in duplicate in two different gels. Proteins were transferred to Hybond nitrocellulose membrane by electroblotting.

For immunoblotting, we used a mouse monoclonal antibody against GFAP (Clone G-A-5, 1:5000; Chemicon Int Inc.). This antibody has been previously characterized in detail (Debus et al., 1983) and it recognizes in Western blot a specific band of ~50 kDa corresponding to GFAP. It shows no cross-reactivity with other intermediate filament proteins. Immunoreactivity was visualized with enhanced chemiluminescence (ECL, Amersham, UK), using peroxidase-conjugated goat antimouse IgG (1:2000; Sigma-Aldrich, St. Louis, MO, USA) as secondary antibody. Densitometry was used to quantify the changes in protein levels in the immunoblots (AIS image analyzer, Imaging Research Inc., Ontario, Canada). Values obtained for each sample run in duplicate in two different gels were averaged to take into account variability due to protein loading. Assay variability in each sample was <10%. Film exposures
with maximal signals below the photographic saturation point were used in the densitometric analysis.
3.5 Evaluation of gene expression

3.5.1 Total RNA extraction and DNase I treatment

Rats were killed by decapitation at selected timepoints. Brains were removed and both hippocampi were dissected out at 4°C and rapidly frozen to — 70°C until assayed. Total RNA was isolated from tissue samples according to the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987). The samples were digested by a denaturing solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, pH 7.0. The lysate was extracted with a mixture of 2 M sodium acetate, pH 4.0/phenol/chloroform-isoamyl alcohol (49:1), with relative ratios of 1:10:2 respectively, and nucleic acids were precipitated with equal volumes of isopropyl alcohol. Total RNA was quantified by spectrophotometer whilst an aliquot was loaded onto 1 % agarose gel in order to visually check for RNA integrity. Before performing RNA reverse transcription to cDNA an aliquot of each sample (1-2 µg) was treated with 1-2 U/µl of DNase I (Invitrogen, S. Giuliano Milanese, Italy) in order to minimize genomic DNA contaminants.
3.5.2 Synthesis of cDNA by total RNA reverse transcription

The preferred method to generate cDNA was reverse transcription of total RNA with a Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) using random primers. The use of random hexamers produces the least bias in the resulting cDNA and is amenable to both relative and standard-curve quantification methods (see below). Moreover, the use of random primers maximizes the number of mRNA molecules that can be analyzed from a small sample of RNA.

Practically, 1-2 µg of DNase-treated total RNA from each sample was used as a substrate for single-stranded cDNA synthesis using murine leukemia virus reverse transcriptase (50 U/µl; Perkin-Elmer, Emeryville, CA, USA), random hexamers (2.5 µM), and deoxyNTP mix (1.25 mM each) in a final volume of 20 µl. The mixture was stored at room temperature for 10 min, then incubation was performed in a thermocycler (Omn-E; Hybaid, Ashford, UK) at 42°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min.

For sample processing (i.e. real-time quantitative PCR) it was noticed that a two-step RT-PCR approach, in which the reverse-transcription reaction is performed in a separate tube to that of the PCR, was preferred to a one-step RT-PCR reaction, in which both reactions are performed in the same tube. Primarily, the two-step method produces cDNA that can be used in many experiments with several different genes and with less handling of the RNA, which can lead to degradation.
3.5.3 Measurement of gene expression by real-time PCR

The levels of expression of mRNAs were determined by real-time quantitative PCR, performed with 5700 SDS instrumentation (Applied Biosystems), exploiting SYBR Green I as fluorescent dye. The specificity of SYBR Green I dye to a specific DNA target is entirely determined by primers, since this dye binds any double stranded nucleotide sequence.

In order to reach high efficiency rate of amplification most of the steps to optimize the reaction of amplification were accomplished according to the manufacturer of the real-time PCR instrument. Indeed, amplicon and primer design were accomplished by Primer Express software (Applied Biosystems) set with default parameters. Thus, the annealing temperature of each primer was restricted to 58-60°C, which corresponds to the optimal working conditions for the used DNA polymerase enzyme (AmpliTaq Gold, Applied Biosystems). General conditions for primer design were also followed, i.e., to keep the G/C content in the 20-80% range, to avoid runs of an identical nucleotide (especially for guanine) and, when possible, no more than two G and/or C bases at 3' end. Software allowed to check for hairpins, loops and potential primer-dimers thus permitting a finer tuning of primer pair selection.

Primers were not designed on different exons or intron-exon boundaries. To avoid co-amplification of contaminating genomic DNA, all RNA samples were treated with RNase-free DNase (see paragraph 3.4.2).

After primer design, the concentrations of each pair of forward and reverse primers that provide optimal assay performance (lowest threshold cycle, Ct, with highest efficiency) was identified.
Since the incorporation of SYBR Green I into real-time PCR allows the
detection of any double-strand DNA generated during PCR, a dissociation curve
analysis (analysis of melting temperature profiles) was performed upon
completion of each PCR run. Dissociation curve analysis is equivalent to
agarose gel analysis in order to assure for amplification of specific products and
the lack of any spurious band due to non-specific amplification.

The following tables 7 and 8 summarize the operative conditions we used
for measurement of gene expression by real-time PCR and list the primer pairs
used for each specific gene of interest.
Table 7. Summary of the operative conditions used for measurement of gene expression by real-time PCR

<table>
<thead>
<tr>
<th>Real-time PCR operative conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>➤ <strong>Replicate number</strong> for each sample loaded on reaction plate: 4</td>
</tr>
<tr>
<td>➤ <strong>Reagents and reaction buffer</strong>: SYBR Green I PCR Master Mix (ABI)</td>
</tr>
<tr>
<td>➤ <strong>Amplification Instrument</strong>: GeneAmp 5700 Sequence Detection System (ABI)</td>
</tr>
<tr>
<td>➤ <strong>Amplification Instrument operative conditions</strong>:</td>
</tr>
<tr>
<td>• 2 min at 50 °C</td>
</tr>
<tr>
<td>• 10 min at 95 °C</td>
</tr>
<tr>
<td>• 5 sec at 95 °C</td>
</tr>
<tr>
<td>• 1 min at 60 °C</td>
</tr>
<tr>
<td>➤ <strong>Dissociation Curve</strong> (melting temperature profile): starting at 60 °C to 90 °C</td>
</tr>
<tr>
<td>➤ <strong>Quantification Method</strong>: standard scale</td>
</tr>
</tbody>
</table>
Table 8. List of the primer pairs used for the amplification of each specific gene of interest

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong> (NM_031512)</td>
<td>ACAGCAATGGTCGGGACATAG</td>
<td>CTTGGCAGAGGACAAAGGCT</td>
</tr>
<tr>
<td><strong>IL-1Ra</strong> (NM_022194)</td>
<td>CTTCTACCTGAGGAACACCAGC</td>
<td>AGACTTGACAAAGACAGGCACA</td>
</tr>
<tr>
<td><strong>TNF-α</strong> (NM_012675)</td>
<td>ATCCGAGATGTGGAACTGGCA</td>
<td>CGATCACCCCAGATGGCAGTAG</td>
</tr>
<tr>
<td><strong>IL-6</strong> (M26744)</td>
<td>TCTGATTGTATCAACAGCGATGAG</td>
<td>CCAGGTAGAAACGGAAACTCCAG</td>
</tr>
<tr>
<td><strong>IL-4</strong> (NM_201270)</td>
<td>ACCTCCGTGCTTGAAGAACAA</td>
<td>CATTACGGTCAGCTTCTC</td>
</tr>
</tbody>
</table>
Table 8 – (continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>GAAGAGAAACCAGGTGCTCCTT</td>
<td>GTCACAGCTTTCGAGACTGGAA</td>
</tr>
<tr>
<td>iNOS</td>
<td>GGAGGAGAGAGATCCGGTTCA</td>
<td>GAGGACTGTGGCTTGACGC</td>
</tr>
<tr>
<td>PKC-γ</td>
<td>CTGTATTTTTGATGGAGTACGTCACT</td>
<td>TCCGCGGCATAGAATGCT</td>
</tr>
<tr>
<td>PKA RIIβ</td>
<td>AGCTTGCCCTGGTCACTAACA</td>
<td>CCCAGAAGCCTCTCAATGCT</td>
</tr>
<tr>
<td>COX-2</td>
<td>TGATCGAAGACTACGTGCAACA</td>
<td>CAATGCCTGTTCTGATACTGGAA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GAAGTGAGACGTTGACATCCGT</td>
<td>CACAGAGTACTTGCCTCAGGA</td>
</tr>
</tbody>
</table>

* Housekeeping gene for samples normalisation
4. Experimental section
4.1 Evaluation of glia activation and cytokine expression in the rat hippocampus by kainic acid-induced status epilepticus during postnatal development

The key determinants of the age-dependent occurrence of seizure-induced injury are still unclear. The present investigation starts by testing the hypothesis that activation of glia and the subsequent production of inflammatory molecules are involved in seizure-induced neuronal damage. To test this, we used rats of different ages [postnatal days (PN) 9, 15, and 21] known to have distinct susceptibility to seizures and neuronal injury. We studied (i) whether microglia and astrocytes are activated after SE induced by kainic acid, because these cells represent the major source of cytokine synthesis in mature rodent brain (Allan and Rothwell, 2001); (ii) whether proinflammatory (IL-1β, IL-6, and TNF-a) and anti-inflammatory (IL-1Ra) cytokines are induced by SE in immature rat brain; and (iii) if there is a relationship between SE-induced cytokine changes in immature brain and the occurrence of neuronal cell injury.
4.1.1 Materials and methods

4.1.1.1 Induction of status epilepticus

Different doses of intraperitoneal kainic acid (1.3, 5, and 7.5 mg/kg) were injected in PN9, PN15, and PN21 rats respectively because the seizure threshold to kainic acid is age-dependent (Cherubini et al., 1983; Albala et al., 1984; Veliskova et al., 1988). As previously described in the chapter 'Materials and methods – general procedures', we choose this model because it is well-established that SE induced by kainic acid is associated with age-dependent onset of neuronal injury (Albala et al., 1984; Ben-Ari et al., 1984; Holmes and Thompson, 1988 (a)). Controls were age-matched rats injected with an equivalent volume of phosphate-buffered saline (PBS, pH 7.4). Rats were monitored for about 3 h after kainic acid injection for behavioural manifestation of SE.

Kainic acid induced behavioural SE at all ages, with an average onset of 30 min and duration of at least 3 h. As previously described in detail (Albala et al., 1984; Friedman et al., 1997), in PN9 and PN15 rats SE was characterized by bilateral clonic seizures of all extremities, whereas in PN21 rats, limbic motor seizures were characterized by continuous forelimb clonus.

Rats were returned to the lactating mother until sacrificed.
4.1.1.2 Immunohistochemistry and Fluoro-Jade staining

Technical details related to immunohistochemical detection of OX-42 and GFAP as well as Fluoro-Jade staining have already been reported in 'Materials and methods – general procedures'.
4.1.2 Results

4.1.2.1 Glia activation by SE during postnatal development

Kainic-acid induced SE age-dependently increased OX-42 and GFAP immunostaining mainly in the hippocampus with no differences between individual hippocampal regions examined (CA1, CA3, dentate gyrus).

4.1.2.2 Changes of OX-42 immunostaining

Figure 7 shows OX-42 immunostaining in area CA3 of hippocampi from representative PN 9, PN 15, and PN 21 rats, 4 h and 24 h after SE onset, and their respective controls (n = 5–10 rats each group). Faint immunoreactivity was found in control sections at all postnatal ages. High magnification analysis of cell morphology at PN 9 (Fig. 6, control panel) revealed the presence of primitive ramified microglia, i.e., microglia with an oval, slightly elongated shape and scanty developed processes, whereas at PN 15 (Fig. 6, control panel) reactive-like microglia (i.e., round to oval shape and coarse processes) was also observed (Dalmau et al., 1998). At PN 21, OX-42 staining in control sections (Fig. 6, control panel) was too faint to allow clear definition of cell morphology. This corresponds to the adult immunoreactivity pattern previously reported (Vezzani et al., 1999). SE induced little activation of microglia in PN 9 rats at either time points. OX-42 immunostaining was enhanced by seizures in PN 15
and PN 21 rats (Fig. 6, 4 and 24 h panels) since strongly immunoreactive cells resembling reactive and ameboid microglia (Kreutzberg, 1996) were observed at both postnatal ages (Fig. 6, high magnification panels). In PN 15 rats, staining of microglia was enhanced also in the subiculum, whereas in 40% of PN 21 rats, microglia staining was increased also in the amygdala, entorhinal cortex, external layers of the temporal cortex, and periventricular thalamic nuclei (not shown).
Representative photomicrographs of horizontal sections of the ventral hippocampus showing OX-42-positive microglia, 4 and 24 h after the onset of kainic acid-induced SE in PN 9, PN 15, and PN 21 rats. CA3, pyramidal layer; h, hilus. Scale bar, 100 μm.

Fig. 6
4.1.2.3 Changes of GFAP immunostaining

GFAP staining was diffusely low in control sections at all postnatal ages (Fig. 7). High-magnification analysis of control sections showed stellate-shaped astrocytes with more pronounced arborization at PN 21. GFAP immunostaining was slightly enhanced by SE in PN 9 rats, 4 h and 24 h after SE onset as depicted in area CA3 of the hippocampus.

In PN 15 and PN 21 rats, a pronounced increase in GFAP signal was observed at both time points. GFAP-positive cells showed markedly enlarged cell bodies and thickening of processes, typical features of an activated phenotype. In PN 15 and PN 21 rats, the pattern of astrocyte activation in other forebrain areas was overlapping with that described for microglia.
Representative photomicrographs of horizontal sections of CA3 area of the ventral hippocampus showing GFAP-positive astrocytes, 4 and 24 h after the onset of kainic acid-induced SE in PN 9, PN 15, and PN 21 rats. CA3, pyramidal layer; h, hilus. Scale bar, 100 μm.

Fig. 7
4.1.2.4 Western blot analysis of GFAP activation

Fig. 8, panel A, shows a Western blot analysis of GFAP from hippocampi of PN 9, PN 15, and PN 21 rats, 4 h after the onset of KA-induced SE, versus respective controls, accomplished in order to provide a semi-quantitative measure of reactive astrocytosis. Bargram depicts mean ± SEM (n = 4) of the optical density (OD) of the 50-kDa band corresponding to GFAP expressed as a percentage of respective control values.

A densitometric analysis of protein bands (fig. 8, panel B) showed a significant age-dependent increase in GFAP concentration in the hippocampus of control rats. At PN 15, GFAP concentration in the hippocampus was 26 ± 1.5% (p < 0.05) higher than at PN 9. At PN 21, GFAP concentration was 60 ± 3% higher than at PN 9 (p < 0.01) and 34 ± 1.5% higher than at PN 15 (p < 0.01). Four hours after SE, GFAP levels did not change at PN 9, whereas they increased by 35% on average at PN 15 and PN 21. An attempt of measuring microglia activation by Western blot using the antibodies OX-42 and ED-1 (Serotec, Oxford, UK) both labelling microglia in immunocytochemistry was unsuccessful since both antibodies did not recognize their specific antigens when using brain homogenates under denaturized conditions.
Panel A. Western blot analysis of GFAP in the hippocampus, 4 h after the onset of kainic acid-induced SE in PN 9, PN 15, and PN 21 rats. Panel B. Representative Western blots showing the increase in the GFAP band after seizures at PN 15 (lane 4) and PN 21 (lane 6) versus respective controls (lanes 3 and 5). No changes in GFAP were found after seizures at PN 9 (lane 2) versus control (lane 1).

*p < 0.05, **p < 0.01 vs control by Mann–Whitney.

Fig. 8
4.1.2.5 Cytokine mRNA expression

The mRNA expression of the various cytokines was evaluated in the hippocampus of PN 9, PN 15, and PN 21 rats, 4 h after the onset of KA-induced SE (Fig. 9). Data are the mean ± SEM, (n = 6–10 rats) and are expressed as percentage of basal values of each cytokine. The amount of mRNA was calculated as the ratio of densitometric measurement of the cytokine samples to the corresponding internal standard (β-actin). Cytokine mRNA expression was unaffected by SE at PN 9, whereas in PN 15 rats, only IL-1β mRNA was increased by 2.2-fold (p < 0.01). In PN 21 rats, all cytokines were increased. Thus, IL-1β and IL-6 mRNA expression was augmented by 3.6-fold above control (p < 0.01) and TNF-α and IL-1Ra by 1.5-fold on average (p < 0.05, p < 0.01). IL-1β mRNA was induced to a significantly larger extent in PN 21 compared to PN 15 rats (p < 0.05). A more detailed time-course of changes in PN 15 rats (2, 4, 18, and 24 h after the onset of SE) showed that IL-1β mRNA expression was not modified at 2 h. The expression of IL-1β was significantly increased over basal levels at 4 h. This increment was transient and returned to control values by 18 h. The expression of the other cytokines did not change at any time point tested (fig. 10).
mRNA expression of IL-1beta, IL-1Ra, TNF-alpha and IL-6 in the hippocampus of PN 9, PN 15 and PN 21 rats 4 hours after SE induced by KA

Densitometric analysis of cytokine mRNA expression in rat hippocampus 4 h after SE induced by kainic acid in PN 9, PN 15, and PN 21 rats. *p < 0.05, **p < 0.01 vs respective age-matched controls by Mann–Whitney. IL-1β mRNA levels after SE at PN 21 were significantly higher than at PN 15 (p < 0.05)

Fig. 9
Time-course of IL-1beta mRNA expression after KA-induced seizures in PN 15 rats

Densitometric analysis of IL-1β mRNA expression 2, 4, 18, and 24 h after the onset of KA-induced SE in the hippocampus of PN 15 rats. *p < 0.05 vs control group by Kruskal Wallis.

Fig. 10
4.1.2.6 Evaluation of neuronal cell injury by Fluoro-Jade staining

Neuronal cell injury was not observed in the hippocampus of PN 9 rats (n = 5–6) (fig. 11, panel A) or in any forebrain area, 4 and 24 h after SE onset. Evidence of Fluoro-Jade-positive neurons was found only 24 h after the SE onset in PN 15 and PN 21 rats. At PN 15 (n = 7), scattered positive pyramidal CA3 neurons were found in the most temporal aspect of the ventral hippocampus (fig. 11, panel B). At this age, few Fluoro-Jade-positive neurons were also detected in the dorsal part of the subiculum (fig. 11, panel C) but not in other forebrain areas. At PN 21 (n = 10), various pyramidal neurons in CA1 (not shown) and in CA3 (fig. 11, panel D) were labelled in the ventral and dorsal hippocampus. At this age, 40% of rats showed Fluoro-Jade-positive neurons in the basolateral amygdala (fig. 11, panel E), entorhinal and temporal cortices, and periventricular thalamic nuclei. In PN 15 and PN 21 rats, consistent glia activation was found in the same brain areas showing injured neurons (not shown).
Fluoro-Jade labelling of hippocampal neurons in horizontal section of the forebrain, 24 h after kainic acid-induced SE in PN 9, PN 15, and PN 21 rats. CA3, pyramidal layer; sl, stratum lacunosum-moleculare; so, stratum oriens; h, hilus. Scale bar, 100 µm.

Fig. 11
4.1.3 Discussion

Results obtained in this experimental section show that activation of microglia and astrocytes, major sources of cytokines in the brain, as well as cytokine neosynthesis induced by SE, are age-dependent. Thus, at PN9 little activation of glia occurs and cytokines are not synthesized despite severe seizure activity. At PN15 and PN21 glia is strongly activated; IL-1β mRNA only is induced at PN15, whereas the profile of cytokine mRNAs induction at PN21 is similar to adult rat brain (De Simoni et al., 2000).

The limited sensitivity of ELISA methods and lack of specific signal in immunocytochemistry with available antibodies in immature brains did not make the measure of cytokine proteins possible. In this regard, it is relevant to observe that the interpretation of our experimental data relies on the assumption that upon mRNA increasing a corresponding protein synthesis occurs. Nonetheless, several evidence has shown that transcription can be an isolated event from translation. A variety of stimuli, including LPS, IL-1β and TNF-α, were shown to increase IL-1β transcription without affecting translation, depending on the system under investigation (Fenton, 1992; Chantry et al., 1989; Sung and Walters, 1991; Knudsen et al., 1986; Dinarello, 1992). Indeed, both positive and negative regulation of translation occur at multiple sites of modulation along the translation pathway (Watkins et al., 1999; Fenton, 1992). Among several factors involved in the regulation of translation, relevant roles were shown to be played by the modulation of the percentage of mRNA sequestered in a functionally inactive state within structures called messenger ribonucleoprotein particles (Hershey, 1991), the modulatory effect mediated by proteins binding at
specific regulatory sites within the 5' UTR (Jansen et al., 1995) and the interactions between this region and the poly(A) tail which is also associated with specific binding proteins (Jackson, 1993). Additionally, the presence of AU rich element in the 3' UTR is known to modulate translation (Kruys and Huez, 1994). In particular, the AU rich elements were shown to modulate the translation stability both by tonically decreasing the efficiency with which protein is formed and by expression of binding sites for proteins that can enhance or suppress the effect mediated by the same AU rich elements (Pain, 1996; Kruys and Huez 1994; Kruys et al., 1989). In this regard, the AU rich elements found in cytokines mRNAs are thought to tonically suppress translation via binding of a constitutive repressor protein.

In principle, the complexity of gene transcription modulation of molecules such as proinflammatory cytokines and the immaturity of the organism under investigation make the possible dissociation between transcription and translation a conceivable hypothesis. Indeed, it cannot be ruled out that the production of several modulators potentially relevant for bridging the gap between transcription and translation may not be appropriately entered into play at young ages, maybe due to the immaturity of signalling pathways and transcriptional/translational machineries.

By comparing the developmental patterns of cytokine induction in the hippocampus with the appearance of Fluoro-Jade-positive neurons, it was evident that de novo cytokine synthesis, and in particular that of IL-1β closely parallels the age-dependent profile of SE-induced cell injury. Noteworthy, IL-1β mRNA at PN15 and PN21 increased in the hippocampus 4 h after SE onset, thus before the appearance of injured neurons (i.e., 24 h after seizures). The increased
expression was more pronounced in PN21 rats in which a greater degree of injury was observed.

At PN15 only IL-1β is synthesized after SE, whereas the entire variety of proinflammatory cytokines is produced at PN21 as in adult brain (De Simoni et al., 2000). It is therefore possible that IL-1β needs to work in concert with other cytokines in order to contribute to the occurrence of significant neuronal damage. Accordingly, IL-1β and TNF-α in combination have been shown to cause marked neuronal injury in neuronal cell cultures although neither cytokine alone was toxic (Chao et al., 1995).

The possibility that the absence, or limited, cytokine induction within the first three postnatal weeks might be due to lack of hippocampal involvement in paroxysmal activity can be excluded, since it has been established that kainic acid induces strong metabolic activation and electrocorticographic seizures in the hippocampus of immature rats by PN9 (Albala et al., 1984; Ben-Ari et al., 1984; Stafstrom et al., 1992).

Thus, the age-dependent profiles of mRNA induction appear to play a pivotal role in favour of the hypothesis that neurodegeneration might occur when seizures are able to induce the genome transcription of TNF-α and, in particular, IL-1β.

It is of interest to observe that the ontogeny of physiological as well as KA-induced activity of nuclear regulatory elements that govern the gene transcription of proinflammatory cytokines in the rat hippocampus might contribute to describe our results.
4.1.3.1 Ontogeny of KA-induced AP-1 mRNAs, proteins and DNA binding activity in the rat hippocampus

The activator protein-1 (AP-1) represents a family of transcription factors which bind to AP-1 regulatory site (TGACTCA) located in the promoter region of target genes hence modulating transcriptional activity. This protein family includes c-fos and fos-related antigens (fra) as well as the jun-related factors. The AP-1 DNA binding complex is usually composed of a fra:jun heterodimer, although jun proteins can also form homodimers or dimers with other jun family members. Chemical agents, such as kainate, or hilar lesions that lead to seizure activity are known to induce the expression of AP-1 transcription factors in the adult hippocampus (Dragunow and Robertson, 1987; Morgan et al., 1987; Sonnenberg et al., 1989 (a, b); Simonato et al., 1991). Indeed, within 1.5 hours of kainate administration, changes in fra-immunoreactivity occur in entorhinal cortex and dentate gyrus, whereas fra-immunoreactivity in CA subfields of the hippocampus requires about 3-4 hours to become significantly different (Popovici et al., 1990).

The pattern of fra activation is significantly different when mRNA expression of transcription factors was evaluated in young rats aged PN7, PN14 and PN21 at 1 and 6 hours after kainate treatment (Pennypacker et al., 1994). Despite seizure activity induced by KA administration at all ages, none of the transcripts encoding for c-fos, fos B, c-jun, jun B and jun D were overexpressed in PN7 rat hippocampus at 1 or 6 hours, while in the hippocampus of PN14 rats the mRNA for all of the transcription factors was significantly induced 6 h after KA administration with the important exception of c-jun and jun D which
remained at basal levels. At PN21, rats exhibited an increase of mRNA expression for all transcription factors at 6 hours, reaching patterns of induction similar to that observed in adult rats.

The same group of investigators also assessed the AP-1 DNA binding activity and immunoreactivity for fos and c-jun proteins. Interestingly, PN7 rats, show elevated basal AP-1 DNA binding activity, despite a lack of KA-induced gene expression of AP-1 constitutive elements. The binding activity was indeed the highest as compared to what measured in PN14, PN21 and even adult rats, clearly showing that AP-1 DNA binding activity decreases markedly during ontogeny (Pennypacker et al., 1994). No KA-induced AP-1 DNA binding activity was evident until the second postnatal week of life. Consistent with these findings, both fra and c-jun immunoreactive proteins were not increased in the hippocampus following KA administration until PN14, where induction occurs later (4 h) compared to PN21 (1.5 h, similar to what occurs in adult rats). The c-jun protein in the rat hippocampus was not induced by KA administration at PN7 although high basal level of c-jun protein was observed at this developmental stage as compared to PN14, PN21 and adult rats. Kainate was able to increase the expression of c-jun protein at PN14 as well as PN21, although at PN14 the increment takes longer to be evident (about 4 h) as compared to PN21 (1.5 h, similarly to adults).

This evidence is consistent with findings by other groups of investigators. For instance, c-fos protein has been shown not to be induced by KA administration in the mouse hippocampus until PN20 (Sakaurai-Yamashita et al., 1991), whereas prolonged seizures caused by high doses of pentylenetetrazole induce c-fos protein immunostaining in the mouse
hippocampus which appears delayed until 4 h after drug administration (Jensen et al., 1993). Schreiber et al., (Schreiber et al., 1992) showed that KA administration was able to increase c-fos mRNA in the rat hippocampus only at PN13 (Dugich-Djordjevic et al., 1992).

Recently, a more detailed study based on immunohistochemistry technique (Silveira et al., 2002) has shown that fra immunoreactivity was increased in CA3 hippocampal subfield already at PN 7, 2-4 h after induction of seizures by KA administration. In older rats (aged PN 13 and PN 20) the induction of fra proteins was extended to all hippocampal subfields and DG, showing a significant increase 1 h after seizure onset which remained significantly elevated above basal level even after 24 h (PN 20 and adult rats) from seizure onset. These results are not in contradiction with those reported above since the discrepancy concerning pups at younger ages is likely due to the loss of sensitivity that affect measurements accomplished on whole tissue (as it is by electrophoretic mobility shift assay, EMSA) as compared to local evaluation of protein expression (as it is by immunohistochemistry).
4.1.3.2 Ontogeny of physiological DNA binding activity of CREB transcription factors in the rat hippocampus

It is of interest to observe that AP-1 transcription factors are usually composed of a fra:jun heterodimer formed through a leucine zipper to confer DNA binding activity. Nonetheless, jun proteins can also form homodimers or dimers with other jun family members. Differences in the individual components of AP-1 are known to affect the specificity as respect to canonical consensus sequences, hence giving rise to the possibility for AP-1 to bind to other sequences with similarities to the AP-1 site. Pennypacker et al., (Pennypacker et al., 1994; Pennypacker et al., 1995) showed that fra immunoreactivity in AP-1 complexes in the rat hippocampus during the first three weeks of development is very low or undetectable. Indeed, the same group of investigators evidenced that CREB antibodies reacted with the AP-1 DNA binding complex at PN7, PN14 and PN21 thus indicating that CREB protein is a component of the basal AP-1 DNA binding activity during the early postnatal development (Pennypacker et al., 1995), just opposite to what encountered in adult animals where AP-1 DNA binding complex contains fra and little or no CREB. Moreover, probably due to the similarity between the AP-1 sequence (TGACTCA) and CREB sequence (TGACGTCA), it was demonstrated that factors in the CREB DNA binding complex are able to recognize both the CRE and AP-1 elements, implying that genes with either of these promoter elements could be modulated by this transcription factor. This CREB containing complex was also shown to be recognized by antibodies reacting with jun-related factors. Since i) c-jun protein is also expressed at high basal levels during early development, ii) fra protein
immunoreactivity is low during the same period and iii) c-jun expression is correlated with AP-1 DNA binding activity, then it is conceivable that the early postnatal AP-1 transcription factor might be composed of a CREB:c-jun dimer (Pennypacker et al., 1995).

These findings could suggest that during early developmental stages the transcription of genes containing binding element for AP-1 and CREB is favoured, and signalling pathways leading to jun protein transcription and activation might play a more relevant role during early postnatal life, maybe due to the constitutive commonality of this element with both transcription factors. Also, it appears that CREB could have a broader influence on gene transcription since it could bind to both CRE and AP-1 DNA binding site (Pennypacker et al., 1995).
4.1.3.3 Ontogeny of KA-induced NF-κB binding activity in the rat hippocampus

Of interest is also the profile of induction of NF-κB following KA-induced SE in the rat hippocampus at different ages. Adult (PN 50) and young rats (PN 15) were compared in their seizure-induced pattern of activation of nuclear factors such as NF-κB, and AP-1 (Rong et al., 1996). Besides confirming previous observations of AP-1 induction at young ages, as reported above, it was shown that following SE induced by kainate administration the transcription factor NF-κB was significantly induced in adult rats at 4 hours from KA injection, reaching the maximal increase at 8-16 hours after treatment and then decreasing to basal levels within 5 days. Conversely, no significant changes in NF-κB binding activity were observed after KA injection in PN 15 rats. Although not directly proven, it is conceivable that the lack of induction of NF-κB following KA-induced seizures might hold true also for younger pups such as those aged PN 7-9.

Thus, it appears that changes related to SE induced by KA administration as well as some physiological features of young pups could account for the age-dependent pattern of both the expression of proinflammatory cytokines and the ontogenetic profiles of some transcription factors which are relevant to IL-1β and TNF-α expression. Interestingly, it could be observed the accordance of time course of induction of AP-1 by KA administration in PN 14 and PN 21 with the time course of transcription of IL-1β mRNA at comparable ages. Thus, IL-1β transcript is transiently increased at 4 h after KA administration in PN 15 rats,
while it is expressed at long-lasting high levels in PN 21 rats, similar to what occurs in adulthood.

The following table 9 aims to show the good accordance between the ontogenetic profile of KA-induced nuclear transcription factors for proinflammatory cytokines and their mRNAs as evidenced in this experimental section.
Table 9

Schematic representation of the ontogenetic profile of expression of KA-induced nuclear transcription factors for proinflammatory cytokines. The hypothetical total effect on gene transcription for proinflammatory cytokines has also been reported

<table>
<thead>
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<th>PN 7</th>
<th>PN 14</th>
<th>PN 21</th>
<th>ADULT</th>
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<tbody>
<tr>
<td>AP-1 (KA-induced)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NF-κB (KA-induced)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gene transcription for proinflammatory cytokines (KA-induced)</td>
<td>None</td>
<td>Weak</td>
<td>Increased</td>
<td>Sustained</td>
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</table>
In relation to seizure-induced neuronal damage that affect the limbic system, all temporal profiles of proinflammatory cytokines and related molecules, as evidenced so far, clearly show how the first two weeks of development mark a significant border-line beyond which some relevant phenomena linked to maturational aspects might come into play. Indeed, physiological events such as synapse formation, maturation of mossy fibres pathway, dendrite formation and innervation from the entorhinal cortex are expected to have a relevant role into the age-dependent occurrence of neurodegeneration.
4.1.3.4 Induction of proinflammatory cytokines and potential mediators of neurodegeneration

The limited activation of microglia and astrocytes after SE in the hippocampus and the lack of cytokine transcription at PN9 appear to be specific to seizures, as it was shown that microglia, astrocytes, and cytokines are activated at PN9 in other pathological conditions, such as hypoxia/ischemia (Szaflarski et al., 1995; Hagberg et al., 1996; Bona et al., 1999).

A general thought supports the hypothesis that if the induction of inflammatory processes by sustained seizures plays a role in age-dependent neuronal death, at least three pathways linked to cytokine-activated signal transduction may be involved, namely induction of iNOS, COX-2, and NMDA receptor subunit phosphorylation. Indeed, the pattern of induction of these potential mediators of neurodegeneration appears to be in good agreement with the time course of induction of proinflammatory cytokine mRNAs.
4.1.3.4.1 Proinflammatory cytokines and iNOS

Proinflammatory cytokines are potent activators of iNOS, thus leading to the production of reactive nitrogen species (Chao et al., 1995; Romero et al., 1996). Activation of iNOS in astrocytes by IL-1β potentiates NMDA-mediated neurotoxicity in mixed cortical cultures (Hewett et al., 1994). In addition, astrocytes exposed to IL-1β release measurable quantities of peroxide (Robinson et al., 1999), likely by activation of membrane-bound NADPH oxidase. Overproduction of free radicals, both oxygen and nitrogen species, during seizures has been suggested to play a pivotal role in neuronal cell injury (Koprowski et al., 1993; Ishida et al., 2001; Takei et al., 2001). As already mentioned (see paragraph 1.3.3) recent evidence has indeed shown that prolonged seizures in immature rats do not increase reactive oxygen species (ROS) as they do in adults (Li and Patel, 2002; Sullivan et al., 2003). Thus, IL-1β induced production of reactive nitrogen species and ROS may be developmentally regulated and contribute to age-dependent onset of irreversible cell injury.
4.1.3.4.2 Proinflammatory cytokines and COX-2

The role of IL-1β in seizure-induced neuronal damage at PN15 and PN21 may also involve its ability to stimulate COX-2 activity (Cao et al., 1995; Tocco et al., 1997). Constitutive expression of COX-2 in brain increases markedly in hippocampal neurons between PN7 and PN14, reaching adult levels at PN21 (Tocco et al., 1997). Despite intense kainic acid-induced seizures, no COX-2 induction was found before PN14 (Tocco et al., 1997). COX-2 mRNA induction in adult rat brain parallels temporally and spatially with the appearance of apoptotic cells in the hippocampus and other limbic areas (Tocco et al., 1997).
4.1.3.4.3 Proinflammatory cytokines and NMDA receptor

It was recently found that IL-1β induces Src kinases family-mediated tyrosine phosphorylation of the NR2A/B subunit of the NMDA receptor (Viviani et al., 2003). This is known to mediate upregulation of NMDA activity by increasing its channel gating properties (AH and Salter, 2001). The NR2A subunit sharply increases on pyramidal hippocampal cells after the first postnatal week (Monyer et al., 1994; Zhong et al., 1995). Thus, the age-dependent induction of IL-1β during seizures may contribute to the occurrence of neuronal damage by inducing post-translational modifications in the NR2 subunit. Interestingly, although NMDA receptors are operative in immature hippocampi, the reactivity of pyramidal cell apical dendrites to NMDA increases from PN5-9 to PN12-30 (Hamon and Heinemann, 1988), suggesting age-dependent modifications in receptor responsiveness to endogenous agonists.

The low reactivity of NMDA receptors at PN 5-9 itself might represents a control mechanism for the up-regulation of gene transcription for proinflammatory cytokines such as IL-1β and TNF-α, whose induction could potentially become self-inducing by the involvement of this subtype of glutamate receptor. NMDA receptors have been shown to be physiologically and age-dependently coupled with CREB. Indeed, it is known that calcium influx through postsynaptic NMDA receptors can stimulate neuronal gene expression via signalling pathways such as the Ras-MAPK and the induction of CREB. It has been shown, in vitro, that the duration of CREB phosphorylation induced by NMDA receptor activation depends on the developmental maturity of cultured...
hippocampal neurons (Sala et al., 2000). A long-lasting (2 hours) increase in phosphorylated CREB was shown to occur at 7 days in vitro, whereas at 14 days in vitro and older a strong stimulation of NMDA receptors only yielded a transient and short-lasting increase (< 30 minutes) in phosphorylated CREB. This feature appears to be specific for NMDA receptors since other stimuli, such as depolarization induced by high concentration of KCl, were shown to induce the same prolonged CREB phosphorylation in young and mature cultures. These observations suggest that NMDA receptors become coupled to a specific CREB phosphatase activity as neurons mature. Indeed, it was shown that PP-1 is the major phosphatase involved in CREB dephosphorylation (Hagiwara et al., 1992; Sala et al., 2000).
4.1.4 A few observations on the hypothesis so far formulated

Evidence and arguments mentioned so far support the hypothesis that neuronal damage could be age-dependently linked to the overproduction of proinflammatory cytokines and related molecules.

Nonetheless, some issues arise. The experimental results obtained so far do not fully harmonize with the physiological and SE-induced developmental patterns as reported above.

Indeed, a look at the promoter region of genes for proinflammatory cytokines and mediators such as IL-1β, TNF-α, COX-2 and iNOS, clearly shows that DNA binding sites for transcription factors such as AP-1, CREB and NF-κB are common to all those genes. The high levels of c-jun protein during early stages of life (despite low levels of its mRNA), together with a prolonged NMDA-mediated activation of CREB as well as formation of CREB:c-jun dimers which are able to also recognize AP-1 DNA binding sites, should increase the probability that gene transcription could be induced at earlier developmental stages of life (such as PN 9), in particular after induction of SE which leads to a prolonged stimulation of glutamate receptors. Genes responsive to CREB are known to be activated only when phosphorylation of CREB is prolonged (Bito et al., 1996; Sala et al., 2000), as occurs during long-lasting synaptic stimulation (e.g. during seizure activity) and, in light of findings mentioned above, it should be age-dependent, showing the strongest induction of CREB only at earlier developmental stages.
Thus, apparently, it seems that at early postnatal stages of life, there are two opposite routes involved in the induction of gene transcription for proinflammatory cytokines such as IL-1β and TNF-α, as well as proinflammatory mediators such as COX-2. An age-dependent AP-1 activation would account for the pattern of induction for IL-1β, TNF-α and COX-2. In opposition, a prolonged activation of CREB following NMDA receptor stimulation, a physiological age-dependent feature which is particularly pronounced in the early postnatal development, represents a potential inductor of IL-1β, TNF-α and COX-2 gene transcription. Such a gene transcription induction would be accomplished by binding to CRE DNA site as well as to AP-1 DNA site since it should be kept in mind that CREB also represents, together with c-jun protein, a constitutive element of AP-1 transcription factors in young pups. At present, there is no explanation for this issue.

However, it should be also observed that the great majority of studies on the induction of nuclear transcription factors relies on techniques such as the ‘electrophoretic mobility shift assay’ (EMSA) which are mainly designed to determine any change in levels of such factors, leaving unanswered the question whether binding to chromatin and induction of target gene transcription actually occurs. It is known that the activation of a transcription factor and its subsequent translocation into the nucleus does not necessary mean that gene transcription is actually induced, as it has been shown for CREB (Gilty, 1997). Indeed, genomic advances have clearly shown that eukaryotic gene expression is mediated by interlinked regulatory mechanisms raising up through the interactions among nucleotide sequences, chromatin structure and 3D spatial organization of the genome inside the cell nucleus (van Driel et al., 2003).
it cannot be excluded that the age-dependent patterns of induction, as evidenced so far, might also be better explained in terms of ontogenetic changes of the complex dynamics governing the genome structure.
4.2 Induction of cytokine expression in the hippocampus of PN 9 rats by flurothyl-induced status epilepticus

At the present step of discussion, it is relevant to ascertain if the pattern of expression of proinflammatory cytokines mRNAs by seizures induced by KA administration is a peculiarity of ictal activity itself or it could be related to the proconvulsant agent specifically used in the animal model under investigation. As the most relevant results occurred at PN 9, rat pups at this age underwent a protocol of SE induced by inhalation of flurothyl and mRNA expression for IL-1β, IL-1Ra and TNF-α was evaluated at 4 and 24 hours after seizure onset. These measurements were also accompanied by histochemical evaluation of neuronal damage in the hippocampus by Fluoro-Jade staining, since it has been shown that also for flurothyl model of SE the induction of neurodegeneration is age-dependent (Sperber et al., 1999).
4.2.1 Materials and methods

4.2.1.1 Flurothyl-induced status epilepticus and evaluation of gene expression

Technical details related to:

- Flurothyl-induced status epilepticus;
- total RNA extraction;
- reverse transcription of total RNA;
- quantitative evaluation of gene expression by real-time PCR
- Fluoro-Jade staining

have already been reported in ‘Materials and methods – general procedures’

For the assessment of neurodegeneration, Fluoro-Jade positive cells evaluation was accomplished 24h after seizure-onset. For each rat, 5 to 6 40μm-thick slices were analysed.
4.2.2 Results

The effect of 1 hour duration of flurothyl-induced SE on the mRNA expression of IL-1β, IL-1Ra and TNF-α was evaluated in the hippocampus of PN 9 rats, 4 and 24 hours after the onset of seizures.

Results are depicted in fig. 12. Differently from that observed in PN 9 pups who underwent KA-induced seizures, statistically significant differences of mRNA expression were detected for both IL-1β (12.3-fold increase) and TNF-α (6.7-fold increase) 4 hours after the onset of flurothyl-induced seizures as compared to control values, whereas the level of transcript of IL-1Ra was unaffected.

Table 10 shows that 1h flurothyl-induced SE does not affect neuronal viability 24 h after seizure-onset in PN 9 rats.
mRNA expression of IL-1 beta, IL-1Ra and TNF-alpha in the hippocampus of PN 9 rats sacrificed after 4 h and 24 h from the onset of flurothyl-induced seizures

Fig. 12

Data are the means ± SEM (n=6). * p<0.05 ** p<0.01 versus control group; § p<0.05 §§ p<0.01 vs 4 h group by Kruskal-Wallis test
<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>(Bregma-2.8 mm)</th>
<th>(Bregma-3.8 mm)</th>
<th>(Bregma-5.3 mm)</th>
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<tr>
<td>PBS</td>
<td>3.8 ± 0.6</td>
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<td>2.3 ± 0.9</td>
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<tr>
<td>PBS+Flurothyl</td>
<td>3.0 ± 0.7</td>
<td>3.1 ± 1.0</td>
<td>2.6 ± 0.4</td>
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</table>

Evaluation of neuronal degeneration in the hippocampus of PN 9 rats. Three experimental groups are represented. Each data represents the average number of Fluoro-Jade positive cells in their respective location inside the hippocampus. Data are the mean ± SEM (n=4 rats). No statistically significant differences occurred by Kruskal-Wallis test.
4.2.3 Discussion

The mRNAs for IL-1β and TNF-α (fig. 12) are considerably expressed by flurothyl-induced SE 4 hours after the onset of ictal activity in rat pups aged as young as PN 9, with the exception of mRNA for IL-1Ra. The induction of IL-1β mRNA was still significantly higher when compared to basal levels 24 hours after the onset of flurothyl-induced seizures. We checked for neurodegeneration by Fluoro-Jade staining in the hippocampus of PN 9 rats which underwent flurothyl-induced SE and then sacrificed 24 h after seizure-onset (table 10, compare PBS group vs PBS+Flurothyl group). Clearly, there was a lack of degenerating neurons as assessed by Fluoro-Jade staining.

These findings confirm and extend results indicating the age-dependency of neurodegeneration induced by SE triggered by inhalation of flurothyl (Sperber et al., 1999). These authors showed that rat pups at PN 9 are preserved from neuronal damage in this model of SE, as in the KA model. Nonetheless, the expression of mRNA for proinflammatory cytokines, such as IL-1β and TNF-α, is strongly induced 4 hours post treatment in rat pups aged PN 9, which is different from what occurs in the KA model of SE.

Studies investigating flurothyl-induced ontogenetic profiles of induction of either transcriptional factors or constituent molecules have been poorly investigated so far, probably because of the high cost of flurothyl, hence it is hard to attempt a plausible explanation for the pattern of expression of proinflammatory cytokines at PN 9 as it was done for the KA model of status epilepticus. At present, available evidence is mainly focused on c-fos and fra mRNA and protein expressions in young rats as compared to adults. Thus, c-fos
protein was shown to be mildly induced in rat pups at PN 10-11 following single or brief flurothyl-induced seizures (Liu et al., 1999; Jensen et al., 1993), whereas a more pronounced immunostaining in DG and hippocampus occurs with more prolonged seizures (Jensen et al., 1993). A mild pattern of induction has been shown to occur at PN 23 following single seizures (Liu et al., 1999) while at PN 19 the levels of c-fos mRNA expression were shown to be variable, being elevated in DG and CA3 hippocampal subfield in four animals out of ten (Szot et al., 2001).

However, these data, although similar to those found in the KA model as reported above, are not useful for the interpretation of the experimental results obtained, in particular because c-fos and fra in rat pups at young ages appear not to be so relevant as in older rats as respect to their contribution as constituent elements in nuclear transcription factors potentially involved in transcriptional activation of genes coding for proinflammatory cytokines and related molecules.

Generally, the model of flurothyl-induced SE seems not to support the hypothesis that neurodegeneration is preceded by an increase of transcription levels of proinflammatory cytokines. It might be that mRNAs production for IL-1β and TNF-α does not yield significant levels of respective proteins in order to give raise to an inflammatory state that could affect the viability of neuronal populations in PN 9 rats. In vitro, the ability of proinflammatory cytokines to affect neuronal survival to an excitotoxic insult has been shown to depend on the concentration of the cytokines. For instance, it has been recently shown that a relatively high concentration of mouse recombinant TNF-α (10 ng/ml) enhances excitotoxicity in organotypic hippocampal slice cultures when...
simultaneously exposed to AMPA and to this cytokine. Conversely, a 10-times lower concentration of TNF-α (1 ng/ml) was shown to protect the cultures against AMPA-induced neuronal death (Bernardino et al., 2005).
4.3 Effect of icv administration of IL-1β on flurothyl-induced seizure-onset and neurodegeneration following 1h flurothyl-induced SE in PN 9 rats

The possibility that IL-1β levels might not be sufficient to induce neurodegeneration in PN 9 rats prompted us to evaluate the effect of the icv administration of a high dose of IL-1β on neuronal viability after flurothyl-induced SE. We also investigated the dose-response of the icv administration of IL-1β on flurothyl-induced clonic and tonic-clonic seizure-onset in PN 9 rats.
4.3.1 Materials and methods

Technical details related to:

- Flurothyl-induced status epilepticus;
- Fluoro-Jade staining

have already been reported in 'Materials and methods – general procedures'.

Each group of rats injected with a dose of IL-1β was matched with a control group (indicated in the bargram's legend as "PBS"). Independently from the dose of IL-1β used, rat pups of the control group were always injected icv with 0.1 % BSA dissolved in PBS. Seizure threshold was tested 5 min after the icv administration of IL-1β. For the assessment of neurodegeneration, Fluoro-Jade positive cells evaluation was accomplished 24h after seizure-onset. For each rat, 5 to 6 40µm-thick slices were analysed.
4.3.2 Results

Table 11 shows the effect of IL-1β on neuronal viability after 1h SE induced by flurothyl. Evaluation was accomplished 24h after the onset of seizure. For a direct comparison, Table 11 also includes data of Table 10. The increment of the number of degenerating neurons in animals treated with icv injection of the cytokine ranges from 2 to 4 fold, on average.

Figure 13 depicts the dose-response of the icv administration of IL-1β on clonic and tonic-clonic seizure onset flowing flurothyl inhalation. None of the doses administered was able to affect seizure susceptibility in PN 9 rats.
Table 11

<table>
<thead>
<tr>
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<tr>
<td>IL-1β+Flurothyl</td>
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<td>10.8 ± 1.4 *</td>
<td>11.8 ± 1.1 **</td>
<td>10.7 ± 1.1 **</td>
<td>10.2 ± 2.0 *</td>
<td>8.8 ± 0.9 *</td>
</tr>
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</table>

Evaluation of neuronal degeneration in the hippocampus of PN 9 rats. Three experimental groups are represented. Each data represents the average number of Fluoro-Jade positive cells in their respective location inside the hippocampus. Data are the mean ± SEM (n=4 rats). *p<0.05; **p<0.001 vs PBS and PBS+Flurothyl by Kruskal-Wallis test.
Dose-response of icv administration of IL-1beta on flurothyl-induced seizures in PN 9 rats

Data are the mean ± SEM (n=8-12). No statistically significant differences occurred by Mann-Whitney test.
4.3.3 Discussion

Our findings support the hypothesis that the lack of neurodegeneration following SE at PN 9 may be ascribed at least in part to the lack of sufficient production of hippocampal IL-1β. Thus, the administration of IL-1β induces cell damage at PN 9 when exogenously applied before the induction of SE. Noteworthy, IL-1β does not affect seizure in immature rat brain differently from adult rats. It should be observed, however, that testing for seizure threshold was performed shortly after the administration of cytokine (5 minutes, since this time interval was shown to be sufficient for IL-1β to affect seizure threshold in adult rats). Therefore, it might be that longer exposure time to cytokines is required to induce any effect on seizure susceptibility at young ages. We investigated this hypothesis in the next experimental section.
4.4 Effect of LPS on the susceptibility to seizures induced by flurothyl inhalation in PN 9 rats

4.4.1 Introduction

As shown in the previous experimental section, the icv administration of IL-1β in rat pups at PN 9 does not affect seizure susceptibility, differently from what reported in adult animals (fig. 13). This might be due to an insufficient concentration of IL-1β in the brain parenchyma to affect seizure susceptibility as in adult rats, although a high dose of the cytokine was injected. The possible requirement of a longer exposure time to cytokines to induce any effect on seizure susceptibility at young ages is indeed relevant. Clinical and experimental studies have shown that perinatal exposure to proinflammatory cytokines such as IL-1β and TNF-α can be associated to neonatal brain damage which might constitute a pathological substrate priming the brain to develop enhanced seizure susceptibility and epilepsy later in life. During the last decade it has been suggested that maternal or placental infection (as well as early postnatal exposure to infections) may cause neonatal brain damage such as white matter disease (periventricular leukomalacia), and that induction of proinflammatory cytokines such as IL-1β and TNF-α owing to maternal infectious diseases may be a mechanism mediating this and several other neurological disorders. In
support of this hypothesis it has been shown that the occurrence of periventricular leukomalacia, which often leads to cerebral palsy, is frequently associated with increased concentrations of proinflammatory cytokines in the infant brain (Deguchi et al., 1996; Deguchi et al., 1997; Yoon et al., 1997), in the cord blood (Yoon et al., 1996) or in the amniotic fluid (Yoon et al., 1997). Additionally, a variety of maternal infections during pregnancy have been associated with an increased incidence of several pathologies such as schizophrenia in offspring, bronchopneumonia and other respiratory infections and varicella zoster (Gilmore and Jarskog, 1997; Watson et al., 1984; Torrey et al., 1988; O'Callaghan et al., 1994; Brown et al., 2000).

However, although increasing evidence has demonstrated the link between inflammatory cytokines and brain injury induced by infection/inflammation or hypoxia-ischemia (Dammann et al., 1997; Leviton, 1993), the detailed role of cytokines in mediating neuronal cell death and white matter injury is still elusive and often controversial. Indeed, neurological alterations that frequently accompany white matter injury such as ventricle dilation, white matter rarefaction, reactive astrogliosis as well as decreased levels of myelin binding protein in the brain of neonates appear to be differently affected by proinflammatory cytokines such as IL-1β and TNF-α. Thus, whereas administration of IL-1Ra has been shown to significantly attenuate LPS-induced white matter injury as well as LPS-induced elevation of caspase-3 activity in the rat brain, administration of TNF-α antibody was shown not to affect caspase-3 activity nor significantly counteract white matter injury elicited in the same experimental conditions although a significant reduction of LPS-induced
increase of IL-1β protein levels in the neonatal rat brain was observed (Cai et al., 2003).

Generally, cytokines are known to regulate normal brain maturation and have been implicated in abnormal brain development (Merrill, 1992; Mehler et al., 1995; Mehler and Kessler, 1997). Moreover, proinflammatory cytokines have been shown to be neurotoxic to a variety of developing neurons in vitro. For instance, IL-1β, TNF-α and IL-6 decrease survival of dopaminergic and serotonergic neurons (Jarskog et al., 1997). Also, IL-1β decreases neuron survival in primary cultures of embryonic rat hippocampus (Arajujo and Cotman, 1995; Viviani et al., 2003) and TNF-α potentiates glutamate excitotoxicity in cultures of fetal cortical neurons (Chao and Hu, 1994) and in organotypic slice cultures (Bernardino et al., 2005).

Therefore, it is conceivable that alterations of neuronal and glial populations and brain circuitries due to hypomyelinated neuronal connections might prime the neonatal CNS to increase the likelihood of seizures and/or epilepsy to occur during later stages of life.

In light of the aforementioned findings, we investigated the effect of inducing an inflammatory response in PN 9 rats on seizure susceptibility. LPS administration was used as the proinflammatory stimulus and seizure threshold was tested 2 hours after the endotoxin challenge. As a measure of the ongoing proinflammatory response IL-1β and TNF-α mRNAs were evaluated by RT-real time PCR.
4.4.2 Materials and methods

LPS (Lipopolysaccharides from Escherichia coli, serotype 055:B5, Sigma, St. Louis, Mo, USA) was dissolved in 0.1 M Phosphate-buffered saline (PBS, 0.1 M, pH 7.4 - 0.154 M NaCl; 0.016 M NaH$_2$PO$_4$·2H$_2$O; 0.089 M Na$_2$HPO$_4$·2H$_2$O) and administered intraperitoneally 120 min before flurothyl delivery.

Technical details related to:

- seizure-threshold testing by flurothyl inhalation;
- mRNA extraction;
- reverse transcription;
- quantitative evaluation of gene expression by real-time PCR

have already been reported in 'Materials and methods - general procedures'.

For seizure susceptibility testing, drugs were administered intraperitoneally 2 hours before flurothyl delivery. For the evaluation of hippocampal levels of IL-1β and TNF-α mRNA expression, rats were sacrificed 2 hours after drugs administration.
4.4.3 Results

Fig. 14 (A, B) depicts the dose-response effect of lipopolysaccharide (LPS) on flurothyl-induced seizures in PN 9 rats. Data are the mean ± SEM (n = 12-16 pups) expressed as % of control (PBS-injected) group. The ordinate axis represents the latency times to onset of clonic (A) and tonic-clonic (B) seizures (the total volume in μl of flurothyl required to induce convulsions). Statistically significant delays of seizure-onset were achieved for all the doses of LPS administered, with the exception of the highest ones (2.0 and 4.0 mg/kg) for clonic seizures and 0.4 mg/kg for tonic-clonic seizures. The most effective dose of LPS in delaying seizure-onset for both clonic and tonic-clonic seizures was 0.1 mg/kg, which showed, on average, 40% increase of latency to ictal events vs PBS-injected controls (p<0.01).

Herein we will refer to any dose of LPS that was shown to delay seizure-onset as being 'protective'.

Fig. 15 (A, B) shows the level of mRNA of IL-1β and TNF-α in the hippocampus induced by the administration of LPS, at protective (0.1 mg/kg and 1.0 mg/kg) and non-protective (4.0 mg/kg) doses. Measurements were done 2 hours after the LPS administration, i.e. at the time of flurothyl delivery, thus, before the challenge with flurothyl-inhalation. The ordinate axis represents the level of mRNA expression as % of control (PBS-injected) group, assessed by quantitative RT-PCR.

The most protective dose of LPS (0.1 mg/kg) induced 235.2 ± 5.5 % level of expression of IL-1β mRNA as compared to PBS-treated rats (p<0.01), whereas 10 times-higher concentration of LPS (1.0 mg/kg) triggered 145.5 ± 4.5
%, p<0.01 vs basal levels of expression (fig.15, panel A). A similar pattern occurred also for TNF-α (fig. 15, panel B). Thus, 0.1 mg/kg LPS induced a 362.7 ± 8.5 % level of expression of TNF-α mRNA as compared to PBS-treated rats (p<0.001), whereas 1.0 mg/kg LPS triggered 271.6 ± 15.5 %, p<0.001 vs basal levels of expression.

After administration of a non-protective dose of LPS (4.0 mg/kg), the levels of transcript of IL-1β were similar to those elicited by 1.0 mg/kg LPS, whereas the TNF-α mRNA expression was markedly increased, 425.0 ± 27.3 %, p<0.01 vs basal levels of expression (fig. 15, panel B).
Fig. 14

Dose-response curve of LPS administration on flurothyl-induced clonic (panel A) and tonic-clonic (panel B) seizure-onset in PN 9 rat pups.

Data are the mean ± SEM (n=12-16). * p<0.05 ** p<0.01 vs PBS; § p<0.05 vs LPS 1.0 mg/kg and LPS 1.5 mg/kg; # p<0.01 vs LPS 2.0 mg/kg and LPS 4.0 mg/kg; ° p<0.01 vs LPS 4.0 mg/kg; ‹"
p<0.01 vs LPS 2.0 mg/kg; ^ p<0.05 vs LPS 4.0 mg/kg; ^^ p<0.01 vs LPS 2.0 mg/kg by Kruskal-Wallis test
mRNA expression of IL-1β (panel A) and TNF-α (panel B) following the administration of different doses of LPS in PN 9 rats

Data are the mean ± SEM (n=6). * p<0.05  ** p<0.01 vs PBS; § p<0.05  §§ p < 0.01 vs LPS 0.1 mg/kg; # p<0.01 vs LPS 1.0 mg/kg by Kruskal-Wallis test
4.4.4 Discussion

Our findings suggest that the delaying effect of LPS on seizure-onset is not related to the pattern of induction of proinflammatory cytokines. Thus, the most pronounced increase in cytokines was provoked by the low protective dose of LPS. On the bases of the literature evidence it is expected that increasing doses of LPS induce increasing levels of proinflammatory cytokines transcripts.

Since this is not the case in our experimental conditions, a candidate mechanism which may explain our results is the activation of the hypothalamic-pituitary-adrenal axis (HPA). As already mentioned, unlike in adult animals which respond to most stimuli by increasing circulating corticosterone levels, rat pups are known to undergo a period (SHRP) characterized by adrenal insensitivity to direct stimulation with ACTH, minimal corticosterone elevations in response to most stressors, and a failure of mild stimuli to elicit ACTH response. Nonetheless, the limited response of the immature HPA system during the SHRP is not absolute and appears to be stimulus-specific and depends on which component of the HPA axis is considered. LPS, which is known to potently activate HPA axis in adult rats, acts similarly in rat pups at very young ages (such as PN 1-2) even during the SHRP (Witek-Janusek, 1988; Dent et al., 1999). Indeed, the HPA axis response to intraperitoneal LPS administration in rat pups was determined at the beginning (PN 6), at the nadir (PN 12) and outside (PN 18) the SHRP. At all three ages, ACTH levels were robust with highest concentrations reached at PN 12, whereas the highest concentrations of corticosterone were reached at PN 18, as shown in fig 17. It is of interest to observe the temporal profile of circulating corticosterone at each age, which
actually parallels the ACTH variations. At PN 6 and PN 12, after 4 hours from LPS administration, the concentration of corticosterone still tends to increase, although at PN 12 the absolute levels are lower than those measured at PN 6. These findings are fully compatible with i) a lack of an effective inhibitory feedback mechanism acting onto the HPA axis in young animals and ii) a decreased sensitivity of adrenal glands to circulating ACTH at PN 12. This is known to occur around this stage of postnatal life, leading to a reduced release of corticosterone into the circulation. Conversely, at PN 18, the levels of both ACTH and corticosterone tends to decrease after 4 hours from LPS administration, thus revealing operative negative feedback mechanisms (Dent et al., 1999).
Plasma ACTH (A) and CS (B) levels in 6-, 12-, and 18-day old pups. Samples were collected at 2 and 4 h after LPS (0.1 mg/kg) or saline (0.9 %, i.p.) injection. Also included is a non-injected control group. Data are the mean ± SEM (n=6-9). *p<0.05 vs saline- and non-injected control groups. Adapted from Dent et al., 1999
Thus, increasing doses of LPS in PN 9 rats may progressively stimulate the HPA axis to release corticosterone. Subsequently, this hormone would exert its well known anti-inflammatory action at genomic level by inhibiting transcriptional activation of molecules such as IL-1β and TNF-α.

Interestingly, our results may have some relevance in relation to the phenomenon known as ‘preconditioning’ or ‘tolerance’. Generally, this term describes an event, usually a short insult, that once occurred in the brain is able to confer a transient resistance of brain tissue to a subsequent deleterious insult. For instance, it is known well that a brief exposure to ischemia provides robust protection against injury from subsequent prolonged ischemia (ischemic preconditioning) (Simon et al., 1993). This has been shown in animal models of stroke, whereas an analogous process has been suggested in humans wherein previous transient ischemic attacks are associated with improved clinical outcome and reduced infarction size from subsequent stroke (Weih et al., 1999; Wegener et al., 2004; Johnston, 2004). Although tolerance has mostly been described for ischemic insults, it has been reported also for other types of insults, including brain trauma and epilepsy (Dirnagl et al., 2003; Rejdak et al., 2001). Indeed, epileptic tolerance is met in several animal models of seizures where, often, the preconditioning stimulus consists of noninjurious epileptic episode such as that induced by normal or rapid hippocampal kindling (Kelly and McIntyre, 1994; Penner et al., 2001), kainic acid-induced seizures (El Bahh et al., 1997; El Bahh et al., 2001; Lere et al., 2002; Najm et al., 1998), bicuculline-induced seizures (Sasahira et al., 1995), or electroshock (Kondratyev, 2001). Usually, the positive effects of preconditioning over prolonged epileptic episodes
are related to a significantly reduced extension of neuronal damage as well as to an increased seizure-threshold to proconvulsant stimuli.

Interestingly, ischemic preconditioning or hypoxia inhibits the lethal effects of subsequent sustained severe seizures on neurons of various regions of brain (Plamondon et al., 1999; Emerson et al., 1999; Emerson et al., 2000; Rejdak et al., 2000; Rejdak et al., 2000), whereas preconditioning seizures partially inhibit neuronal death associated with severe ischemia (Plamondon et al., 1999). This evidence suggests that ischemic preconditioning and epileptic tolerance may share common mechanisms of action. Generally, preconditioning stimuli have been shown to generate a wide variety of metabolites and ligands that may be involved in the tolerance phenomenon (Blondeau et al., 2000; Dirnagl et al., 2003; Rejdak et al., 2001; Emerson et al., 2000; Leker and Neufeld, 2003; Nvue et al., 2004; Plamondon et al., 1999; Xu et al., 2002). In relation to our results, it is of interest to note that ischemia, seizures and LPS are usually expected to enhance gene expression and protein synthesis of proinflammatory molecules. Indeed, the activation of the transcription factor NF-κB and signalling pathways involving p38 MAPK were shown to be crucial steps underlying the development of brain seizure and ischemia tolerance (Blondeau et al., 2001; Jiang et al., 2005). These molecules play a pivotal role in the induction of transcription and mRNA stabilization of immediate early genes products such as inflammatory mediators, thus making the involvement of proinflammatory molecules in preconditioning phenomenon a conceivable hypothesis. In this regard, it has recently shown that within a short time window of 3 hours ischemic preconditioning followed by stroke strongly up-regulates genes related to inflammation and stress responses as compared to stroke.
alone. This pattern of gene induction at 3 hours turned to a significant down-regulation as compared to stroke alone when evaluated within a longer time window as 24 hours (Stenzel-Poore et al., 2004). It could be argued that our results show an inhibition of gene expression of LPS-induced proinflammatory markers such as IL-1β and TNF-α. In this regard, it is of interest to note that NF-κB and p38 MAPK have been shown to enhance the transcription of COX-2 mRNA, leading to a subsequent increase of PGE₂ production (Akundi et al., 2005; Rhee and Hwang, 2000) which could actually account for the diminished expression of IL-1β and TNF-α mRNA, being PGE₂ shown to down-regulate the gene transcription of these cytokines. Accordingly, an indirect confirmation of increased PGE₂ levels in our experimental system may be inferred by considering that LPS-induced activation of the HPA axis has been shown to involve PGE₂ (see paragraph 1.4.5). In principle, although the anticonvulsant effect of LPS are expected to be mediated by the occupancy of membrane mineralocorticoid receptors by corticosterone, it cannot be ruled out an anticonvulsant role of PGE₂. Several evidence from literature has shown the potential anticonvulsive and neuroprotective properties of endogenous prostaglandins in animal models of seizures. So, apparently, it might be that a possible phenomenon of preconditioning in our experimental system could be potentially mediated by corticosterone and PGE₂. Nonetheless, a role of PGE₂ per se as a protective agent over seizure susceptibility may not be consistent with our experimental results. Indeed, our data show that the more the dose of LPS increases, the lower the seizure-threshold. Since several evidence from literature shows that PGE₂ production is dose-dependently related to LPS, the less protective doses of LPS (i.e. the highest doses administered) are expected
to be associated with high levels of PGE$_2$ production, hence the exclusion of a protective role of PGE$_2$ over seizure susceptibility.

In light of the present results, the exploitation of a proinflammatory stimulus such as LPS as a tool for seizure preconditioning needs substantial information which cannot be inferred from our data. A relevant parameter to be established is the duration of the protective effect over seizure susceptibility. Usually, the temporal scale within which the preconditioning shows its efficacy ranges from days to weeks. In our experimental system we detected the effect of LPS on seizure threshold within a time window as short as 2 hours, an interval which would suggest LPS administration as an efficacious 'pretreatment' rather than a useful tool for 'seizure preconditioning'. However, it is worth reminding that due to the immaturity of negative feedbacks involved in the HPA axis activation in rat pups, the increase over basal levels of LPS-induced corticosterone in the circulation was shown to be a long lasting phenomenon. Thus, elevation of corticosterone levels may represent a potential preconditioning stimulus, whereas the effect of PGE$_2$ may be limited to the HPA axis activation with a possible affection of IL-1$\beta$ and TNF-$\alpha$ gene transcription. However, it is also conceivable that the down-regulation of IL-1$\beta$ and TNF-$\alpha$ gene expression might be ascribed to the conventional anti-inflammatory effect mediated by corticosteroids. This effect, in light of IL-1$\beta$ as a potential proconvulsant stimulus, may strengthen the resistance of the organism to develop seizures and should be a long lasting effect, being the translation of IL-1$\beta$ protein down-regulated.
Thus, to sum up, at this step of the investigation it is possible to speculate that the activation of the HPA axis by the systemic LPS administration and the subsequent involvement of the membrane mineralocorticoid receptors could represent a possible explanation of the LPS-induced delayed onset of flurothyl-induced seizures in rat pups.

The assessment of this working hypothesis is the target of the next experimental section.
4.5 Involvement of corticosteroid receptors in the LPS modulation of flurothyl-induced seizures in PN 9 rats

4.5.1 Introduction

We tested the hypothesis that the delaying effect of LPS administration on flurothyl-induced seizure onset is mediated by an interaction of circulating corticosterone with membrane mineralocorticoid receptors. However, since our evaluation of seizure susceptibility and mRNAs expression of proinflammatory cytokines were made 2 hours after endotoxin administration it is possible that some genomic effects of corticosterone may have occurred, possibly contributing to modulate seizure susceptibility. Genomic effects of corticoids include the transcription of molecules which have been shown to be involved, at least in adulthood, in neurodegenerative phenomena such as IL-1β, TNF-α, COX-2, iNOS. All these molecules are characterized by a NF-κB consensus sequence in their promoter region. Any significant modulation of corticoids on these molecules might contribute to alter developing neuronal circuitries, producing a priming of the immature brain to show a predisposition to elicit ictal events and related neurodegeneration later in life.
To verify the influence of corticosterone on seizure-threshold in our experimental conditions, canrenoic acid and RU486 were used as selective antagonists to mineralocorticoid and glucocorticoid receptors respectively. Drug delivery and testing of seizure susceptibility were accomplished in rat pups at PN 9.
4.5.2 Materials and methods

Technical details related to seizure-threshold testing by flurothyl inhalation have already been reported in 'Materials and methods - general procedures'.

The concentration of LPS used was 0.1 mg/kg and flurothyl delivery always occurred 2 h after the endotoxin challenge.

Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg) 30 min before flurothyl delivery (i.e. 90 min after the systemic LPS administration).

RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg) 30 min before flurothyl delivery. Controls were rat pups treated with 0.1 mg/kg LPS and then administered with PBS or 25% Tween 80 30 min before flurothyl delivery.
4.5.3 Results

Figure 17, (A, B) depicts the effect of corticosteroid receptors on LPS-induced delay of seizure onset following fluoroethyl inhalation. The administration of canrenoic acid per se did not affect the susceptibility to seizures, but it abolished the delay of seizure onset induced by a protective dose of LPS (0.1 mg/kg).

Figure 17, (C, D) shows the effect of the glucocorticoid receptor antagonist RU486 on the LPS-induced delay of seizure onset following fluoroethyl inhalation. The vehicle used to dissolve RU486 (25% Tween 80) significantly affected the onset to fluoroethyl-induced seizure when administered alone by increasing by 25% the latency to clonic seizures.

A further 30% increase above Tween-injected rats (p<0.01) in the latency to both clonic (panel C) and tonic-clonic (panel D) seizures was induced by RU486. There were not statistically significant differences between the RU486 alone and the LPS + RU486 administered group (panels C and D).
Fig. 17

Effect of canrenoic acid (panels A and B) and RU486 (panels C and D) on LPS-induced delay of flurothyl-induced seizures in PN 9 rats
Data are the mean ± SEM (n=9-12 for panels A and B; n=6-10 for panels C and D). * p<0.05 ** p<0.01 vs PBS; § p<0.01 vs LPS 0.1 mg/kg; # p<0.01 ## p<0.001 vs 25% Tween 80 and LPS 0.1 mg/kg (25% Tween 80) by Kruskal-Wallis test.
4.5.4 Discussion

These experiments show that seizure susceptibility is modulated by corticosterone. In particular, corticosterone receptors appear to mediate opposite effects since antagonism of mineralocorticoid receptors blocks the protective effect of LPS whereas antagonism of glucocorticoid receptors enhances seizure threshold per se but does not affect the LPS effect.

Thus, corticoid receptors appear to play a dual role on seizure susceptibility. A protective effect mediated by mineralocorticoid receptors, and a proconvulsant effect mediated by glucocorticoid receptor.

A relevant role for mineralocorticoid receptors in our experimental conditions is in line with the ontogenetic profile of these receptors. Protein levels of mineralocorticoid receptors are similar to adults by PN 7 and are much more abundant when compared to glucocorticoid receptors. Moreover, mineralocorticoid receptors show affinity an order of magnitude higher to the endogenous ligand corticosterone than glucocorticoid receptors after birth and during brain maturation (Bohn et al., 1994; Sarrieau et al., 1988; Vazquez et al., 1998).

As far as glucocorticoid receptors are concerned, there is no evidence in the literature to support a proconvulsant effect emerging from the specific activation of these receptors in rat pups. Indeed, these receptors were shown not to modulate the occurrence of ictal events at young ages (Edwards et al., 2002). In our experiments RU486 affects seizure susceptibility despite the effect of vehicle alone (25% Tween 80) (panels C, D). In particular, the delaying effect of RU486 on seizure onset is present in rats treated with this antagonist alone.
The lack of a statistically significant difference between the RU486 administered group and the LPS + RU486 group (panels C and D) suggests a plateau effect when RU486 is administered in rats that cannot be further increased by endotoxin.

It cannot be excluded that antagonism to glucocorticoid receptors results in an enhancement of the anticonvulsant effect of circulating corticosterone acting on mineralocorticoid receptors. Glucocorticoid receptors are expressed to a much lesser extent than mineralocorticoid receptors and their affinity to corticosterone binding is 10 times lower. Additionally, although binding to glucocorticoid receptors by RU486 has been shown to translocate the receptorial complex into the nucleus, there is a substantial lack of a specific binding to GRE consensus sequences as demonstrated by the random distribution of the receptorial complex inside the nucleus (Htun et al., 1996). Therefore, it is likely that blockade of glucocorticoid receptors increases the probability of free corticosterone to significantly augment the percentage of cytoplasmic and membrane mineralocorticoid receptors occupancy.

In summary, HPA axis activation seems effective in modulating seizure susceptibility in LPS challenged animals, presumably through activation of membrane mineralocorticoid receptors. It is likely that cytoplasmic corticosteroid receptors exert also their classical genomic action, hence giving support to the hypothesis of a corticosterone-mediated modulation of IL-1β and TNF-α mRNA expression following systemic LPS administration in PN 9 rats. Therefore, it is conceivable that, in PN 9 rats, increasing doses of LPS progressively tend to increase mRNA levels of proinflammatory cytokines while at the same time stimulate the HPA axis to release corticosterone. Subsequently, this hormone
would exert its well known anti-inflammatory action at genomic level by inhibiting transcriptional activation of molecules such as IL-1β and TNF-α.

It is of interest to observe that the activation of the HPA axis may be ascribed as a possible mechanism affecting the age-dependent expression of proinflammatory cytokines after KA and flurothyl administration. Excitatory aminoacids have been shown to activate the HPA axis not only in adults but also in rat pups, even during the stress hypo-responsive period (SHRP) (Bardgett et al., 1992; Chautard et al., 1993). In the study by Kent (Kent et al., 1996) experiments were accomplished in rat pups at PN 6 (near the beginning of SHRP), PN 12 (at the nadir of responsiveness) and PN 18 (after the end of SHRP). The pattern of induction of ACTH and corticosterone clearly shows that KA is a potent activator of the HPA axis in rat pups. The temporal profile of induction are very similar to those shown following LPS administration (Kent et al., 1996). The release of ACTH, which stimulates the release of corticosterone, is induced by KA at a greater extent at earlier ages (PN 6 and PN 12) and it appears even stronger than that induced by LPS. Similarly to endotoxin administration, negative feedback starts only at PN 18; thus, at earlier ages, corticosterone release by the adrenal glands stimulated by ACTH induces feedback inhibitory control. Interestingly, the authors showed that the elevation of levels of circulating hormones following KA administration was not due to ictal activity, since the raise of ACTH and corticosterone concentrations in animals who experienced SE was found long before the onset of any behavioural manifestation, and was similar to that found in animals that did not show any abnormal behaviour (Kent et al., 1996). These observations suggest that the lack or limited induction of mRNAs for cytokines at earlier ages (PN 9 and PN 15)
following KA-induced SE might be a consequence of the anti-inflammatory effect of high levels of corticosterone triggered by KA administration itself. At PN 21, the raise of cytokines would be affected by the negative feedback mechanism acting on HPA axis, hence limiting elevation of corticosterone with a subsequent decrease of anti-inflammatory effect and resulting in a pronounced elevation of transcripts for proinflammatory cytokines.

In regard to the activation of the HPA axis following the administration of flurothyl, inhalation of ether vapor at PN 9 has been shown to be ineffective or mildly stimulating corticosterone levels (Suchecki et al., 1995; Walker et al., 1993). This evidence may explain why rat pups exposed to flurothyl-induced SE showed elevation of transcripts for IL-1β and TNF-α (fig. 12).

It should be mentioned, however, that a major limitation of data obtained in this experimental section is in that the interpretation of results relies on the assumption that the pattern of induction of corticosterone levels in our experimental conditions is similar to that reported in the literature for similar experimental paradigms. Indeed, our experimental system and the experimental paradigms share common features such as age, gender and treatment. However, the accordance of basal and LPS-induced levels of corticosterone and ACTH in our experimental system to those reported in the literature should be confirmed and extended. Two parameters, in particular, would be amenable of attention, i) the temporal profile of corticosterone induction shortly after the administration of a protective dose of the endotoxin and ii) the pattern of induction of corticosterone levels induced by a non-protective dose of LPS. As it regards the first parameter, the evaluation of corticosterone levels at time-points as short as 15-30 minutes after LPS administration would allow to verify the
hypothesis of the involvement of membrane mineralocorticoid receptors in the quick modulation of seizure susceptibility in our experimental system, being this time-window hardly compatible with effects mediated by an action of corticosteroids at genomic level. As it concerns the second parameter, i.e. the induction of corticosterone by a non-protective dose of LPS, it cannot be ruled out that the lack of a protective effect of the highest doses of LPS might be ascribed to an inadequate amount of corticosterone due to an inefficient machinery of the immature HPA axis when facing an intense stimulation, as shown to occur in regard to the ACTH levels in young pups (Vazquez, 1998).
4.6 LPS-induced IL-1β and TNF-α mRNA expression in the hippocampus of PN 9 rats and its modulation by corticosteroid receptors

4.6.1 Introduction

Our data indicate that corticosterone released by the LPS-induced HPA axis activation modulates seizure susceptibility by activation of membrane mineralocorticoid receptors. Gene transcription of proinflammatory cytokines such as IL-1β and TNF-α is NF-κB-dependent and susceptible to modulation by steroid hormones. Thus, it cannot be excluded that antagonism to mineralocorticoid receptor by canrenoic acid affects mRNA levels of these proinflammatory cytokines. It is possible that the effect of canrenoic acid administration on LPS-induced decrease in seizure susceptibility in PN 9 rats, is due to an enhancement of transcription of proinflammatory cytokines. In particular, IL-1β has been shown to increase seizure susceptibility in adult rodents. It could be argued that we showed IL-1β not to affect seizure susceptibility in PN 9 rats (fig. 13, paragraph 4.3.2) and we hypothesized it might be due to an insufficient increase in the brain parenchyma of IL-1β levels, mainly because of the short interval between the icv administration of IL-1β and seizure induction (5 min). However, the experimental protocol we used for testing
seizure susceptibility was aimed to evaluate the levels of proinflammatory cytokines 2h after the administration of LPS. Moreover, the involvement of cerebral vascular system due the systemic administration of LPS is expected to induce an ubiquitous production of proinflammatory cytokines in the brain parenchyma. In principle, this widely distributed induction of IL-1β may actually affect seizure susceptibility negatively, as it occurs in adult rodents.

Thus, we investigated the pattern of LPS-induced gene expression of IL-1β and TNF-α and its modulation by antagonists of corticosteroid receptors in our experimental system.
4.6.2 Materials and methods

Technical details related to mRNA extraction, reverse transcription and evaluation of gene expression by real-time PCR have already been reported in 'Materials and methods - general procedures'.

The concentration of LPS used was 0.1 mg/kg. The experimental animals were sacrificed 2 h after the PBS or LPS injection for the subsequent evaluation of levels of transcripts in hippocampal tissue.

Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg) alone or 90 min after the systemic LPS.

RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg) alone or 90 min after the systemic LPS.
4.6.3 Results

Figure 18, (A-D), depicts the effect of systemic administration of canrenoic acid and RU486 on the LPS-induced IL-1β and TNF-α mRNA expression in PN 9 rats.

**Effect of canrenoic acid**

Systemic administration of 0.1 mg/kg LPS induced a 12-fold increase of IL-1β and a 25-fold increase of TNF-α above basal levels (p<0.01).

Antagonism of mineralocorticoid receptors by canrenoic acid alone did not affect the mRNA expression of IL-1β and TNF-α. Conversely, canrenoic acid administration markedly diminished the LPS-induced expression of IL-1β and TNF-α by 68% (p<0.05, panel A) and 60% (p<0.05, panel B) respectively.

**Effect of RU486**

Antagonist of glucocorticoid receptors by RU486 alone, or its vehicle, did not alter cytokine transcripts. Conversely, RU486 administration markedly diminished the LPS-induced expression of IL-1β and TNF-α by 78% (p<0.05, panel C) and 84% (p<0.001, panel D).
Fig. 18

Effect of canrenoic acid and RU486 administration on LPS-induced IL-1β (panels A and C) and TNF-α (panels B and D) mRNAs expression in PN 9 rats
Data are the mean ± SEM (n=6). Panels A and B. * p<0.05 ** p<0.01 vs PBS; §§ p<0.01 vs Canrenoic acid; # p<0.05 vs LPS 0.1 mg/kg; ^ p<0.05 vs Canrenoic acid by Kruskal-Wallis test. Panels C and D. * p<0.05 ** p<0.01 vs PBS; § p<0.05 §§ p<0.01 vs RU486; # p<0.05 ## p<0.01 vs LPS 0.1 mg/kg and LPS 0.1 mg/kg (25% Tween 80) by Kruskal-Wallis test.
4.6.4 Discussion

The basal levels of mRNA expression of IL-1β and TNF-α were not modified by either canrenoic acid or RU486. As expected, LPS administration increased the mRNA expression of these cytokines and this effect was strongly reduced following treatment with either canrenoic acid or RU486.

Our results show that the inhibitory effects of canrenoic acid and RU486 on LPS-induced levels of IL-1β and TNF-α are independent on the effects of these drugs on seizure susceptibility. These data support previous evidence that these cytokines are unlikely to play a role in the susceptibility to seizures in PN 9 rats differently from adult rodents. In addition, our results show that the classical anti-inflammatory effect mediated by corticosteroids is not operative since antagonists of corticosterone receptors decrease LPS-induced IL-1β and TNF-α mRNA expression.

As a possible explanation of our findings, it is worth considering that, however, the production of proinflammatory mediators by glial cells is expected to be accompanied by production of regulators which limit in space and time the action of such inflammatory mediators. Among these regulators are IL-10 and IL-4, often referred to as anti-inflammatory cytokines. In peripheral tissues, these cytokines can be produced by a variety of immune cells (e.g. T cells and macrophages) and are known to suppress the secretion of proinflammatory cytokines and NO by activated macrophages. In the CNS, anti-inflammatory cytokines are produced by microglial and astroglial cells under various pathological conditions, including multiple sclerosis (Cannella and Raine, 1995; Peress et al., 1996), experimental allergic encephalomyelitis (EAE) (Jander et
al., 1998; Kiefer et al., 1998; Laman et al., 1998), ischemia (Knuckey et al., 1996; Lehrmann et al., 1998), AIDS (Johnson and Gold, 1996; Wahl et al., 1991), and Alzheimer's disease (Flanders et al., 1995; Peress and Perillo, 1995). Since their production is associated with lesions or affected sites in the brain, IL-10 and IL-4 may have anti-inflammatory actions in the CNS similar to those in the periphery, i.e. inhibition of the production of inflammatory mediators.

As already discussed in the introductory part of the thesis, another candidate mechanism that could account for our results involves PGE2. It is worth reminding that PGE2 was shown to reduce microglia activation in vivo (Caggiano and Kraig, 1996) and in vitro (Théry et al., 1994; Caggiano and Kraig, 1996; Caggiano and Kraig, 1998; Minghetti et al., 1997) by down-regulating the expression of major histocompatibility complex class II and the synthesis of proinflammatory cytokines. In particular, PGE2 was shown to largely prevent the expression of IL-1β and TNF-α mRNA elicited by tissue injury (Zhang and Rivest, 2001).

Therefore, in the following two experimental sections we investigated the involvement of anti-inflammatory cytokines and PGE2 as possible candidate mechanisms capable to account for our findings.
4.7 LPS-induced IL-4, IL-10, iNOS and IL-1Ra mRNAs expression in the hippocampus of PN 9 rats and their modulation by corticosteroid receptors

4.7.1 Introduction

As mentioned in the previous section, in the present experimental investigation we turn our attention to the levels of gene expression of anti-inflammatory cytokines such as IL-4 and IL-10 in the same samples processed for IL-1β and TNF-α evaluation. Indeed, it cannot be ruled out that the pattern of expression of IL-1β and TNF-α mRNAs as shown in the previous experimental section might be ascribed to the induction of anti-inflammatory cytokines such as IL-10 and IL-4.

Biologically active IL-10 is a homodimers that affects many aspects of inflammatory and immune responses and acts on both haematopoietic and non-haematopoietic cells. Studies accomplished by cultures of microglia, co-cultures of rat astroglial and microglial cells and by in vivo administration of IL-10 have shown that exogenous IL-10 suppressed the LPS-induced production of NO, IL-6, IL-1β and TNF-α at level of both proteins production and gene expression,
whereas antibody to IL-10 yielded the opposite results (Kremlev and Palmer, 2005; Ledeboer et al., 2000; Ledeboer et al., 2002; Di Santo et al., 1995). IL-10 was also shown to enhance production of IL-1Ra by delaying mRNA degradation (Cassatella et al., 1994; Jenkins et al., 1994; de Waal Malefyt et al., 1991). Interestingly, IL-10 inhibits its own mRNA expression, thus indicating an autoregulatory loop for IL-10 (Ledeboer et al., 2002). Computer-assisted studies inherent to the identification of consensus sequences for nuclear transcription factors have shown that the gene of IL-10 possesses putative AP-1 binding sites, glucocorticoid response element (GRE) and a cAMP response element (CRE) which appears to be functional, as agents that raise intracellular cAMP levels enhance the expression of IL-10 in monocytes. Interestingly, IL-10 was shown to inhibit PGE\textsubscript{2} production induced by LPS-stimulated monocytes (Niiro et al 1994). This latter observation seems to suggest a regulatory loop between IL-10 and PGE\textsubscript{2} since, on the one hand, IL-10 gene expression is up-regulated by cAMP elevating agents (such as PGE\textsubscript{2} itself), while, on the other hand, IL-10 inhibits PGE\textsubscript{2} production following LPS stimulation.

Also IL-4 is an anti-inflammatory cytokine capable to reduce the production of inflammatory mediators, including IL-1\textbeta, TNF-\alpha, iNOS, COX-2 induced by activated microglia in vivo and in vitro (Furlan et al., 2000; Ledeboer et al., 2000). IL-4 production is controlled post-transcriptionally through stabilization of IL-4 mRNA (Dokter et al., 1993). Interestingly, in activated human T lymphocytes, accumulation of IL-4 mRNA is down-regulated by PGE\textsubscript{2} and cAMP-dependent pathways, thus indicating the activation of PKA as a negative regulatory mechanism in IL-4 expression (Borger et al., 1996).
Evidence shown above prompted us to associate the evaluation of IL-4 and IL-10 gene expression with the measurement of transcription levels of IL-1Ra and iNOS, both molecules being relevant in the context of a proinflammatory state. We will take advantage from the evidence that these molecules are affected by the protein levels of IL-4 and IL-10, thus their modulation could be exploited as a marker of the functional involvement of the two anti-inflammatory cytokines.
4.7.2 Materials and methods

Technical details related to mRNA extraction, reverse transcription and evaluation of gene expression by real-time PCR have already been reported in 'Materials and methods - general procedures'.

The concentration of LPS used was 0.1 mg/kg. The experimental animals were sacrificed 2 h after the PBS or LPS injection for the subsequent evaluation of levels of transcripts in hippocampal tissue.

Canrenonic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg) alone or 90 min after the systemic LPS.

RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg) alone or 90 min after the systemic LPS.
4.7.3 Results

Figure 19, (A-F), depicts the effect of systemic administration of canrenoic acid and RU486 on the LPS-induced IL-10, IL-1Ra and iNOS mRNAs expression in PN 9 rats. Likely due to the paucity of expression of IL-4 mRNA, RT-PCR did not yield any amplification for this cytokine. Indeed, the samples fluorescence was never significantly different from no-template controls (data not shown).

Effect of canrenoic acid

Panel A

Systemic administration of 0.1 mg/kg LPS induced a 2-fold increase of IL-10 vs PBS injected group (p<0.01) whereas canrenoic acid +LPS induced a 2.5-fold increase of IL-10 above basal levels (p<0.01). The experimental groups treated with LPS and canrenoic acid + LPS were not statistically different. Canrenoic acid per se did not affected the basal levels of expression of IL-10.

Panel B

The administration of 0.1 mg/kg LPS induced a 2.2-fold increase of IL-1Ra vs PBS injected group (p<0.01) whereas canrenoic acid +LPS induced a 1.7-fold increase of IL-1Ra above basal levels (p<0.01). The expression of IL-1Ra in the experimental group treated with canrenoic acid + LPS was
significantly reduce by 21% as respect to the experimental group treated with LPS alone (p<0.01). Canrenoic acid per se did not affect the basal levels of expression of IL-1Ra.

Panel C

LPS administration (0.1 mg/kg) induced a 4-fold increase of iNOS vs PBS injected group (p<0.01) whereas canrenoic acid +LPS induced a 3.4-fold increase of iNOS above basal levels (p<0.01). The experimental groups treated with LPS and canrenoic acid + LPS were not statistically different. Canrenoic acid per se did not affected the basal levels of expression of iNOS.

Effect of RU486

Panel D

Systemic administration of 0.1 mg/kg LPS induced a 2-fold increase of IL-10 vs PBS injected group (p<0.05) whereas RU486 +LPS induced a 2.7-fold increase of IL-10 above basal levels (p<0.01). RU486 per se and RU+LPS induced a significant increase of the basal levels of expression of IL-10 (230% p<0.01, 170% p<0.01, respectively). The increments of levels of IL-10 mRNA induced by RU486 and RU486+LPS were also statistically different from that induced by LPS (p<0.01, p<0.05 respectively). The experimental groups treated with LPS and RU486 + LPS were not statistically different.
Panel E

The administration of 0.1 mg/kg LPS induced a 2-fold increase of IL-1Ra vs PBS injected group (p<0.01) whereas RU486 +LPS induced a 4-fold increase of IL-1Ra above basal levels (p<0.01). The levels of IL-1Ra mRNA induced by RU486+LPS were significantly higher than those induced by LPS (p<0.01). RU486 per se did not affect the basal levels of expression of IL-1Ra.

Panel F

LPS administration (0.1 mg/kg) induced a 4-fold increase of iNOS vs PBS injected group (p<0.05) whereas RU486 +LPS induced a 13-fold increase of iNOS above basal levels (p<0.01). The levels of iNOS mRNA of experimental groups administered with LPS and RU486+LPS were statistically different (p<0.01). RU486 per se did not affect the basal levels of expression of iNOS.
Fig. 19

Effect of the administration of Canrenoic Acid (panels A, B, C) and RU486 (panels D, E, F) on LPS-induced IL-10, IL-1Ra and iNOS mRNAs expression in PN 9 rats

**IL-10 mRNA expression**

A

![Bar chart showing IL-10 mRNA expression](chart)

**IL-1Ra mRNA expression**

B

![Bar chart showing IL-1Ra mRNA expression](chart)
Fig. 19 – (continued)

**C** iNOS mRNA expression

- **ORBS**
- **LPS 0 mg/kg**
- **Canrenoic Acid**
- **LPS+Canrenoic Acid**

**D** IL-10 mRNA expression

- **ORBS**
- **LPS 0.1 mg/kg**
- **RU486**
- **LPS+RU486**
Fig. 19 – (continued)

Data are the mean ± SEM (n=6). Panels A, B, C. * p<0.05  ** p<0.01 vs PBS; § p<0.05 §§ p<0.01 vs Canrenoic acid; ## p<0.01 vs LPS 0.1 mg/kg, by Kruskal-Wallis test. Panels D, E, F. * p<0.05  ** p<0.01 vs PBS; § p<0.05 §§ p<0.01 vs RU486; # p<0.05  ## p<0.01 vs LPS 0.1 mg/kg, by Kruskal-Wallis test.
4.7.4 Discussion

One of the immediate conclusions coming from our results is the irrelevant influence of IL-4. The amount of IL-4 mRNA expression is so low, even after stimulation by LPS, that a sensible technique such as real-time PCR cannot detect a significant amount of transcript in any of the experimental groups. This result is not surprising since other investigators have met undetectable amount of IL-4 both in terms of mRNA expression and protein synthesis, suggesting that in several experimental systems IL-4 cannot be readily produced in functionally relevant concentrations (Xiao et al., 1996; Brodie et al., 1998; Suzumura et al., 1994; Ledeboer et al., 2000), despite several studies indicate that IL-4 can be expressed by both microglial and astroglial cells in neuropathological conditions such as multiple sclerosis and EAE (Cannella and Raine, 1995; Laman et al., 1998; Ledtke et al., 1998). It cannot be ruled out that the short time-window of our testing, together with undetermined age-dependent feature of hippocampal tissue, may account for the low pattern of expression of IL-4 transcript.

Additionally, these results suggest that also IL-10 is not relevant in governing our experimental system. A few observations and several evidence from the literature support this conclusion. The first trivial observation is that the pattern of LPS-induced IL-1β and TNF-α mRNA expression modulated by CA and RU486 is always toward a decrease of transcript levels. If such a decrease was ascribed to the modulation of IL-10 than it is expected a similar pattern of LPS-induced IL-10 mRNA induction modulated by either corticosteroid receptors
antagonists. This is not the case, as the administration of CA does not affect IL-10 mRNA expression, whereas RU486 per se increases the levels of transcript of this anti-inflammatory cytokine. Indeed, this effect seems to be maximal for the system since LPS administration does not further increase the levels of IL-10 transcripts. A second relevant consideration comes from the experimental observation that following LPS-administration both IL-10 gene expression and IL-10 protein synthesis takes much longer to be induced than those of IL-1β and TNF-α. In principle, this observation does not rule out that a significant amount of IL-10 protein is synthesized, hence leading to a subsequent decrease of the expression of proinflammatory cytokines. Nonetheless, in co-cultures of rat microglial and astroglial cells, although LPS induces an up-regulation of IL-10 mRNA expression, several hours are required for IL-10 protein to become apparent in the medium (Ledeboer et al., 2002). A similar slow onset of IL-10 mRNA and protein responses has been reported for human monocytes (de Waal Malefyt et al., 1991; Donnelly et al., 1995) and microglial cells (Sheng et al., 1995; Williams et al., 1996; Kitamura et al., 2000). Indeed, the relatively delayed increase of IL-10 production after LPS activation is markedly different from the early onset of production of proinflammatory cytokines such as IL-1β and TNF-α, whose secretion was shown already detectable at 2h (Ledeboer et al., 2002; Sheng et al., 1995; Chao et al., 1995). For that reason the delayed production of IL-10 as compared to that of IL-1β and TNF-α is consistent with the concept of a modulating role of IL-10 in the late phase of the inflammatory cascade (Zhai et al., 1997). Thus, it is hardly conceivable that our experimental system is facing a relevant amount of IL-10 sufficient to account for a strong down-regulation of IL-1β and TNF-α gene expression, despite an increased level
of IL-10 mRNA as shown following RU486 administration. It is of interest to observe that another confirmation of the lack of significant amount of IL-10 protein production in our system may come from the increased expression of LPS-induced iNOS gene expression following RU486 administration. This finding is inconsistent with evidence from literature showing that IL-10 is likely to down-regulate the iNOS levels of transcript in cultures of rat microglia (Minghetti et al., 1998). However, it could be argued that IL-10 was shown to induce the production of IL-1Ra and our data with RU486 seems to support this finding. Nonetheless, in our opinion, the evidence supporting the lack of functionally relevant levels of IL-10 protein in our experimental conditions makes this presumed direct induction of IL-1Ra by IL-10 unlikely.

The evaluation of IL-1Ra as a potential anti-inflammatory modulator in our experimental system seems to suggest two different patterns of expression modulated by either corticosteroid receptors antagonists, being IL-1Ra gene expression enhanced when corticosterone is prevented to act on glucocorticoid receptors by RU486, whereas mineralocorticoid receptors appear to tonically sustain the expression of IL-1Ra mRNA. Clearly, these different patterns of IL-1Ra expression are not compatible with the down-regulation of LPS-induced gene expression of IL-1β and TNF-α which is a common feature observed following the administration of either corticosteroid receptors antagonists. Interestingly, dexamethasone (an agonist of glucocorticoid receptors) was shown to inhibit IL-1Ra macrophage mRNA expression in LPS-treated rats (Ulich et al., 1992). Similar results were also obtained in LPS-stimulated human monocytes (Arzt et al., 1994). The effect was reversed by blocking the glucocorticoid receptors with the antagonist RU486. Indeed, this paper showed
that inhibition by dexamethasone concomitantly affected both mRNA expression and protein levels of IL-1Ra, thus suggesting that IL-1Ra gene expression is regulated by glucocorticoid receptors (Arzt et al., 1994). This evidence may explain the decrease of LPS-induced IL-1Ra mRNA expression mediated by CA administration, as shown in our experiments. The occupancy of the abundant population of mineralocorticoid receptors by CA could increase the amount of corticosterone available to bind to glucocorticoid receptors, hence the decrease of IL-1Ra gene expression as shown following stimulation of this type of corticosteroid receptor. However, such an explanation may account for the down-regulation of LPS-induced IL-1Ra mRNA modulated by CA, but cannot be used as a possible explanation for the enhanced transcription of LPS-induced IL-1Ra mRNA following the administration of RU486. Indeed, RU486 was shown to counteract the effect of dexamethasone but not to increase the IL-1Ra gene expression when administered alone (Arzt et al., 1994).

Thus, the patterns of expression of conventional anti-inflammatory molecules such as IL-4, IL-10 and IL-1Ra lead to exclude a role for any of these molecules in mediating the down-regulation of LPS-induced IL-1β and TNF-α mRNA expression following the administration of CA and RU486, as shown in our experimental conditions.

As far as the iNOS gene expression in concerned, it is not surprising to observe an insensitivity of this proinflammatory molecule to modulation by mineralocorticoid receptors, being iNOS mainly produced by glial cells. The antagonism of glucocorticoid receptors by RU486 enhances the LPS-induced gene expression of iNOS, apparently suggesting a conventional anti-inflammatory role of glucocorticoid receptors. Thus, from this point of view, it is
expected that blockade of glucocorticoid receptors by RU486 increases the expression of NF-κB-dependent inflammatory molecules such as iNOS. However, this hypothesis does not account for the down-regulation of LPS-induced IL-1β and TNF-α mRNA expression following the administration of CA and RU486, as shown in our experimental conditions. According to evidence from literature, this result may be due to the insensitivity of microglia to circulating corticosterone, an insensitivity which seems not to affect iNOS production. Indeed, our data accentuate the discrepancy among the different patterns of gene expression of IL-1β, TNF-α and iNOS following the administration of either corticosteroid receptors antagonists, being all of them NF-κB-dependent immediate early genes. Thus, our data further exclude that corticosteroid receptors antagonists might be directly involved in the induction of patterns of gene expression so different from each other. Rather, CA and RU486 are expected to induce the production of a common mediator which should account for the majority (if not the whole) patterns of gene expression seen in our experimental conditions. Such a common mediator, as suggested by our evaluations, does not belong to the category of conventional anti-inflammatory cytokines such as IL-4, IL-10 and IL-1Ra.

It is of interest to note that a quick survey on present data allows a distinction of the functional involvement of neuronal and glial cell populations, in that mineralocorticoid receptors are located on neurons whereas glucocorticoid receptors are mainly located on glial cells. Thus, it is not surprising to find out a substantial lack of effect of canrenoic acid as respect to the effects modulated by RU486, being IL-10, iNOS and IL-1Ra molecules originating from glial cells mainly. In this respect, it could be argued that also the patterns of inhibition of
LPS-induced IL-1β and TNF-α mRNA expression modulated by CA and RU486 appear similar to each other, thus potentially suggestive of an irrelevant role of mineralocorticoid receptors. Nonetheless, the similarity is limited to the qualitative pattern since the relative amount of the inhibition of IL-1β and TNF-α mRNA expression by RU486 is much more pronounced as respect to the inhibition induced by CA, thus suggesting a specific involvement of RU486 whose effect might sum to or synergize with a presently unknown factor.

According to evidence from literature, we formerly proposed also PGE$_2$ as a candidate mediator of the down-regulation of proinflammatory cytokines expression, and our recent results keep this hypothesis conceivable. Indeed, COX-2 and iNOS are immediate early genes whose products of translation (PGE$_2$ and NO, respectively) are promptly synthesized in LPS-stimulated glial cells. NO was shown to enhance the enzymatic activity of COX-2, without affecting the levels of its mRNA expression (Salvemini et al, 1993). Therefore, PGE$_2$ production under a proinflammatory stimulus such as LPS appears to be favoured also in our experimental conditions. However, such a mechanism would only account for the mechanism of action of RU486, since CA does not affect iNOS mRNA expression. Nonetheless, our experimental system shows that both corticosteroid receptors antagonists down-regulate the IL-1β and TNF-α mRNA expressions. Thus, it may be that CA and RU486 lead to an enhancement of prostanoid production through the involvement of different pathways, as the receptors upon which they act are localized on distinct cellular populations, that is neurons (mineralocorticoid) and glia (glucocorticoid).
4.8 Modulation of LPS-induced COX-2 mRNA expression by canrenoic acid and RU486 in the hippocampus of PN 9 rat

In this experimental section we focused on the involvement of PGE$_2$ in the modulation of IL-1$\beta$ and TNF-\(\alpha\) mRNA expression as shown in our experimental findings. Specifically, we evaluated the hypothesis that CA and RU486 would enhance PGE$_2$ production by up-regulating the COX-2 gene expression. Such a hypothesis is indeed conceivable since gene transcription of COX-2 is NF-\(\kappa\)B-dependent. Therefore, inhibition of corticosterone action by antagonists is expected to boost the expression of NF-\(\kappa\)B-dependent genes. From this point of view, this is an intriguing issue since in our experimental system the expression of IL-1$\beta$ and TNF-\(\alpha\) mRNAs seems not to be affected by a direct action of corticosterone, although gene transcription of both of these proinflammatory cytokines is NF-\(\kappa\)B-dependent.
4.8.1 Materials and methods

Technical details related to mRNA extraction, reverse transcription and evaluation of gene expression by real-time PCR have already been reported in ‘Materials and methods - general procedures’.

The concentration of LPS used was 0.1 mg/kg and after drug delivery the PN 9 rats were always sacrificed 2 hours after the endotoxin challenge for the successive evaluation of levels of transcripts in hippocampal tissue.

Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg) 30 minutes before sacrifice (i.e. 90 minutes after the systemic LPS administration).

RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg) 30 minutes before sacrifice.
4.8.2 Results

Fig. 20 depicts the effect of canrenoic acid administration on LPS-induced COX-2 mRNA expression in PN 9 rats. Data are the mean ± SE (n = 6 pups). The ordinate axis represents the level of mRNA expression as percentage of the control (PBS-injected) group, assessed by quantitative RT-PCR.

The protective dose of LPS (0.1 mg/kg) significantly increased the expression of COX-2 mRNA (303.1 ± 15.4) %, p<0.01 vs PBS-injected control group). This increment was not affected by the systemic administration of canrenoic acid, a mineralocorticoid receptor antagonist (406.4 ± 54.0) %. Canrenoic acid did not affected the basal level of COX-2 mRNA expression when administered alone.

Fig. 21 shows the effect of systemic administration of the glucocorticoid receptor antagonist RU486 on LPS-induced COX-2 mRNA expression in PN 9 rats. As above, data are the mean ± SE (n = 6 pups) and the ordinate axis represents the level of mRNA expression as a percentage of control (PBS-injected) group, assessed by quantitative RT-PCR. The vehicle used to dissolve RU486 (25% Tween 80) did not affect the levels of COX-2 transcripts when administered alone and when injected 90 minutes after 0.1 mg/kg LPS.

The systemic administration of RU486 appeared to not affect the basal and LPS-induced expression of COX-2 mRNA. The only statistically significant differences were strictly related to the LPS-induced increment of COX-2 transcription vs respective control groups, i.e. 280.1 ± 29.2 % vs 100.0 ± 7.3 % (p<0.05).
Fig. 20

Effect of Canrenoic acid administration on LPS-induced COX-2 mRNA expression in PN 9 rats

Data are the mean ± SE (n=6). * p<0.01 ** p<0.001 vs PBS; # p<0.01 ## p<0.001 vs Canrenoic acid. Kruskal-Wallis followed by Dunns multiple comparison test
Fig. 21

Effect of RU486 administration on LPS-induced COX-2 mRNA expression in PN 9 rats

Data are the mean ± SE (n=6). * p<0.05 ** p<0.01 vs PBS group; § p<0.05 ° p<0.01 vs 25% Tween 80 and RU486 groups. Kruskal-Wallis followed by Dunns multiple comparison test
4.8.3 Discussion

Systemic LPS administration induces COX-2 mRNA expression in the rat hippocampus at PN 9 but this effect appears to be insensitive to modulation by corticosterone. Since LPS is expected to activate gene transcription by NF-κB pathway, such insensitivity might suggest an apparent NF-κB-independent mechanism. Nonetheless, it is difficult to reasonably exclude an NF-κB-dependent mechanism of expression for COX-2, in particular when mediated by LPS administration, as several papers have demonstrated (Lacroix and Rivest, 1998; Laflamme and Rivest, 1999; Laflamme et al., 1999; Zhang and Rivest, 2001). Moreover, the inhibitory effect of glucocorticoids onto microglial COX-2 expression has been clearly shown in vitro (Bauer et al., 1997).

Nonetheless, it is important to note that, in spite of a high responsiveness to glucocorticoids in vitro, microglia were shown not to readily respond to manipulation of glucocorticoid levels in vivo (Castaño et al., 1996). According to available studies, this could be due to the density of corticosteroid receptors in microglia in vivo which is negligible in comparison with that found in neuronal cells and astrocytes, where they have been mainly localized (Aronsson et al., 1988; Chou et al., 1991; Cintra et al., 1994). In addition, in vivo microglial glucocorticoid receptors appear to be down-regulated (Minghetti and Levi, 1998).

If it is assumed that, in our experimental system, COX-2 expression is mainly of microglial origin and that its transcript is indeed NF-κB-dependent, our data are in agreement with a manifest insensitivity to circulating corticosterone.
of microglia in vivo. Indeed, the similarity of pattern of induction of COX-2 following administration of either type I or type II corticosteroid receptor antagonists supports this hypothesis.

The insensitivity of COX-2 expression and, presumably, PGE₂ production to circulating corticosterone, increases the probability that the proconvulsant effect following the administration of canrenoic acid might be exclusively due to the lack of occupancy of membrane mineralocorticoid receptors, apparently without a significant contribution of PGE₂.

Nonetheless, although PGE₂ does not appear directly involved in the modulation of the effect of canrenoic acid administration on the LPS-induced delay of seizure-onset in rat pups, in adult animals the increase of its levels has been shown as a fundamental step for the activation of the HPA axis following the injection of a proinflammatory stimulus. It is conceivable that also in rat pups the increase of PGE₂ following LPS administration could activate the HPA axis, thus leading to the age-dependent anticonvulsant effect of corticosterone.

So, on the one hand, PGE₂ could play an indirect anticonvulsant role through the activation of the HPA axis, while, on the other hand, evidence from literature shows that (in adult rats) the administration of a COX-2 inhibitor delayed flurothyl-induced seizure-onset, hence supporting a proconvulsant role of PGE₂. In the next experimental section we went on investigating on the possibility of a functional involvement of PGE₂ in our experimental system. We focused our attention on alternative mechanisms capable to boost PGE₂ production despite unchanged levels of expression of COX-2 mRNA.
4.9 Modulation of the expression of the PKA regulatory subunit RIIβ and PKC-γ by corticosteroid receptors

4.9.1 Introduction

Our experimental results suggest that despite the insensitivity of microglia to circulating corticosterone, canrenoic acid and RU486 administration somehow modulate the down-regulation of IL-1β and TNF-α expression (fig. 18). This modulation is likely to involve an effector originating from neurons and/or astrocytes following blockade of corticosterone action.

We speculated that, despite unchanged levels of COX-2 expression, the treatment with canrenoic acid or RU486 could lead to an increase of PGE₂ production from non-microglial cells. This would lead to the decreased expression of proinflammatory cytokines resulting from the deactivating role of PGE₂ over microglia.

Therefore, we wondered whether corticosteroids might affect the gene expression of some proteins usually involved in the modulation of the functionality of the COX-2/PGE₂ system, leading to an overexpression of PGE₂ production, in particular in non-microglial cells.
As discussed in the introductory section (see paragraphs 1.4.5.1 and 1.4.5.2), the expression and functionality of COX-2 and PGE$_2$ are strictly interrelated with the activity of two relevant kinases, PKA and PKC. Therefore, as a working hypothesis, we wondered whether antagonists to corticosteroid receptors could affect the expression of these two enzymes and therefore account for the postulated overproduction of PGE$_2$ observed, in spite of the unchanged level of expression of COX-2 mRNA.

As PKA and PKC are characterized by several isoforms and variations of constitutive regulatory and catalytic subunits, it is of interest to ascertain whether specific isozymes as well as subunits might be of particular relevance in order to target their mRNA expression.
4.9.1.1 PKA: a relevant role of the regulatory subunit

RIIβ

In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two catalytic subunits (C) bound to a dimer which constitute the regulatory subunit (R). Activation of PKA occurs when four molecules of cAMP bind to the R subunit dimer, two to each subunit, in a positive cooperative fashion. As a consequence of cAMP binding, the R subunits adopt a configuration with low affinity for the C subunits and the holoenzyme dissociates. Active C subunits then phosphorylate serine and threonine residues on specific substrate proteins (Skålhegg et al., 2000). The two major forms of PKA are termed type I and II as they were shown to contain C subunits associated with two different R subunits, termed RI and RII. Cloning of cDNAs for regulatory subunits have identified for each R subunit two isoforms termed α and β, hence giving rise to RIIα, RIIβ, RIIα and RIIβ. Generally, PKA type I is soluble and preferentially located in the cytosol whereas type II preferentially binds to the particulate fraction (Meinkoth et al., 1990). The tissue distribution of regulatory subunits is such that RIIα and RIIα are present ubiquitously, whereas RIIβ is present in the brain. However, the predominant isoform and principal mediators of cAMP-induced activity in mammalian CNS is RIIβ, which shows a high affinity for specific proteins located on membranes of intracellular structures such as the Golgi complex and perinuclear area. These proteins are generally named A-kinase anchoring proteins, and are required to mediate specific, localized effects of cAMP to
subcellular domains (Sarkar et al., 1984). In cortical cells, RIIβ was shown abundant in neurons and astrocytes (Stein et al., 1987).

Interesting observations have been made on investigation of the differential response of PKA to cAMP in discrete brain areas in relation to the abundance of RIIβ mRNA expression (Ventra et al., 1996 J). Indeed, the investigators pointed out that cerebral areas with high levels of membrane-bound PKA and low levels of cytosolic catalytic subunit also have high levels of RIIβ expression, suggesting that areas containing membrane-bound PKA maintain PKA prevalently undissociated, thus showing a correlation between RIIβ mRNA level measurements and PKA intracellular distribution. In comparison with cerebral areas such as neocortex and corpus striatum, the hippocampus expresses low levels of RIIβ mRNA in terms of relative ratio to the other R subunits, R1α and RIIα. Nonetheless, this limbic area shows a very high level of PKA catalytic activity in the cytosol, activity which is the highest among all the cerebral areas investigated (Ventra et al., 1996 J). Furthermore, the same paper showed a relevant differential feature between cerebral areas with low levels of RIIβ mRNA expression (such as cerebellum) and those expressing high levels (such as neocortex), in that although PKA responsiveness to cAMP-stimulation of areas with lower RIIβ mRNA is highly sensitive, there is a substantial lack of translocation of catalytic subunits into the nucleus, as also confirmed by immunoblot analysis aimed to evaluate the amount of nuclear phosphorilated-CREB. Conversely, high RIIβ mRNA expressing areas were shown poorly sensitive to cAMP-stimulation, leaving PKA mainly in the undissociated state. However, despite the low degree of PKA dissociation, the response in terms of nuclear translocation of catalytic
subunits is quick and sustained, as confirmed by the consistent levels of phosphorylated-CREB (Ventra et al. 1996).

Regarding the gene expression of PKA regulatory subunits, the lack of DNA-binding sites sensitive to direct or indirect modulation of RIIβ gene expression by glucocorticoids (GRE, NF-κB, AP-1) would apparently suggest a weak relationship between corticosteroids and PKA. Nonetheless, several studies have suggested that glucocorticoids can up- or down-regulate adenylyl cyclase activity depending on the type of neurotransmitter receptors affected (Mobley et al., 1983; Harrelson et al., 1987; Gannon et al., 1990; Duman et al., 1989; Johnson et al., 1983; Saito et al., 1989). Of interest to our purposes, in the cortex and hippocampus of adult rats, it was shown that removal of corticosterone by adrenalectomy increased PKA activity and cAMP binding sites to PKA, along with an increase of protein expression for the specific subunits Rlα and RIIβ as well as catalytic subunit β (Dwivedi et al., 2000). These increments were preventable by simultaneous treatment with corticosterone, which caused just the opposite effect on administration to intact animals.

It is worth noting that the relationship between the catalytic activity of PKA and RIIβ might be relevant in view of the modulating role of the PKA C subunit upon both transcriptional activity of NF-κB and cross-repression by NF-κB and the glucocorticoid receptor (Zhong et al., 1997; Doucas et al., 2000).

The majority of available data comes from studies accomplished in adult animals which underwent prolonged treatment with corticosteroids, thus dealing with experimental protocols which are substantially different from our experimental conditions. Nonetheless, they show that glucocorticoids can
modulate the cAMP-PKA system, including the expression of regulatory and catalytic subunits of the kinase. However, these studies would suggest an indirect effect of glucocorticoids since interactions between the HPA axis and cAMP-PKA system become significantly relevant only on chronic treatments with augmented or abolished levels of circulating corticosterone, as, for instance, in the case of corticosterone-supplemented naïve animals or adrenalectomized animals.
4.9.1.2 PKC: a relevant role of the isoform γ

Generally, protein kinase C is a serine/threonine protein kinase ubiquitously found in a variety of animal tissues and shown to be encoded by a family of closely related genes, hence giving rise to several PKC isoforms. Structurally, PKC consists of a regulatory domain and a catalytic domain (Nishizuka et al., 1991). To date, at least 12 PKC isozymes are known and have been classified as conventional (α, βI, βII and γ), novel (δ, ε, η, θ and μ), or atypical (ζ, λ and i). Novel PKCs differ from conventional ones because they do not require Ca²⁺ for activation whereas atypical PKCs, besides being Ca²⁺ -independent, are also insensitive to DAG and to phorbol ester activation (Dwivedi et al., 1999). Each PKC isozyme differs in its distribution, biochemical characteristics and substrate specificity. PKC isozymes α, δ and ζ are present in nearly all tissues, whereas PKC ε, η and θ are restricted to a few tissues, and PKC γ is present only in the brain and spinal cord (Nishizuka, 1988; Nishizuka et al., 1991; Casabona, 1997). The PKC γ-isoform, which is exclusively located in neuronal cells and totally absent in microglia and astrocytes (Gott et al., 1994; Roisin et al., 1997; Masliah et al., 1991; Slepko et al., 1999), is highly concentrated in cerebellum, hippocampus, amygdala and cerebral cortex (Huang et al., 1987). During the rat brain development this kinase was shown to be expressed at a low level in the neonate one-week after birth, whereas a rapid synthesis occurs between two and three weeks of age (Yoshida et al., 1988), a period which correlates with commencement of synaptogenesis and myelination. In keep with the aforementioned observation, it has been suggested that the PKC-γ gene might be developmentally regulated.
in that a specific binding motif in the 5′-flanking region is targeted by nuclear proteins whose levels of expression are evident in PN 3 – 6 rats but much less expressed at PN 14, and by the third week of age become undetectable. As the decrease of binding proteins to such a specific site in developing rats is inversely associated to the expression of PKC-γ, it was speculated that these binding proteins possess a negative regulatory function (Chen et al., 1993).

Several studies have pointed out the relevant role of PKC-γ in the brain, especially in cerebral areas into which neuronal plasticity occurs, as in the hippocampus where PKC-γ is particularly abundant in pyramidal cells (Saito et al., 1988). At the cellular level, PKC-γ, differently from the other isoforms, is nearly exclusively localized postsynaptically (Kose et al., 1990) and it is present abundantly in dendritic spines (Kose et al., 1990; Tsujino et al., 1990). This specific localization is indeed supportive of pharmacological and electrophysiological evidence showing that several neuronal functions such as long-term potentiation (LTP) are specifically PKC-γ-dependent (Angenstein et al., 1994; Akers et al., 1986; Bliss et al., 1993).

Several studies, spanning different models of epileptogenesis as well as status epilepticus, have described how PKC-γ can be modulated by seizures, without showing any peculiar pattern of variation for this PKC isoform (Tang et al., 2004; Guglielmetti et al., 1997; Chen, 1994; Beldhuis et al., 1992), probably owing to the different triggering stimuli and experimental protocols adopted. However, the available data usually deal with measurement of PKC-γ levels after convulsive phenomena and refer to experiments in adult animals, usually rodents. Nonetheless, in a different paper (McNamara et al., 1999), it was
reported that following KA administration a transient increase of PKC-γ was shown to precede seizure activity.

As far as modulation of PKC by corticosterone is concerned, repeated administration of dexamethasone was shown to up-regulate the protein levels of isoform γ in the cortex and the hippocampus of adult rats, whereas a single administration did not produce any effect (Dwivedi et al., 1999). These data are supportive of an indirect effect of glucocorticoids on PKC expression since only repeated administrations were able to induce significant changes. This finding is compatible with the observation that the promoter region of PKC-γ lacks DNA-binding sites sensitive to direct or indirect modulation of gene expression by glucocorticoids (GRE, NF-κB, AP-1), whereas a consensus sequence for AP-2 and Sp1 has been identified (Chen et al., 1990; Chen et al., 1993; Takanaga et al., 1995).

Thus, owing to the aforementioned evidence, we were interested to ascertain in rat pups at PN 9 whether i) the protective endotoxin challenge was able to modulate mRNA expression of the PKA RIβ subunit and PKC-γ isoform by itself and ii) the effect on such modulation of antagonists on both corticoid receptor subtypes.
4.9.2 Materials and methods

Technical details related to mRNA extraction, reverse transcription and evaluation of gene expression by real-time PCR have already been reported in 'Materials and methods - general procedures'.

The concentration of LPS used was 0.1 mg/kg. The experimental animals were sacrificed 2 hours after the PBS or LPS injection for the successive evaluation of levels of transcripts in hippocampal tissue.

Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and injected intraperitoneally (50 mg/kg) alone or 90 minutes after the systemic LPS administration, depending on the experimental group.

RU486 (Sigma, USA) was dissolved in 25% Tween 80 and administered subcutaneously (8.5 mg/kg) alone or 90 minutes after the systemic LPS administration, depending on the experimental group.
4.9.3 Results

Figures 22-25 show the effect of the systemic administration of canrenoic acid and RU486 on the LPS-induced RIIβ and PKC-γ mRNA expression in PN 9 rats. Measurements were made 2 hours after the endotoxin challenge. Data are the mean ± SE (n = 6 pups). In each bargram the ordinate axis represents the level of mRNA expression as the percentage of control (PBS-injected) group, assessed by quantitative RT-PCR.

Effect of canrenoic acid

Canrenoic acid did not affect the basal and 0.1 mg/kg LPS-induced mRNA expression of the PKA regulatory subunit RIIβ (fig. 22). Indeed, the amount of transcript elicited by LPS (171.0 ± 10.5 % vs PBS-injected controls, p<0.05) was left unchanged in the presence of the mineralocorticoid receptor antagonist (208.0 ± 23.1 % vs PBS-injected controls, p<0.01).

Conversely, the systemic administration of canrenoic acid markedly increased the mRNA expression of PKC-γ in the presence of the endotoxin stimulus (0.1 mg/kg), despite the fact that the administration of LPS alone did not induce variations in the level of PKC-γ transcript (fig. 23). The PKC-γ mRNA expression induced by canrenoic acid in the presence of the endotoxin was 332.6 ± 19.1 % (p<0.001) compared with PBS-injected controls as well as LPS and canrenoic acid both individually injected (fig. 23).
Effect of RU486

The vehicle used to dissolve RU486 (25% Tween 80) did not show any effect on basal and LPS-induced expression of RIIβ mRNA (fig. 24). Also the systemic administration of RU486 alone left the levels of transcript unchanged. Conversely, the RU486 administration markedly diminished the LPS-induced expression of RIIβ mRNA, even to below the basal level of PBS-injected controls. Indeed, the extent of reduction was, on average, 39% (p<0.001) compared with 0.1 mg/kg LPS (25% Tween 80), and 29% (p<0.001) compared with PBS-injected controls.

Fig. 25 shows the lack of any modulating effect of the systemic administration of RU486 onto PKC-γ mRNA expression, both when administered alone and in the presence of the LPS challenge. Indeed, the levels of transcript in all the experimental groups were not statistically different from those of PBS-injected controls.
Fig. 22

Effect of Canrenoic acid administration on LPS-induced RIIbeta mRNA in PN 9 rats

Data are the mean ± SE (n=6). * p<0.05 ** p<0.01 vs PBS; § p<0.05 vs Canrenoic acid. Kruskal-Wallis followed by Dunns multiple comparison test.
Fig. 23

Effect of Canrenoic acid administration on LPS-induced PKC-gamma mRNA in PN 9 rats

Data are the mean ± SE (n=6). ** p<0.001 vs PBS; §§ p<0.001 vs Canrenoic acid; ## p<0.001 vs LPS 0.1 mg/kg. Kruskal-Wallis followed by Dunns multiple comparison test.
Effect of RU486 administration on LPS-induced RII-beta mRNA expression in PN 9 rats

Data are the mean ± SE (n=6). * p<0.05 ** p<0.001 vs PBS; §§ p<0.001 vs 25% Tween 80, RU486, LPS+RU486; ## p<0.001 vs 25% Tween 80, RU486, LPS+RU486. Kruskal-Wallis followed by Dunns multiple comparison test.
Fig. 25

Effect of RU486 administration on LPS-induced PKC-gamma mRNA expression in PN 9 rats

Data are the mean ± SE (n=6). No statistically significant differences. Kruskal-Wallis test.
4.9.4 Discussion

The administration of LPS induces transcriptional activation of the PKA RIIβ subunit, whereas PKC-γ isoform mRNA expression is left steady at basal levels. Interestingly, selective antagonists to corticosterone receptors appear to delineate two differential patterns of mRNA expression, one for each specific receptor subtype. Mineralocorticoid receptors seem to exert an inhibitory tone on PKC-γ isozyme transcriptional activation, leaving RIIβ levels unaltered, whereas glucocorticoid receptors appear to sustain the expression of RIIβ mRNA without affecting the expression of the PKC-γ isoform.

It could be observed that PKC-γ is exclusively modulated by mineralocorticoid receptors, hence supporting the neuronal location of the type I receptor, whereas data related to RIIβ expression point to astrocytes as an exclusive target of modulation by type II receptors. Type I receptors are mainly localized in neuronal cells, as our results confirm by showing the modulation of gene expression of neuron-specific PKC-γ. Type I receptors do not modulate RIIβ mRNA expression, whereas type II receptors, also distributed in glial cells, are shown to affect transcription levels. Since, in vivo, microglia seems to be insensitive to corticosterone (as also supported by our results) then astrocytes appear to be the main target for modulation of RIIβ subunit by corticosterone.

In regard to the modulation of IL-1β and TNF-α mRNA expression by canrenoic acid and RU486, we hypothesized that such an effect could be mediated by an increase of PGE₂, despite unchanged levels of COX-2 mRNA following LPS injection after canrenoic acid or RU486 administration. In
addition, we hypothesized that the *de novo* synthesis of PGE$_2$ should occur in non-microglial cells.

In our opinion, the pattern of induction of PKC-γ and RIIβ following the administration of type I and type II corticosteroid receptor antagonists could support our hypothesis.

The enhancement of PKC-γ expression by blockade of type I receptors would lead to an increase of synaptic strength by LTP induction, which implicates a long-lasting elevation of intracellular Ca$^{2+}$ levels on synapse activation. Owing to the prolonged increase of intracellular calcium concentration, an enhancement of translocation of cPLA$_2$ to neuronal membranes occurs and, subsequently, levels of free arachidonic acid increase, leading to an overproduction of PGE$_2$ via the enzymatic activity of neuronal COX-2. Then, extracellularly transported PGE$_2$ would act on neighbouring microglial cells leading to a down-regulation of IL-1β and TNF-α expression.

As far as the type II receptor is concerned, a comparison between the patterns of induction of RIIβ mRNA by antagonists to both corticosteroid receptor subtypes suggest that the modulation of this PKA subunit mostly, if not exclusively, occurs in astrocytes. Astrocytes are the main source of arachidonic acid in the brain (Moore et al., 1991). Once synthesized, arachidonic acid is released in the extracellular fluids and then taken-up by neurons where it is very quickly incorporated into the endoplasmic reticulum, plasma and mitochondrial membranes (Katsuki et al., 1995). Importantly, the release of arachidonic acid from primary astrocyte cultures was shown to be PKA-dependent in that it is significantly reduced by PKA inhibitors (Strokin et al., 2003). This finding could help explaining how RU486 contributes to increased
PGE₂ production without affecting COX-2 expression. The levels of RIIβ mRNA expression were shown to be inversely correlated to the amount of PKA catalytic subunits in the cytoplasm (see paragraph "PKA: a relevant role of the regulatory subunit RIIβ"). Thus, on the decrease of RIIβ mRNA expression, the subsequent increase of dissociated PKA catalytic subunits is expected to enhance the release of arachidonic acid from astrocytes. This should lead to an increase in levels of arachidonic acid incorporated into the membrane phospholipids of both neurons and microglial cells, hence increasing the availability of substrates for cPLA₂. Elevation of PGE₂ production then ensues, due to an elevation of intracellular arachidonic acid. Then, PGE₂ of microglial as well as neuronal origin should act in autocrine and paracrine fashion yielding a down-regulation of IL-1β and TNF-α mRNAs expression.

Thus, the aforementioned hypotheses do not require a further increment of COX-2 expression in order to achieve an enhancement of PGE₂ production.

These results show that CA and RU486 can modulate LPS-induced IL-1β and TNF-α mRNA expression through different mechanisms, yielding, however, the same effect, that is an enhancement of PGE₂ production. Obviously, any mechanism affecting, for instance, the efficiency of enzymatic activity of COX-2, the rate of cAMP production or the sensitivity of EP2 receptor to PGE₂ itself as well as to its coupling with down-stream signalling could be evoked as a possible explanation for an enhanced PGE₂ production. Nonetheless, any direct modulation of these mechanisms by blockade of both corticosteroid receptors should be excluded since it would require an action of corticosterone directly onto microglial cells.
It is worth speculating on the different amount of inhibition of LPS-induced IL-1β and TNF-α gene expression when comparing the effect of CA as respect to RU486, being the down-regulation of proinflammatory cytokines much more pronounced with the latter antagonist. Evidence from the literature suggests a few mechanisms that may contribute to explain this feature. We already observed an increment of the LPS-induced immediate early gene iNOS following RU486 administration and this makes conceivable the hypothesis that production of NO enhances the enzymatic activity of COX-2 without affecting its mRNA expression. However, one more mechanisms is worth to be mentioned. Glucocorticoid receptors have been shown to mediate a tonic anti-inflammatory effect by inhibiting the formation of lipocortin-1 (also known as annexin-1), a molecule capable to enhance the activity of PLA₂ and, subsequently, to increase the release of arachidonic acid. Thus, upon RU486 administration, it is expected an increment of lipocortin-1 production and, eventually, an increment of PGE₂ production mediated by an enhanced release of arachidonic acid.
4.10 Dose-response of LPS-induced COX-2 mRNA expression in PN 9 rats

4.10.1 Introduction

Starting from this experimental section we investigated the role of PGE\textsubscript{2} in our experimental system as a modulator of susceptibility to seizures. At this step of investigation our experimental data indirectly support the role of PGE\textsubscript{2} as a down-regulator of proinflammatory cytokines expression also in rat pups. However, there are no clear indications concerning the role of PGE\textsubscript{2} on seizure susceptibility in rat pups. Indeed, it is of interest to observe the pattern of LPS-induced seizure-threshold to flurothyl inhalation (fig. 14). The more the dose of LPS increases, the less the LPS administration delays the seizure onset. The highest doses do not show any protective effect over seizure susceptibility. Generally, it is expected that increasing amounts of the endotoxin dose-dependently stimulate the mRNA expression of NF-\textkappa B-dependent genes such as COX-2, hence promoting the PGE\textsubscript{2} production in a dose-dependent manner. Therefore, it is conceivable to hypothesize that the increasing amount of PGE\textsubscript{2} in our experimental system might be responsible for the dose-dependent reduction of the protective effect of LPS administration. Therefore, from this point of view, it cannot be excluded that PGE\textsubscript{2} might preserve its own
proconvulsant effect as shown in adult rats but this effect would be partially
masked by the concomitant activation of the HPA axis by the prostaglandin
itself. Upon a strong increase of PGE$_2$ production (as it is expected to occur at
high doses of LPS) the proconvulsant effect of PGE$_2$ would prevail over the
anticonvulsant effect mediated by the HPA axis activation.

We started investigating the validity of such working hypothesis by testing
whether the levels of COX-2 transcript were dose-dependently increased by
LPS. The assay was done using the same samples where levels of IL-1$\beta$ and
TNF-\(\alpha\) transcripts were measured after LPS (fig. 15, panels A and B).
4.10.2 Materials and methods

Technical details related to mRNA extraction, reverse transcription and evaluation of gene expression by real-time PCR have already been reported in 'Materials and methods - general procedures'.

The concentrations of intraperitoneally administered LPS (dissolved in 0.1 M PBS) were 0.1 – 1.0 – 4.0 mg/kg. The experimental animals were sacrificed 2 h after the PBS or LPS injections for the subsequent evaluation of COX-2 level of transcript in hippocampal tissue.
4.10.3 Results

Figure 26 depicts the pattern of COX-2 mRNA induction following the systemic administration of three doses of LPS, two of which were shown protective (0.1 mg/kg and 1.0 mg/kg) whereas the highest dose (4.0 mg/kg) was ineffective on seizure threshold (fig. 14, panels A and B).

LPS induced a dose-dependent increase of COX-2 mRNA ranging approximately from 90% to 220% above control levels at 0.1 and 4.0 mg/kg respectively.
Fig. 26

mRNA expression of COX-2 following the administration of increasing doses of LPS in PN 9 rats

![Graph showing COX-2 mRNA expression](image)

Data are the mean ± SEM (n=6). ** p<0.01 vs PBS; §§ p<0.01 vs LPS 0.1 mg/kg; # p<0.01 vs LPS 1.0 mg/kg by Kruskal-Wallis test
4.10.4 Discussion

The dose-dependent activation of COX-2 following LPS supports the hypothesis that PGE₂ production may be involved in LPS effects on seizure threshold to flurothyl and this finding is also compatible with the concomitant changes in proinflammatory cytokines expression. Generally, the accumulation of free arachidonic acid and the formation of prostaglandins and lipoxigenase reaction products in the brain during seizures is well established (Cole-Edwards and Bazan, 2005). However, we are interested in investigating the role of high levels of PGE₂ on seizure induction. It was shown that ibuprofen, a non-selective COX inhibitor, delays the onset of flurothyl-induced seizures in adult rats (Wallenstein and Mauss, 1984), suggesting a proconvulsant role of PGE₂. On the one hand, this evidence appears to be in contradiction with our results related to the administration of low doses of LPS (a delay of seizure onset), while, on the other hand, this finding could account for the lack of protection on seizure susceptibility of the highest doses of LPS (see fig. 14, panel A and B). We focused our attention on the role of PGE₂ on seizure susceptibility following the administration of a protective dose of LPS. We showed that the protective effect of low doses of LPS in rat pups is mediated by the occupancy of mineralocorticoid receptors by corticosterone. This effect is triggered by the activation of the HPA axis, presumably by the LPS-induced production of PGE₂. Thus, it is conceivable that the prevention of PGE₂ production could significantly increase the susceptibility to seizure in our experimental conditions. In the following experimental section we tested the relevance of PGE₂ production as a
key mechanism for LPS-induced protection on seizure susceptibility by inhibiting the activity of COX-2.
4.11 Role of COX-2 in LPS-mediated effect on flurothyl-induced seizures in PN 9 rats

We studied whether a selective COX-2 inhibitor modulates flurothyl-induced seizure susceptibility in PN 9 rats. Pups were pre-treated with a selective COX-2 inhibitor (SC58125) and the effect of LPS administration alone or in combination with canrenoic acid on flurothyl-induced seizure onset was measured.
4.11.1 Materials and methods

Technical details related to seizure-threshold testing by flurothyl inhalation have already been reported in 'Materials and methods - general procedures'.

LPS was dissolved in 0.1 M PBS and injected at 0.1 mg/kg. Flurothyl delivery occurred 2 h after the endotoxin challenge. The selective COX-2 inhibitor SC58125 (Cayman chemicals, USA) was dissolved in 0.1 M PBS and injected intraperitoneally (3 mg/kg) 30 min before flurothyl delivery (i.e. 90 min after the systemic LPS administration) alone or co-administered with canrenoic acid. Canrenoic acid (Sigma, USA) was dissolved in PBS 0.1 M and co-injected with SC58125 intraperitoneally (50 mg/kg) 30 min before flurothyl delivery (i.e. 90 min after the systemic LPS administration).
4.11.2 Results

Figure 27, (A, B), shows the effect of the selective COX-2 inhibitor SC58125 on the LPS-induced delay of flurothyl-induced seizure in PN 9 rats. In the same experimental section, seizure-threshold was also tested in a group of animals which were treated with canrenoic acid, an antagonist to mineralocorticoid receptors.

SC58125 abolished the protective effect of LPS (panels A and B) but did not modify seizure-threshold when administered alone.

Canrenoic acid alone did not affect the susceptibility to seizures. When administered with SC58125 Canrenoic acid reversed LPS action by anticipating seizure onset by approximately 20% vs PBS-injected rats (p<0.01, panels A and B).
Fig. 27

Effect of COX-2 inhibitor SC58125 ± Canrenoic acid on LPS effect on flurothyl-induced clonic (panel A) and tonic-clonic (panel B) seizures in PN 9 rats

Data are the mean ± SEM (n=6). * p<0.05 ** p<0.01 vs PBS; § p<0.01 vs SC58125, LPS+SC58125, Canrenoic acid and LPS+Canrenoic
acid+SC58125; # p<0.01 vs LPS+SC58125 and Canrenoic acid; ° p<0.05  °° p<0.01 vs SC58125 by Kruskal-Wallis test
4.11.3 Discussion

These results support a relevant role of prostaglandins in the LPS effect on seizure susceptibility, since rat pups pre-treated with the selective COX-2 inhibitor SC58125 and subsequently challenged with the most protective dose of LPS showed a seizure-threshold similar to control rats. In addition, in all experimental groups, inhibition of COX-2 significantly enhanced seizure susceptibility in rat pups treated with canrenoic acid and LPS.

It is of interest to observe that inhibition of COX-2 by SC58125 does not appear to affect seizure susceptibility in the absence of a stimulus capable of increasing COX-2 mRNA expression such as LPS administration, as is evident by comparing seizure-threshold of the control and SC58125 administered groups. So, it appears that elevation of PGE$_2$ levels is necessary to exert the anticonvulsant effect shown by a protective dose of LPS.

Generally, the use of COX-2 inhibiting drugs on seizure modulation provided controversial results in the literature. As mentioned, inhibition of COX-2 activity by ibuprofen was shown to increase flurothyl-induced seizure susceptibility in adult rats (Wallenstein and Mauss, 1984). In addition, Kim and Jang (Kim and Jang, 2006) showed that inhibition of COX-2 protein expression by celecoxib delayed flurothyl-induced seizure onset in PN 10 rats which undergo a protocol of recurrent seizures and were sacrificed at PN 15 and PN 22. These data are in agreement with others showing that COX-2 inhibitors reduce the severity of seizure and protect the brain from irreversible damage.
induced, for instance, by KA administration in adult rats (Baran et al., 1994; Kunz et al., 2001).

However, several studies have reported that prostaglandin exert protective effects on seizures (Forstermann et al., 1982; Baik et al., 1999). In particular, if levels of cerebral prostaglandins were enhanced by a preceding electroshock, the toxicity of pentylenetetrazole was significantly reduced and seizure onset was markedly delayed. Both these effects (neuroprotection and increased latency to seizure-onset) were reversed when prostaglandins production was prevented by administration of indomethacin, or by increasing the time interval between the electroshock and pentylenetetrazole administration so that the electroshock-induced prostaglandins production returned to basal levels (Forstermann et al., 1982). Accordingly, our results showed that the administration of a protective dose of LPS induces COX-2 mRNA expression, thus resulting in increased levels of prostaglandins before flurothyl delivery. After impairment of prostaglandins production by the COX-2 inhibitor, the worsening effect of canrenoic acid on LPS-induced seizure-threshold was potentiated.

In general, the controversial results on seizures obtained using COX inhibitors (also called non-steroidal anti-inflammatory drugs, NSAIDs) may be ascribed to i) mechanisms of action of NSAIDs which extend beyond inhibition of COX enzymatic activity and ii) the emerging role of PGE₂ as an anti-inflammatory molecule. As far as NSAIDs mechanisms of action are concerned, it has been shown that some COX inhibitors also exert effects on transcriptional factors. For instance, acetyl salicylic acid and its derivative salicylate were shown to inhibit NF-κB activity (Kopp and Ghosh, 1994) as well as other transcription factors including AP-1 and CREB (Beauparlant and Hiscott, 1996).
However, other NSAIDs appear to affect NF-κB activation only at certain drug concentrations and in certain cell types (Aeberhard et al., 1995).

As far as PGE$_2$ is concerned, although this prostaglandin has frequently been considered as a possible cause of brain damage and degeneration, it may exert beneficial effects in the CNS. For instance, indirect evidence for its protective role in vivo derives from the detection of increased cerebral levels of PGE$_2$ during the remitting phase of EAE (Khoury et al., 1992) and from the beneficial role of cAMP elevating agents, including PGE$_2$ itself, in ischemia or EAE (Kato et al., 1995; Genain et al., 1995; Sommer et al., 1995; Wiegmann et al., 1995). Furthermore, PGE$_2$ might exert a beneficial role also in multiple sclerosis and other human brain diseases where increased levels of this prostaglandin have been detected (Fretland et al., 1992; Griffin et al., 1994; Froldi et al., 1992).

Experiments in vitro also support a protective role of PGE$_2$. This prostaglandin was reported to protect cultured neurons from several kinds of noxious insult, including hypoxia/reoxigenation, glutamate-induced injury (Cazevieille et al., 1994; Akaike et al., 1994) and microglial toxic products (Théry et al., 1994), all these effects being mediated by cAMP elevation.
4.12 Involvement of EP2 receptors in the modulation of PGE$_2$ effects on seizure susceptibility in PN 9 rats

4.12.1 Introduction

Our experiments support an indirect role of PGE$_2$ in the modulation of seizure susceptibility in rat pups through the activation of the HPA axis, thus promoting, on the one hand, a protection against seizure induction. However, on the other hand, PGE$_2$ has been shown to enhance synaptic excitability by acting through its cognate receptors located in key areas of the brain. It is of interest to observe that our experimental system supports a PGE$_2$-mediated down-regulation of proinflammatory cytokines production. Evidence from literature has shown the involvement of EP2 receptor in modulation of PGE$_2$-induced down-regulation of IL-1$\beta$ and TNF-$\alpha$ mRNA expression. Therefore, it is conceivable that EP2 receptors may play a relevant role also in our experimental conditions. In principle, the pharmacological targeting of the EP2 receptor could constitute a potential therapeutic tool when proinflammatory molecules in the CNS need to be limited. However, the usefulness of such a hypothetical pathway of therapeutic intervention depends on the effect that the specific activation of EP2 receptor exerts on seizure susceptibility. Recently, the specific
involvement of EP2 receptors in modulating the state of excitability of nervous tissue was investigated in hippocampal slices and in cultured hippocampal neurons (Sang et al., 2005). Indeed, it was shown that postsynaptically synthesized PGE\(_2\) modulates hippocampal synaptic transmission via activation of a presynaptic EP2 receptor. Interestingly, another recent paper (Zhu et al., 2005) showed the localization and the level of expression of EP receptor in the hippocampus. In general, all four EP receptors have been found in the rat hippocampus and it has been shown that EP1, EP2 and EP4 are localized at presynaptic sites whereas EP3 is mainly located at postsynaptic sites, although a minority can be found at the presynaptic terminal (Zhu et al., 2005). Astrocytes have been shown to express EP receptors as well, although to a much lesser extent. However, PGE\(_2\) was shown to stimulate Ca\(^{2+}\)-dependent glutamate release from astrocytes. This would lead to a subsequent increase of intracellular Ca\(^{2+}\) concentration in neighbouring neurons through the activation of neuronal glutamate receptors (Bezzi et al., 1998), among which the ionotropic receptors were shown to depolarize neuronal membranes (Sanzgiri et al., 1999).

Generally, since EP1 receptor is coupled to activation of PLC with a subsequent increase of intracellular Ca\(^{2+}\) concentration (whereas both EP2 and EP4 receptors are coupled to adenylyl cyclase activation) then it appears that the main effect mediated at the synapse by PGE\(_2\) in the hippocampus might be, almost exclusively, excitatory. However, under physiological conditions, EP2 and EP3 are the most expressed receptor populations (EP3 being the most abundant), whereas EP1 and EP4 are
barely detectable. Thus, it might be that in physiological conditions the effect mediated by presynaptic EP2 activation might be counteracted by the concurrent activation of postsynaptic EP3 receptors which are known to down-regulate adenylyl cyclase activity.

Nonetheless, it is interesting to observe how these receptors are modulated by inflammatory stimuli such as IL-1β. Indeed, IL-1β was shown to selectively up-regulate mRNA and protein levels of EP2 and EP4 receptors in primary cultured hippocampal neurons, leaving EP1 and EP3 receptors unaffected (Zhu et al., 2005), hence priming the system to a more excitatory action of PGE₂ in the hippocampus following a proinflammatory stimulus. Interestingly, the same group of investigators showed how this selective up-regulation also occurs following LTP induction.

These data refer to EP receptor distribution and responsiveness to inflammatory stimuli in a specific rat brain area such as the hippocampus. However, relevant changes in EP expression following systemic administration of an inflammatory stimulus such as IL-1β also occur in other brain areas (Zhang and Rivest, 1999). In particular, the hypothalamic PVN (paraventricular nucleus) was shown to undergo a selective overexpression of EP4 receptors (Zhang and Rivest, 2000) which accounts for the HPA axis activation by PGE₂ under IL-1β and LPS challenges. Thus, it is conceivable that only two receptor populations are relevant in our experimental conditions, namely EP2 and EP4.

We evaluated the effect of pharmacological intervention of EP2 receptors on seizure susceptibility modulation by administration of a protective dose of LPS. Butaprost was used as a selective EP2 agonist. In
addition, we investigated the role of EP1 receptors in that the activation of its signaling pathway leads to an increase of intracellular Ca\(^{2+}\) thus contributing to increase the level of excitability. Nonetheless, this receptor population being faintly expressed both in physiological conditions and following a proinflammatory stimulus, it is expected to not significantly affect seizure susceptibility in our experimental system. We used SC19220 as a selective EP1 antagonist.
4.12.2 Materials and methods

Technical details related to seizure-threshold testing by flurothyl inhalation have already been reported in 'Materials and methods - general procedures'.

LPS (0.1 mg/kg) was dissolved in 0.1 M PBS. Flurothyl delivery occurred 2 h after the endotoxin challenge.

Butaprost and SC19220 (Cayman Chemicals, USA) were dissolved in 25% Tween 80 and injected subcutaneously (3 mg/kg, each), alone or 90 min after the endotoxin challenge.
4.12.3 Results

Figure 28, (A, B), depicts the effect of butaprost, an EP2 receptor agonist, and SC19220, an EP1 receptor antagonist, on flurothyl-induced seizures in PN 9 rats.

Effect of butaprost

25% Tween 80, used as a vehicle to dissolve butaprost, affected seizure-threshold *per se* (29% above PBS, p<0.01). This effect was not statistically different from that observed with butaprost (39% above PBS, p<0.001).

When butaprost was administered with LPS, it reversed the protective effect of the endotoxin on clonic seizure-threshold (panel A). Tonic-clonic seizures were observed only in 2 out of 6 pups treated with butaprost and LPS. These seizures were apparently less severe than those occurring in PBS-treated rats (panel B).

Effect of SC19220

The EP1 receptor antagonist SC19220 did not affect seizure susceptibility in PN 9 rats (panels A and B). The amount of flurothyl required to elicit clonic
and tonic-clonic seizures was not significantly different from vehicle and LPS treated rats.
Fig. 28

Effect of Butaprost and SC19220 on flurothyl-induced clonic (panel A) and tonic-clonic (panel B) seizure-onset in PN 9 rats

Data are the mean ± SEM (n=6). * p<0.05 ** p<0.01 vs PBS; § p<0.001 vs LPS 0.1 mg/kg; ^ p<0.05 vs 25% Tween 80; #
p<0.05 vs LPS 0.1 mg/kg (25% Tween 80); ° p<0.001 vs Butaprost; nd = not determined by Kruskal-Wallis test
4.12.4 Discussion

In comparison with LPS dissolved in PBS, the administration of LPS dissolved in 25% Tween 80 reduces the protective effect onto seizure susceptibility. Meanwhile the seizure-onset in rats administered with 25% Tween 80 alone increases. Indeed, this vehicle makes the seizure-onset of these two experimental groups comparable, as it was also seen during the evaluation of the effect of RU486 onto seizure susceptibility (fig. 17). Nonetheless, the individual roles of EP2 and EP1 receptors in our experimental system can be sketched out.

As far as the EP1 receptor is concerned, there is a substantial lack of effect of the antagonist SC19220.

On the other hand, the EP2 agonist seems to suggest a dual role of PGE$_2$. Indeed, the EP2 agonist abolishes the protective effect of LPS onto clonic seizure-onset and affects the pattern of seizure spread in that the occurrence of tonic-clonic seizures (a hallmark of massive involvement of brain motor areas) cannot be determined.

We are aware that data regarding the patterns of distribution and inflammation-induced responsiveness of EP receptors in a specific brain area such as the hippocampus might only partially account for our results which are related to the involvement of the whole brain (see paragraph 4.12.1). Nonetheless, it could be that the lack of a substantial effect of the EP1 antagonist SC19220 in our experimental system, compared to basal and LPS challenged groups, might be ascribed to the ordinary paucity of EP1 receptors both under physiological conditions and following LPS.
challenge. Conversely, the EP2 agonist butaprost acts on a receptorial population which is significantly expressed under physiological conditions and overexpressed following an inflammatory stimulus.

Thus, the activation of EP2 receptors appears to exert a proconvulsant action, as shown in our experiments with butaprost, and this effect was shown strong enough to overwhelm the protective effect mediated by the systemic administration of LPS (which is expected to indirectly involve the activation of the EP4 receptor subtype).

Although speculative, a possible explanation of our results might come from the aforementioned experimental evidence (Sang et al., 2005). Thus, the pre-treatment with a relatively selective EP2 agonist such as butaprost might lead to an increase of glutamate release via activation of presynaptic EP2 receptors. The subsequent increase of glutamate, through the activation of its cognate postsynaptic receptors, yields a general increase of neuronal excitability, thus contributing to foster seizure susceptibility. This proconvulsant effect appears not to be counteracted by the activation of mineralocorticoid receptors following systemic LPS administration, and therefore ictal events can be more easily induced by flurothyl inhalation.

In our opinion, these findings support our working hypothesis that the dose-dependent decrease of the protective effect of LPS on seizure susceptibility might be due to an overproduction of PGE$_2$. From this point of view, PGE$_2$ seems to preserve its intrinsic proconvulsant activity also in rat pups but, differently from adults, this effect is overwhelmed by the concomitant activation of the HPA axis, provided the levels of the
prostaglandin are not strongly expressed. From a more general perspective, it might be that in rat pups a moderate inflammatory state might be beneficial to counteract seizure susceptibility.

At present, we have no a reasonable explanation for the apparent lack of a tonic-clonic phase during the occurrence of ictal events (fig. 28, panel B).
5. Conclusions
5.1 General discussion

We sum up the principal findings as they emerged from our study:

1. the pattern of expression of proinflammatory cytokines following SE might be due to the age-dependent stimulus-specific expression of their transcriptional factors. The temporal profile of induction of proinflammatory cytokines, per se, cannot be regarded as a general mechanism which underlies the appearance of neurodegeneration;

2. there are no clear indications emerging from our data concerning the role of proinflammatory cytokines in modulating seizure susceptibility in rat pups;

3. microglia appear insensitive to corticosteroids and production of proinflammatory molecules such as IL-1β and TNF-α might be down-regulated on elevation of parenchymal PGE₂ levels. PGE₂ is expected to originate from non-microglial cells;

4. PGE₂ production and the activation of the HPA axis play a relevant role in the modulation of seizure susceptibility in rat pups.

Obviously, our results are not intended as definitive. Indeed, several limitations may affect the interpretation of our findings, as it will be discussed in the following paragraph. Nonetheless, our findings are worth a few comments from a more general perspective. Indeed, a more general overview to our results
leads to define two main findings in relation to ictal events and neurodegeneration in pups, i) an uncertain role of proinflammatory cytokines such as IL-1β and TNF-α and ii) a possible relevant role of the functional relationship between COX-2/PGE2 and the HPA axis in pups. Interestingly, it is worth wondering what functional mechanism, if any, may significantly contribute to account for the molecular and behavioural differences among rats aged PN 9-15-21. Likely, this is a multifactorial phenomenon. However, any mechanistic explanation could only be expressed in terms of a developmental continuum into which our experimental animals span through. Indeed, we already observed that the pattern of expression of IL-1β and TNF-α induced by SE in pups is dependent on the proconvulsant agent used and the age of occurrence of ictal events. Interestingly, KA and flurothyl induce ictal events by different mechanisms (see 'material and methods – general procedure'). This is indicative of how the behaviour of cerebral areas involved in the insurgence of ictal events is affected by the degree of maturation of fundamental systems such as neuronal connections, signalling pathways and transcriptional/translational machineries. It is important to note that in rodents the most striking developmental changes occur in the early two postnatal weeks. From this point of view, we speculated that the pattern of induction of proinflammatory cytokines could be ascribe to the age-dependent expression of specific transcriptional factors. Nonetheless, during this developmental time-window, other relevant changes occur concomitantly and they may act synergistically with the age-dependent induction of proinflammatory molecules. In particular, developmental changes affecting GABAergic and glutamatergic synapses are worth to be mentioned, as they are relevant to brain excitability regulation and predisposition.
to neurodegenerative phenomena. It is known that GABA\textsubscript{A} receptors switch to be inhibitory during the first 7-10 postnatal days (Ben-Ari, 2001). During this period of time synaptic density reaches the highest level then progressively decreases while sub-serving the establishment of functional circuitries of the mature brain (Insel et al., 1990; McDonald et al., 1992). Usually, GABA\textsubscript{A} receptors are expected to be definitively hyperpolarizing by PN 15. Also glutamatergic synaptic transmission undergoes significant changes during development. It has been shown that during the first two postnatal days, glutamatergic transmission is exclusively mediated by NMDA receptors, without any significant contribution of AMPA receptors (Durand et al., 1996). Interestingly, the voltage-dependent Mg\textsuperscript{2+} block of NMDA channels is fully functional in neonatal brain as it is in adults and, consequently, these premature synapses are actually ‘silent’ at resting membrane potential (Durand et al., 1996; Ben-Ari, 2001). However, the proportion of pure ‘silent’ NMDA synapses decreases during the first week of postnatal development, shifting from approximately 80% at PN 2 to about 30% at > PN 7 (Durand et al., 1996). Generally, the NMDA receptor density peaks late in the first postnatal week, while AMPA receptor density peaks later in the second postnatal week (approximately at PN 10). Kainate receptors have been shown to be already present at birth. Their density is initially low and gradually increases to adult levels by the fourth postnatal week (Chapman, 1998).

The developmental profile of expression related to GABA\textsubscript{A} and, in particular, ionotropic glutamate receptors during the first two postnatal weeks may partially account for the progressive relevance that proinflammatory cytokines gain in ictal and neurodegenerative phenomena the more the age...
approaches adulthood. For instance, as already stated in this work, it is conceivable that the age-dependent strengthening of transcriptional and translational machineries of a proinflammatory cytokine such as IL-1β may age-dependently affects the level of tissue excitability by the modulation of the NMDA channel conductance. Subsequently, seizure susceptibility and predisposition to excitotoxic insult may become age-dependently affected by the establishment of a proinflammatory state in the brain.

Also developmental changes affecting the HPA axis are indeed relevant and may significantly contribute to account for the difference among rats aged PN 9-15-21. From this perspective, as relevant parameters, we already discussed the age-dependent decrease of the ratio between MR and GR, the reversal of the functional role of MR receptors (shown as proconvulsant in adult rodents) and the maturation of negative feedbacks which limit the elevation of circulating corticosterone levels upon stimulation of the HPA axis. However, it is important to note that in adult rats persistent elevation of glucocorticoid levels increases the likelihood of neurodegeneration to occur. Indeed, glucocorticoids have been shown to impair glutamate removal from the synaptic cleft, Ca²⁺ extrusion from the cytoplasm of the postsynaptic neuron and blunt the compensatory increase in antioxidant enzyme activity during insults. All these effects act against the capability of neurons to face neurological insults, in particular those implying excitotoxicity as it occurs during seizures. Indeed, a significant finding has been shown in relation to the effects of elevation of glucocorticoid levels on KA-induced inflammation in the CA3 area of rat hippocampus (Dinkel et al., 2003). Although glucocorticoid are known for their anti-inflammatory and immunosuppressive properties both in periphery and in
the CNS, chronically high levels of circulating corticosterone yielded a surprising proinflammatory effect, showing an anticipation of neuronal damage at shorter time points, higher infiltration of inflammatory cells in the KA-administered CA3 area as well as much higher levels of both transcript and protein of IL-1β and TNF-α. Indeed, this finding is not surprising. Although successfully employed to treat inflammatory diseases within the CNS such as edema arising from brain tumors (Barnes and Adcock, 1993), bacterial or viral encephalitis (Coyle, 1999) or to improve the rate of recovery from acute exacerbation in multiple sclerosis patients (Filippini et al., 2000), in the context of various neurological insults GCs have been shown to exacerbate neuron loss especially in concomitant with excitotoxic insults.

Thus, in regard to seizure susceptibility and seizure-induced neurodegeneration, the concomitant maturational changes affecting the proinflammatory cytokines production, the glutamatergic transmission and the HPA axis activation may indeed account for the molecular and behavioural differences among pups at different ages and between young pups and adult rodents.

As it regards the functional relationship between PGE₂ and the HPA axis activation, it should be mentioned that the functional features mediated by PGE₂ in pups appear similar to those observed in adult rodents. Indeed, PGE₂ activates the HPA axis, down-regulates the expression of proinflammatory markers such as IL-1β and TNF-α and preserves a potentially proconvulsant effect. Conversely, the HPA axis activation in rat pups is peculiar. In particular, two features have been shown relevant at young ages, i.e., the long-lasting increase of circulating corticosterone levels and the anticonvulsant effect
mediated by the occupancy of membrane MR. Our experiments have prompted the possibility that functional mechanisms shown to occur in adult rats are operative also at young ages. However, upon a moderate proinflammatory stimulus that does not induce overwhelming levels of parenchymal PGE$_2$, this mechanistic similarity in pups takes the advantage of being protective against the occurrence of ictal events. This effect is modulated by the age-dependent anticonvulsant feature of membrane MRs. In our opinion, the proposal of this mechanistic hypothesis represents the most intriguing result coming out from our study. Figure 29 (page 317, see also legend on page 318) provides a schematic representation of this mechanistic hypothesis.
Fig. 29

Legend:  ► stimulation, activation
         ─── inhibition

LPS → COX-2 → PGE₂

SC58125, COX-2 inhibitor

HPA axis

Butaprost, EP2 agonist
EP2, proconvulsant

Seizure susceptibility

membrane MR, anticonvulsant

Canrenonic acid, MR antagonist
Legend to fig. 29

The proinflammatory stimulus LPS induces an increase of PGE$_2$ production by enhancing the gene transcription of the enzyme COX-2. In regard to seizure susceptibility in pups, PGE$_2$ may play a dual role, being proconvulsant by activating EP2 receptors and (indirectly) anticonvulsant by activating the HPA axis leading to the subsequent occupancy of membrane MR by corticosterone. This mechanistic hypothesis highlights that a key parameter in the regulation of seizure susceptibility in pups might be the levels of parenchymal PGE$_2$ produced following LPS administration. Subsequently, this working hypothesis also suggests that seizure susceptibility in pups might be affected by the balance between the proconvulsant potential of EP2 receptors and the anticonvulsant potential of membrane MR receptors.

Fig. 29 also reports the main drugs used and their pharmacological property (SC58125, Butaprost, Canrenoic acid). These molecules were exploited to test the conceptual validity of the proposed mechanistic hypothesis. See respective experimental sections for detailed discussions.
5.2 Major limitations of the study

As already stated, the interpretation of the great majority of our experimental data relies on the assumption that upon mRNA increasing a corresponding protein synthesis occurs. Nonetheless, several evidence has shown that transcription can be an isolated event from translation. LPS, IL-1β and TNF-α, were shown to increase IL-1β transcription without affecting translation, depending on the system under investigation. Indeed, the complexity of gene transcription modulation of molecules such as proinflammatory cytokines and the immaturity of the organism under investigation make the possible dissociation between transcription and translation a conceivable hypothesis. From this point of view, it cannot be ruled out that the production of several modulators potentially relevant for bridging the gap between transcription and translation may not be appropriately entered into play at young ages, maybe due to the immaturity of signalling pathways and transcriptional/translational machineries.

Another major limitation of our study is the lack of measurement of circulating corticosterone levels. We are aware that our interpretation of results obtained and the subsequent working hypothesis were confident on the assumption that the pattern of induction of corticosterone levels in our experimental conditions was similar to that reported to occur in the literature in similar experimental paradigms. As already stated, it should be also observed that the lack of a protective effect of the highest doses of LPS might be ascribed to an inadequate amount of corticosterone due to an inefficient machinery of the
immature HPA axis when facing an intense stimulation, as shown to occur in regard to the ACTH levels in young pups (Vazquez, 1998). Thus, the measurement of corticosterone levels released during our experiments is indeed a relevant parameter needed to strongly validate our hypothesis.

Finally, it should be also observed that 2 hours interval between proinflammatory stimuli administration and the evaluation of mRNA expression of molecules of interest is indeed a short time-window. The time interval of observation should be significantly extended. Longer time-windows would significantly increase the probability that several relevant molecules would come into play in our experimental system. In particular, it is conceivable that the production of IL-1β and TNF-α following LPS administration could become affected by the induction of gene expression and translation of classical anti-inflammatory molecules such as IL-4 and IL-10, which are known to be synthesised following longer time-exposure to proinflammatory stimuli. Additionally, PGE₂ production would also increase, thus likely affecting the balance among brain excitability, HPA axis activation and the expression of proinflammatory molecules in concert with anti-inflammatory cytokines. It should be also mentioned that the limited time of observation of our experimental protocols could also affect the validity of our hypothesis that makes the activation of the HPA axis pivotal in limiting seizure susceptibility in pups. Indeed, it cannot be excluded that upon prolonged stimulation by proinflammatory stimuli the immature HPA axis could significantly limit corticosterone release.
5.3 Future work

The goal of future work is to establish whether the hypothesis depicted in fig. 29 holds true. In particular, we will focus on the evaluation of corticosterone levels released in association with the administration of a proinflammatory stimulus such as LPS. Specifically, we will investigate the temporal profile of corticosterone induction shortly after the administration of a protective dose of the endotoxin. In particular, the evaluation of corticosterone levels at time-points as short as 15-30 minutes after LPS administration would allow to verify the hypothesis of the involvement of membrane mineralocorticoid receptors in the quick modulation of seizure susceptibility in our experimental system, being this time-window hardly compatible with effects mediated by an action of corticosteroids at genomic level. Additionally, we will also evaluate the pattern of induction of corticosterone levels induced by a non-protective dose of LPS as it cannot be ruled out that the lack of a protective effect of the highest doses of LPS might be ascribed to an inadequate amount of corticosterone due to an inefficient machinery of the immature HPA axis when facing an intense stimulation, as shown to occur in regard to the ACTH levels in young pups. Related to the latest experimental proposal, we will test the hypothesis that the lack of proconvulsant activity of the icv administration of IL-1β in rat pups might be due to the involvement of mineralocorticoid receptors by the quick activation of the HPA axis following the injection of the cytokine. This goal may be simply achieved by pre-treating pups with canrenoic acid.
The experiments above mentioned will be paralleled by others aimed to better characterize the role of the COX-2/PGE₂ system in our experimental conditions. As a first step toward this direction, we will evaluate the dose-response effect of the COX-2 inhibitor SC58125 on the protective effect of LPS administration on seizure susceptibility, alone and in combination with canrenoic acid treatment. The rationale of this experiment is in that PGE₂ seems to play a dual role in our experimental system and the critical parameter appears to be the amount of PGE₂ produced. Therefore, by keeping under pharmacological control the PGE₂ production induced by a proinflammatory stimulus, it is conceivable to hypothesize the existence of a range of concentrations of SC58125 suitable to sustain and to reinforce the protective effect on seizure susceptibility of LPS administration.

In principle, these experiments could disclose new therapeutic pathways for seizure control at young ages. Indeed, it is conceivable that new pharmacological tools could arise from a better understanding of the effect of the modulation of PGE₂ levels in conjunction with pharmacological intervention on the HPA axis and mineralocorticoid receptors.
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