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Microglial Response to Inflammatory Stimuli-Impact of Protein Acetylation and Phosphorylation

Doctoral dissertation

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ABSTRACT

Inflammation and immune responses have been shown to contribute to a number pathological conditions - including those neurodegenerative diseases which affect the brain. Microglial cells represent the resident immune cell population that constitute the immune system of the CNS. Acetylation of proteins is one of the chemical modifications by which microglial cells regulate the transcription of genes that respond to pro- and anti-inflammatory signals through multiple pathways. In addition to acetylation, the intensity and duration of the inflammatory response is regulated by changes in the phosphorylation state of the signaling pathways involved in the regulation of inflammation.

One of the major intracellular cell-signaling pathways for inflammation, the NF-κB system, regulates the expression of many genes involved in immune responses. The transcriptional response of intranuclear NF-κB is also tightly regulated, not only by post-translational modifications of NF-κB itself but also by modification of histones. The dynamic switches between activity states of chromatin are modified by the interplay of two competing enzymatic activities, histone acetyltransferases (HATs) and histone deacetylases (HDACs), which regulate the acetylation state of proteins. By counteracting the effects of HATs at the site of inflammatory gene transcription, HDACs play a major role in the regulation of the NF-κB-dependent inflammatory response.

Our aim was to shed light on changes occurring in the regulation of the inflammatory response by manipulating the acetylation and phosphorylation status of different neural inflammation models. The pro- and anti-inflammatory responses were characterized using established cell culture models.

The results show that an increase in the level of protein acetylation, as well as enhancement of protein phosphorylation, leads to a proinflammatory response. The potentiated response by hyperphosphorylation occurred earlier than the acetylation induced response. Moreover, the inflammatory response showed a synergistic potentiation when both acetylation and phosphorylation were enhanced simultaneously with compounds affecting separate pathways, indicating that different signaling pathways govern the overall acetylation and phosphorylation mediated immune responses. The proinflammatory responses could be inhibited by using specific blockers targeted against the different signaling pathways.

This thesis provides more insight into the research, development and characterization of anti-inflammatory drug candidates that are targeted to modulate the HAT-HDAC balance, phosphorylation status and the regulation of NF-κB pathway

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Medical Subject Headings: Central Nervous System/immunology; Inflammation; Immune System; Microglia/immunology; Acetylation; Phosphorylation; NF-kappa B; Histone Deacetylases; Histone Acetyltransferases; Cells, Cultured

"A man should keep his little brain attic stocked with all the furniture that he is likely to use, and the rest he can put away in the lumber-room of his library, where he can get it if he wants it."

-Sir Arthur Conan Doyle-

(Sherlock Holmes in "The Five Orange Pips")

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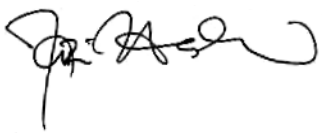

Jari Huuskonen

ABBREVIATIONS

Ab	antibody
AD	Alzheimer's disease
AP	activating protein
APC	antigen-presenting cell
Ara-C	1-β-D-cytosine-arabinofuranoside
AKT	protein kinase B
BBB	blood-brain barrier
CA	*cornu ammonis*
cAMP	cyclic adenosine monophosphate
CAPE	caffeic acid phenethyl ester
CBP	CREB-binding protein, a transcriptional coactivator
CD11b	antigen part of the CD11b/CD18 heterodimer (Mac-1), also known as the C3 complement receptor
CD14	antigen, receptor for LPS and LBP
CNS	central nervous system
CR	calorie restriction
CR3	type 3 complement receptor
CRE	cAMP response element
CREB	CRE-binding protein, a transcription factor
CRP	C-reactive protein
DAB	3,3'-diaminobenzidine
DCX	doublecortin
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
FBSi	inactivated fetal bovine serum
GFAP	glial fibrillary acidic protein
H7	protein kinase C inhibitor
H89	protein kinase A inhibitor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitors
HIV	human immunodeficiency virus
IB_4	*Griffonia simplicifolia* isolectin
IKK	inhibitory kappa kinase
IL-6	interleukin-6
iNOS	inducible nitric oxide
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
LBP	LPS-binding protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase

M344	4-dimethylamino-*N*-(6-hydroxycarbamoyl-hexyl)-benzamide
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NES	nuclear export signal
NF-kB	nuclear factor kappa B
NLAC	National Laboratory Animal Center
NLS	nuclear localization signal
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
ODN1826	oligodeoxynucleotides (CpG, TCCATGACGTTCCTGACGTT), containing CpG motifs, TLR9 ligand
OKA	okadaic acid
P7-P8	postnatal day 7-8
Pam3CSK4	TLR2 ligand, a synthetic triacylated lipopeptide
PAMP	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PMA	phorbol myristate acetate
PP	protein phosphatase
PRR	pattern-recognition receptors
RNA	ribonucleic acid
SAHA	suberoyl anilide bishydroxamide
Ser	serine
SCFA	short-chain fatty acid
SD	standard deviation
SIRT	silence information regulator
SLTA	lipoteichoic acid from *Staphylococcus aureus*, TLR2 ligand
SPSS	Statistical Package for Social Sciences
TBS-T	Tris-buffered saline
TLR	Toll-like receptor
TNF-α	tumour necrosis factor- α
TSA	trichostatin A

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by Roman numerals I-IV.

I. Huuskonen J, Suuronen T, Miettinen R, van Groen T, Salminen A. A refined in vitro model to study inflammatory responses in organotypic membrane culture of postnatal rat hippocampal slices. J Neuroinflammation. 2005; 2:25

II. Suuronen T, Huuskonen J, Pihlaja R, Kyrylenko S, Salminen A. Regulation of microglial inflammatory response by histone deacetylase inhibitors. J Neurochem. 2003; 87:407-416.

III. Huuskonen J, Suuronen T, Nuutinen T, Kyrylenko S, Salminen A. Regulation of microglial inflammatory response by sodium butyrate and short-chain fatty acids. Br J Pharmacol. 2004; 141:874-880.

IV. Suuronen T*, Huuskonen J*, Nuutinen T, Salminen A. Characterization of the pro-inflammatory signaling induced by protein acetylation in microglia. Neurochem Int. 2006; 49:610-618.

* Both authors contributed equally to this article

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1. INTRODUCTION

Surely none of us at one time or another has been able to escape the grasp of infection as we continuously battle against "invaders". Fortunately, our body is endowed with a defence mechanism that, in most of the cases, wins the fight and the inflamed tissue heals quickly. This complex defence mechanism is called the immune system and has evolved to protect us from the attack of the "invaders"- millions of bacteria, viruses, parasites and toxins- that constantly try to find their way into our body.

Like the rest of the body, the brain also is challenged by proinflammatory mediators. If things go awry these substances can damage and/or cross the blood-brain barrier - "the firewall"- and these inflammatory agents are known to play an important role in a wide number of disorders. Indeed, an increasing number of individuals are suffering from aging-associated diseases that affect the brain as we live longer than intended in our "evolutionary design". These neurodegenerative diseases, such as Alzheimer's disease (AD), represent a huge challenge to the scientists trying to discover better therapeutics to prevent, cure or slow down the disease processes.

The past 15-20 years of intensive basic and clinical research has revealed that inflammation and inflammatory responses significantly contribute to the pathogenesis of neurodegenerative diseases. Thus, a better understanding of the immunoregulatory systems of the brain may represent the key to finding novel anti-inflammatory approaches, e.g. identifying novel drug candidates for the treatments of these devastating disorders.

Acetylation of histone proteins is one of the chemical modifications by which the cells regulate the transcription of genes that respond to the pro- and anti-inflammatory signals mediated through multiple pathways. This is a phenomenon which is ubiquitous in all eukaryotic cells. While it is rarely possible to perform invasive studies in the human brain, many cell culture and animal models have been generated to reveal the principle regulatory mechanisms of the cells involved in the inflammatory responses within the central nervous system.

The present thesis deals with the question of how histone deacetylase inhibitors regulate the microglial inflammatory responses. The pro- and anti-inflammatory responses were characterized using established cell culture models. An organotypic slice culture model was established, refined and characterized for these studies. Rat hippocampal slices were used to compare the results from straightforward cell culture studies with a culture model that mimics the response of the whole tissue.

2. REVIEW OF THE LITERATURE

2.1 Brain inflammation

The defence battle

As diverse as life is, one exceptionally common phenomenon unites all life forms from plants to humans, i.e, the immune system. This system, or network, has evolved to fight against pathogens and in that way to protect the individual. The constant struggle against bacteria, viruses and other pathogens would be a "lost cause" unless the immune system could not counterattack against its myriad of enemies via a variety of inflammatory responses. These responses, as we understand today, are orchestrated by a network of complex chemical and molecular interactions in order to limit tissue damage and to facilitate rapid recovery and tissue healing.

Similarly to the rest of the human body, the brain also needs protection. The neurons are fragile and sensitive to insults and the maintenance of the neuronal well-being is truly a matter of life and death. To facilitate the control of the internal milieu of the brain, the CNS is protected by the blood-brain barrier (BBB) which serves as a "gateway" that controls the entry of peripheral infiltrates. The blood-brain barrier is a specialized system of miles of narrow capillaries throughout the brain, all filled with tightly-packed endothelial cells that are exceedingly selective in determing which compounds gain access to the brain and which are excluded. Together with immune cells of the brain, the BBB forms the defence system that confronts and combats the threats (Figure 1).

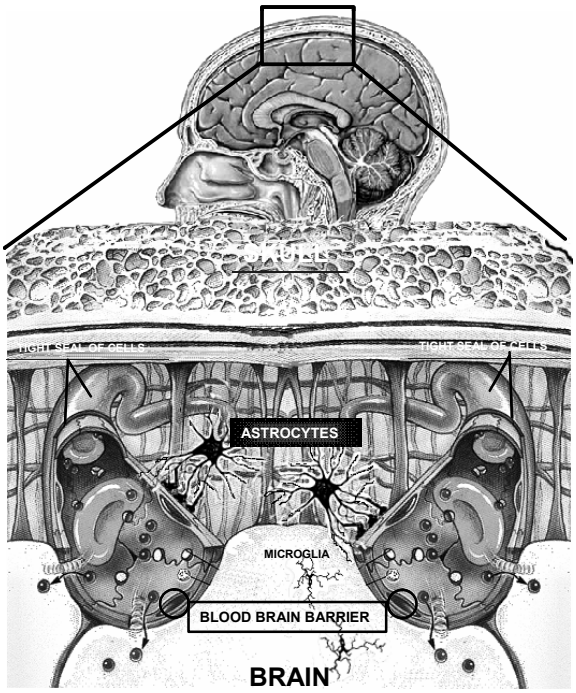

Figure 1. The Blood Brain Barrier

The cellular locus of the the Blood Brain Barrier is the endothelial cell of the brain capillary. The specialized system of membranes of the brain capillary endothelial cells forms a biological interface between the local blood vessels and most parts of the central nervous system. This physical barrier protects the brain by stopping many substances from travelling across it. Astrocyte end-feet encircle endothelial cells and microglia are localized at a site close to the Blood Brain Barrier.

Death of a Dogma

For nearly a century one general belief in the field of neuroscience was that the mammalian brain is an "immunologically privileged" organ, i.e. completely separated from the immune system of the rest of the body (Dermietzel et al., 2006). The blood-brain barrier - "the firewall" of central nervous system - was thought to prevent entry of harmful molecules into the CNS parenchyma and thus was responsible for maintaining homeostatis within the brain. This of course still holds some seeds of the truth but every now and then, as so often happens in science, the traditional views need to be revised. Recent applications of modern research techniques have provided new information in the field of neuroinflammation. It is known that the capability of the CNS to tolerate all consequences of the defence battle is limited and excessive insults can lead to destruction or compromise of the blood-brain barrier (Andjelkovic and Pachter, 1998). However, the brain and the immune system are known to communicate with one another and today it is recognized that the CNS possesses a capacity to mount an immune response.

Intensive neuroinflammation research focusing on the brain macrophages, i.e. the microglial cells, has led to the wide acceptance that these cells truly represent a resident immune cell population that constitute the immune system of the CNS (Streit, 2002, van Rossum and Hanisch, 2004). There is evidence that microglia and perivascular macrophages "talk to each other". Moreover, under pathological conditions, microglia are capable of secreting cytokines and serving as antigen-presenting cells (APCs) (Hanisch, 2002), thus sharing properties with the monocytes in the periphery.

Indeed, over the last 15-20 years, neuroimmunologists have presented many examples of immunological responses to brain antigens, neuronal expression of major histocompatibility complex (MHC) class I genes, microglial expression of MHC class II genes, and also neurological autoimmunity has been recognized (Nakajima and Kohsaka, 2004, Perry,1998, Rivest S, 2003, Shrikant and Benveniste, 1996).

Thus, our original impression of an "immunoprivileged" status of the brain must be qualified by stating that this depends on conditions. The brain clearly has some inimitable immunologic properties, and thus the claim that it is isolated from the immune system is a fallacy.

The Affected Brain

The remarkable power of the protection provided by immune system comes at a cost, the inflammatory response has also the capacity to evoke damage. The consequences of an overwhelming inflammatory response are not always predictable and nowadays it is agreed that dysregulation of the innate or acquired immune response is involved in different brain pathologies (Marchetti and Abbracchio, 2005).

Multiple sclerosis (MS) has long been recognized as an inflammatory disease of the brain (O'Connor et al., 2001). In this devastating disease, vascular inflammatory infiltrates can cause breakdown of the blood–brain barrier which in turn leads to demyelination, gliosis, axonal damage, varying degrees of edema etc. and eventually to permanent neurological disability (Hafler et al., 2005).

In recent years it has also been suggested that a chronic or overwhelming inflammatory response may contribute to other CNS diseases such as stroke, traumatic brain injury, HIV-related dementia, Alzheimer's disease, Parkinson's disease and prion disease (Kadiu et al., 2005). The recognition of the various inflammatory components involved in the pathology of these different diseases has emerged from the new techniques and reagents available for studying inflammation mechanisms in brain pathology (Buckwalter and Wyss-Coray, 2004).

The brain in Alzheimer's disease exhibits a chronic inflammatory response characterized by activated glial cells and increased expression of cytokines and complement factors surrounding the amyloid deposits (McGeer and McGeer, 2003). Similarly, reactive microglia and the presence of activated complement proteins have been demonstrated in the affected brain regions in Parkinson's disease (Hague, Klaffke and Bandmann, 2005).

One of the acute phase proteins of the complement system, the C-reactive protein (CRP), is considered to be one of the critical inflammatory markers (Vermeire, Assche and Rutgeerts, 2005). Together with other markers, such as cytokines, increased levels of peripheral CRP have been shown to be associated with increased risk of incident stroke (Kuo et al., 2005). Evidence is also being gathered to suggest that aged individuals with chronic inflammation, as well as those with chronic or acute infections, are at an elevated risk of suffering from multiple degenerative diseases. There is an increasing body of evidence that the resident macrophages of the central nervous system, i.e. the microglial cells, play a very crucial part in most of the brain disorders (Streit et al., 2005) and neuroinflammation together with microglial pathology contribute to the severity of these diseases.

Microglia: the frontline cells

The smallest cell population of glial cells in the brain, the microglia, were originally distinguished from other CNS supporting cells by del Rio-Hortega (del Rio-Hortega, 1932). In the 1980's, with a development of cell staining techniques, it was shown that these cells are related to macrophages and account for up to 20 % of the total glial cell population, and that they uniformly scattered throughout the adult brain (Barron, 1995, Jordan and Thomas, 1988, Kreutzberg, 1996, Lawson et. al., 1991, Moore and Thanos, 1996, Perry and Gordon, 1988, Streit, 2001). Today, microglia are considered to be derivatives of the hematogenous monocyte/macrophage lineage of mesodermal origin (Kaur et al., 2001, Ling et al., 2001). They enter the brain in early development and appear to form two (sub)populations, one of "resident" microglia (a.k.a. "parenchymal" or "juxtavascular") that form a relatively stable population associated with the microvessels, but these cells are also able to migrate through the brain parenchyma, and "perivascular" microglia/macrophages that are enclosed by the basement membrane of the vessel wall (Gehrmann et. al., 1995, Lassman et. al., 1991. Mato et. al., 1986, 1996). Furthermore, they represent a dynamic population able to exchange with the monocytes in the blood (Gehrmann et. al., 1995, Grossmann et al., 2002, Guillemin G. and Brew B., 2004, Nakajima and Kohsaka, 2004).

Microglia possess the potential to undergo dramatic morphological and functional changes from "resting" ramified forms to "activated" amoeboid cells (Figure 2.). Recent studies indicate that microglia cells actually never rest but instead they constantly monitor their immediate environment by extending and retracting their projections over a minute-to-minute time scale, thus performing an immune surveillance role (Nimmerjahn et al., 2005, van Rossum and Hanisch, 2004). Activated microglia have been shown to secrete a host of proinflammatory soluble factors such as cytokines, free radicals and fatty acid metabolites (Giulian et al., 1994b, Lee et. al, 1993, Prinz et. al., 1999, Smith et. al., 1998), all of which are thought to be harmful to neurons. For example, loss of dopaminergic neurons in rat substantia nigra has been shown to be mediated via microglial activation (Arimoto and Bing, 2003). Furthermore, dying neurons release factors that may stimulate microglia and astrocytes to produce more cytokines (Ferrari et al., 1997, Hide et al., 2000, Liu et al., 2000), leading possibly to a cycle of chronic stimulation of these cells. On the other hand, microglia are thought to possess a supportive and protective role. There is evidence that microglia are able to deliver growth factors and remove cell debris from injured neurons (Elkabes et al., 1996, Nakajima et al., 2001, Nakajima and Kohsaka, 2004). It has also been demonstrated in live hippocampal slice cultures that microglia rapidly engulf any dead cells in their nearby vicinity (Petersen and

Dailey 2004). So, while these cells appear to be there to battle against microbial attacks and to protect the CNS from ischemic or traumatic injury, they do seem to inflict brain damage when they are overburdened. Therefore, the role of microglial activation may be described as a "double-edged sword". Whereas some growth factors secreted by reactive microglia may promote the repair phase after brain trauma, several cytotoxic molecules produced by these cells might affect the well-being and survival of damaged neurons.

The innate immunity in the brain is mainly the responsibility of the microglial cells (Olson and Miller, 2004). Cell surface receptors that mediate immune responses as well as intracellular transcription factors that regulate inflammatory responses have been identified in these cells. There is also extensive evidence that microglia interact with other immune cells (Deng and Sriram, 2005, Giuliani, Hader and Yong, 2005, Markovic et al., 2005) and these complex interactions are tightly regulated within the immune system. The dynamic nature of microglia allows these cells to move around in the CNS parenchyma, especially to any site of infection, and therefore it is no great surprise that these cells can stimulate astrocytes and neurons. In addition, by extending their processes into the perivascular basement membrane, microglia can directly make contact with the pericytes and endothelial cells of the BBB. The literature contains an abundance of evidence for the great variety of effects induced by microglia (for a review see (Farber and Kettenmann, 2005)), but the role of these cells in the regulation of the endothelium and the permeability of the blood-brain barrier is still poorly understood. It has been proposed that microglia, together with astrocytes, release factors that may promote endothelial cell growth and survival (Prat et al., 2001). Activation of microglia has also been suggested to evoke an in situ inflammatory reaction that may modulate BBB function as well causing injury, even death, of endothelial cells.

Moreover, one has to take into account the bi-directional interaction of microglia with other brain cells, and that the other cell types surrounding the brain microvasculature also contribute to BBB integrity. Therefore the overall role of microglia as an immune- or immunoeffector brain macrophage appears to have a dualistic nature. On one hand, microglia play a role in facilitating functional plasticity of CNS and seem to act as the primary sensor for insults but on the other hand, excessive microglial activation can lead to severe brain damage. As a whole, activated microglia appear to play a significant role in inflammatory states of the brain, expressing both or either cytotoxic and neurotrophic functions. Since microglia can produce a wide range of biologically active molecules which can result in either beneficial or detrimental outcomes, therapeutic interventions targeted on microglia and their products may open new avenues for controlling inflammatory brain diseases.

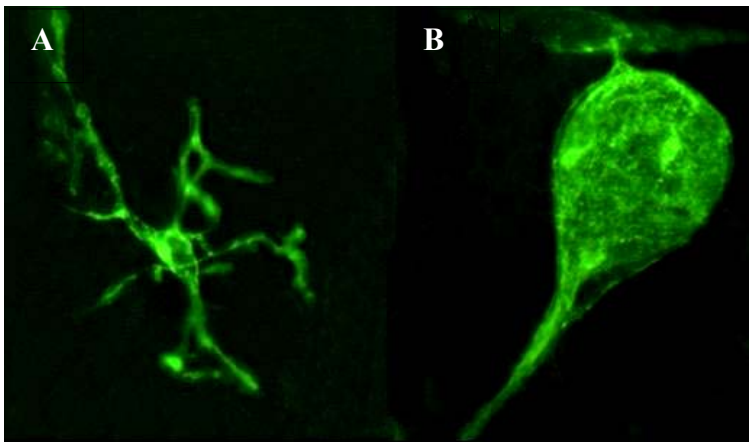

Figure 2.

A. Microglial cell showing typical "resting", ramified morphology with small cell body and branching processes.
B. "Reactive" microglia with a rounded,"amoeboid" phenotype.
Picture: Jari Huuskonen ©

2.2 Innate immunity

Running in the blood

Although it can be concluded from early writings that man knew about disease and its ravages, the history of immunity (L: *immunis* – free of) and immunology goes back to the "Fathers of Immunology"- Louis Pasteur (1882-1895) and his fellow Pastorians Ilya I. Mechnikov (a.k.a. Elias Metchnikoff, 1845-1916) and Paul Ehrlich (1854-1915). After observing that the "mobile cells"of transparent larvae of starfish accumulated around lesions, Mechnikov considered that these cells might participate in defense system of these organisms (Metchnikoff, 1884). To test the value of his hypothesis he carried out further experiments with the transparent water flea *Daphnia* and in that way discovered phagocytosis, a phenomenon which laid the foundation for the theory of cell-based immunity. At the same time, his friend Ehrlich proposed his side-chain theory of immunity – immunity was due to humoral substances, i.e. antibodies, and together with Mechnikov they shared the 1908 Nobel Prize in Physiology or Medicine for their "work on immunity".

However, the field of innate immunity was more or less left in the doldrums until the the late 1950's and early 60's when T and B cells became recognized. Those years are characterised in the literature as the beginning of modern immunology. The basis for humoral and cellular immunity was proven beyond reasonable doubt but the development of molecular and genetic techniques in the past decades has truly remodeled and deepened our understanding of how the immune system operates.

One of the latest breakthroughs of innate immunity saw daylight in 1989 when Charles A. Janeway wrote a paper about his idea of how the immune system alerts the body's T and B cells (Janeway, 1989). Sadly his paper was largely ignored by the scientific community for some time but together with young Russian biochemist Ruslan Medzhitov they were able to unveil the molecular scouts that today have proved to be the key players in the theory of "Pattern Recognition Hypothesis" (Medzhitov and Janeway, 2000b, Medzhitov and Janeway, 2000c).

Today, the term innate immunity is used to describe the defence mechanisms that, in a non-specific manner, respond to the attack of pathogenic organisms. In addition to the physical barrier, the skin, the innate immune system is composed of specialized cells that are able to detect, recognize and respond to pathogens. The cells involved in this counterattack are the white blood cells and macrophages that express specialized receptors, e.g. the microglia in the brain.

The Road to *Toll*

Janeway and Medzhitov coined the phrase "first line of defence" (Janeway and Medzhitov, 2002) which is nowadays a widely used term to describe the innate immune system. This evolutionary ancient system operates through a broad range of defence mechanisms that recognize invariant molecular structures of pathogens. These molecular structures, called "pathogen-associated molecular patterns (PAMPs)" (Medzhitov and Janeway, 2000a), are detected by the cells of the immune system.

In order to be able to elicit a rapid and appropriate defensive response, a large number of potential microbial pathogens need to be discriminated from non-infectious self.

Nature has met this challenge by the evolution of a variety of germ-line encoded cell-surface receptors called ***Toll-like*** receptors (TLRs), originally identified from the fruit fly *Drosophila* (Hashimoto, Hudson and Anderson, 1988). Monocytes, macrophages and their derivatives (i.e. microglia in the brain) express these receptors that are referred to as "pattern-recognition receptors (PRRs)" (Medzhitov and Janeway, 2000a). These receptors are type I transmembrane receptors that function as either homodimers or they may form heterodimers with another TLR. They are generally divided into three groups; 1) secreted molecules that circulate in blood and lymph; 2) surface receptors on phagocytic cells such as macrophages that bind the pathogen prior to engulfment, and; 3) cell-surface receptors that bind the pathogen initiating a signal leading to the release of effector molecules (cytokines).

The mammalian TLR family shares common structural features: multiple leucine-rich repeats and one or two cysteine-rich regions in the large and divergent ligand-binding ectodomain; a short transmembrane region; and a conserved cytoplasmic domain that is highly homologous among the individual TLRs and contains a Toll/IL-1R (TIR) domain similar to the cytoplasmic domain of the interleukin 1 receptor (IL-1R) (Figure 3.). Of the eleven mammalian TLRs described so far (as of September 2006), the ligands for ten of the receptors have been identified (Table 1.). Each TLR has a specific ligand, but for a full sensitivity they may also depend on other co-receptors, for example in the case of TLR4's, recognition of molecular structure of Gram negative bacteria (see Figure 4.)

The PRRs perform their role by mediating phagocytosis or they can function as signal-transducers. Phagocytosis leads to killing and degradation of infectious agent within the maturing phagosome and delivery of cytokines, thus triggering the inflammatory response. As signal-transducers, the PRRs are capable of inducing transmembrane signals that activate intracellular pathways. A common pro-inflammatory pathway involved in the activation of the host defence, the nuclear factor kappa B (NF-kB), is one of the first downstream signaling pathways that was linked to Toll-like receptor pathways (Hawiger J, 2001, Medzhitov and Janeway, 1999) and intensive work in the field has revealed the connection of many other inflammatory pathways to Toll signaling.

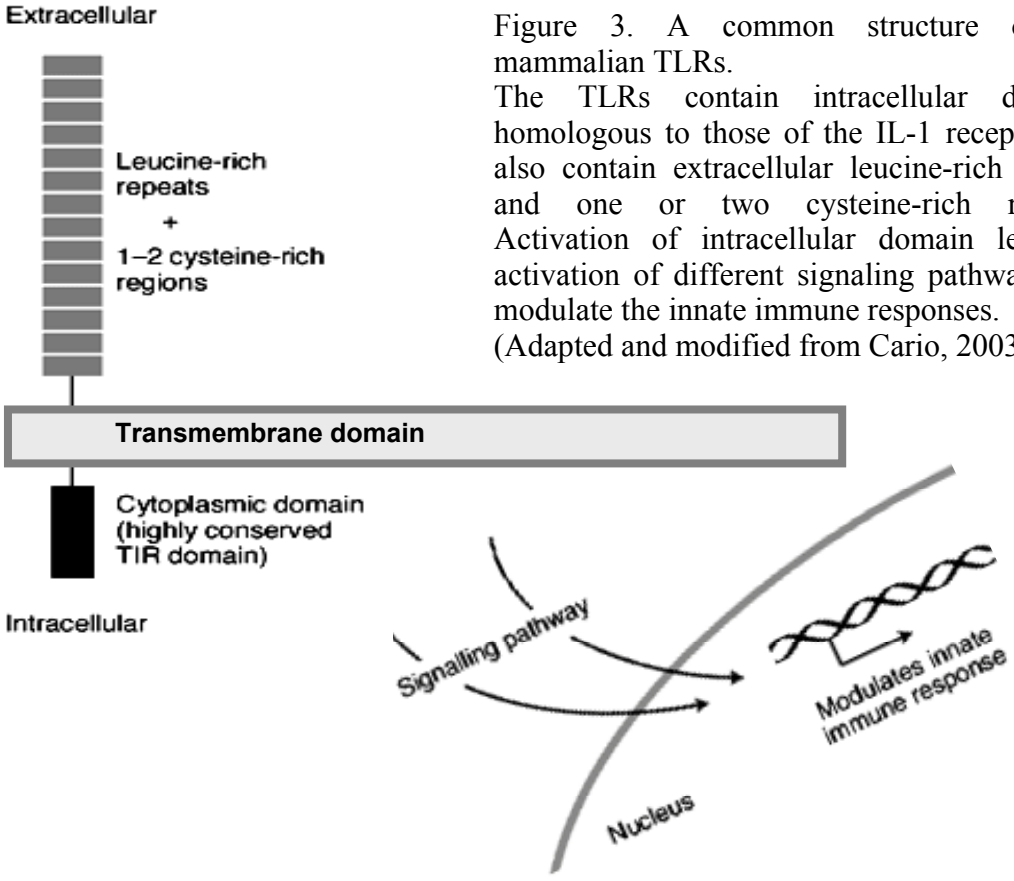

Figure 3. A common structure of the mammalian TLRs.
The TLRs contain intracellular domains homologous to those of the IL-1 receptor and also contain extracellular leucine-rich repeats and one or two cysteine-rich regions. Activation of intracellular domain leads to activation of different signaling pathways that modulate the innate immune responses.
(Adapted and modified from Cario, 2003).

Table 1. Ligands for the activation of the different mammalian TLRs

Receptor	Ligand PAMP(s)	Reference
TLR 1	triacyl lipoproteins	Hornung et al., 2002, Ozinsky et al.,2000
TLR 2	lipoproteins; gram positive peptidoglycan; lipoteichoid acids; fungi; viral glycoproteins	Hertz et al., 2003, Komai-Koma et al., 2004, Underhill et al., 1999
TLR 3	double-stranded RNA	Guillot et al., 2005, Wang et al., 2004, Alexopoulou et al., 2001, Kariko et al., 2004
TLR 4	lipopolysaccharide; viral glycoproteins	Hornef et al., 2003, Andonegui et al., 2002, Sabroe et al., 2003, Fan et al., 2003, Roger et al., 2001, Ohashi et al., 2000
TLR 5	flagellin	Gewirtz et al., 2001, Didierlaurent et al., 2004, Hawn et al., 2003
TLR 6	diacyl lipoproteins	Hornung et al., 2002, Ozinsky et al., 2000
TLR 7	small synthetic compounds; single-stranded RNA	Heil et al., 2004
TLR 8	small synthetic compounds; single-stranded RNA	Heil et al., 2004
TLR 9	unmethylated CpG DNA	Sivori et al., 2004, Latz et al., 2002, Eaton-Bassiri et al., 2004, Bauer et al., 2001
TLR 10	unknown	
TLR 11	profilin-like molecule	Yarovinsky et al., 2005

The Lipopolysaccharide connection

The outer membrane wall component of Gram negative bacteria, lipopolysaccharide (LPS), is an endotoxin that triggers inflammation. Systemic administration of LPS is a generally used model of infection since it increases the release of proinflammatory cytokines by circulating monocytes and macrophages. The innate immune system in the brain recognizes LPS through Toll-like receptor 4 (TLR4) (Lee and Lee, 2002, Lehnardt et al., 2002) which is expressed by microglia. Circulating LPS first binds to LPS-binding protein (LBP) and the CD14 receptor, which are also expressed by microglia. TLR4 binds to this complex and evokes intracellular cascade of events that are regulated by a nuclear factor kappaB (NF-κB) signaling pathway. Activation of microglia and the subsequent pathway induced by LPS lead to release of cytotoxic factors such as nitric oxide and proinflammatory cytokines which can contribute to neurodegeneration (Lehnardt et al., 2003) (Figure 4.). Furthermore, LPS is involved in protein remodelling by inducing acetylation and phosphorylation of histones (Qin et al., 2005, Rahman, 2002). However, as the inflammatory response of microglia may be considered as the normal, defensive response of these cells, the injuries that result from bacterial infection might be more or less an unwanted bystander effect of microglial activation.

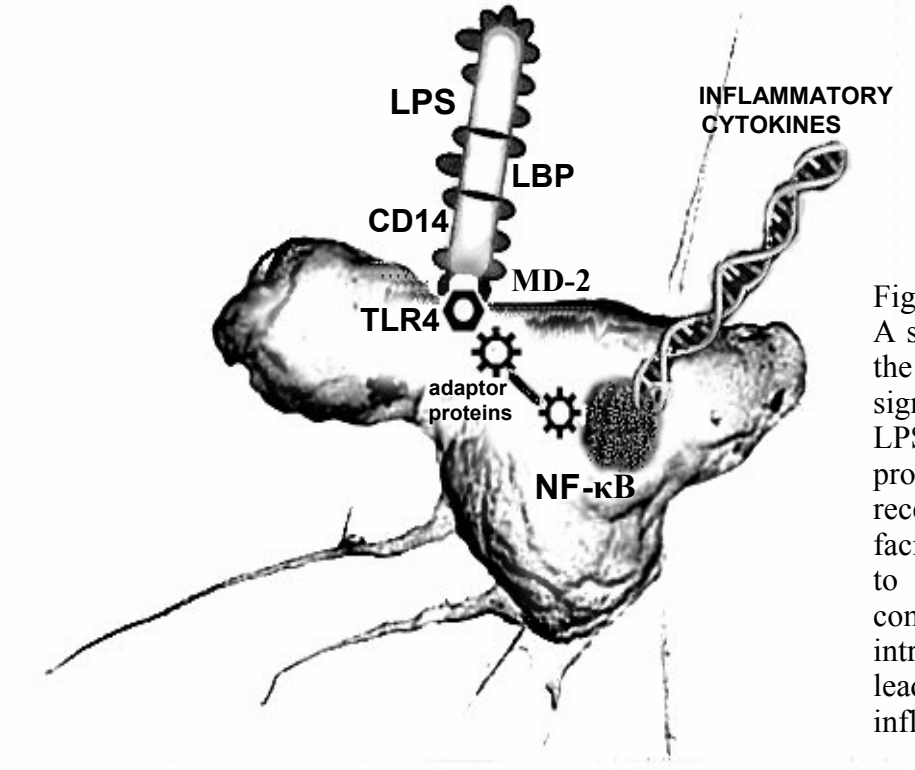

Figure 4.
A schematic drawing to illustrate the LPS induced NF-κB signaling pathway in microglia. LPS first binds to LPS-binding protein (LBP) and the CD14 receptor. LBP and CD-14 facilitate the presentation of LPS to MD-2, TLR4 binds to this complex and induces an intracellular cascade of events leading to production of inflammatory cytokines.

2.3 NF-κB - The Intercity

Preface

In order to produce proteins the cell needs to transfer the genetic information, that is coded in the DNA, into a sequence of messenger RNA (mRNA) molecules. This process, called transcription, is followed by synthesis of proteins, i.e. translation. Transcription factors are proteins that are involved in the regulation of gene expression and thus they control the activation of transcription. Transcription factors are composed of two essential functional regions: a DNA-binding domain and an activator domain. The DNA-binding domain consists of amino acids that recognize specific DNA bases near the start of transcription. Transcription factors are typically classified according to the structure of its DNA-binding domain. The activator domains of transcription factors interact with the components of the transcriptional apparatus (RNA polymerase) and with other regulatory proteins, thereby affecting the efficiency of DNA binding. The regulation by transcription factors takes place at specific sites, i.e., transcription begins at the promoter, which is a sequence specifying the beginning of transcription. It then proceeds through the coding region, a sequence that includes coding information for the polypeptide chain specified by the gene. The transcription ends at the terminator, a sequence that specifies the end of the mRNA transcript. (Stryer, 1995, Lachman, 1995).

Conductor of inflammation

The eukaryotic nuclear factor kappaB (NF-κB), first described in 1986 (Sen and Baltimore, 1986), is a general term used to describe a number of dimeric combinations of the Rel protein family members of gene regulators that possess transcriptional activating properties (Ghosh, May and Kopp, 1998). The mammalian NF-κB family members include p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). Today, NF-κB is recognized as the major cell-signaling molecule for inflammation that regulates the expression of a number of genes involved in multi-level regulation of immune responses. For example, p65/TNFR1 double knockout mice exhibit increased susceptibility to bacterial infection (Alcamo et al., 2001), RelB-deficient mice show decreased baseline activity of NF-κB, a loss of cellular immune responses and deficits in adaptive immunity (Weih et al., 1995, Burkly et al., 1995). Furthermore, a hypozygous mouse model of NF-κB-deficiency in which mice lack p50 and are heterozygous for p65 ($p50^{-/-}p65^{+/-}$; 3X), show increased sensitivity to LPS-induced shock (Gadjeva et al., 2004), a property of innate immune system.

Initial work carried out with animal models of systemic inflammation as well as studies applying brain-derived cell cultures have revealed that activation of NF-kB occurs in numerous tissues (Blackwell et al., 1994, Essani et al., 1996, Manning et al., 1995), including the brain (O'Neill and Kaltschmidt, 1997). Subsequent studies using different models have allowed identification of many of the mediators involved in the development of inflammatory injuries.

Central to activation of NF-κB and the subsequent inflammatory cascade is the translocation of NF-κB from cytosol to nucleus. NF-κB resides in the cytoplasm of unstimulated cells and its activity is tightly regulated by the interaction with its inhibitory IκB proteins, IκBα, IκBβ and IκBε . These inhibitory proteins are believed to mask the nuclear localization signals on NF-κB subunits and thus prevent their translocation. Furthermore, it has been suggested that there is a continuous shuttling of signaling molecules between nucleus and cytoplasm and therefore the state of localization is dynamic (Birbach et al., 2002, Huxford et al., 1998, Huang et al., 2000, Huang and Miyamoto, 2001, Johnson et al., 1999, Malek et al., 2001).

Several different inflammatory stimuli release NF-κB from the inhibitor and the signaling cascade is activated, leading to the complete degradation of IκB. (Chen and Greene, 2004, Greene and Chen, 2004). Degradation of IκB is initiated upon phosphorylation by inhibitory kappa kinases (IKKs), followed by ubiquitination and further degradation through 26S proteasome machinery. This process liberates NF-κB dimers from IκB complexes and allows NF-κB to move into the nucleus where it binds to its target genes, including IκBα, and regulates their transcription (DiDonato et al., 1996, Karin and Ben-Neriah, 2000). The NF-κB activation is terminated and its location reversed by newly synthesized nuclear IκBα, which removes NF-κB from its target genes and shuttles back to cytoplasm (Arenzana-Seisdedos et al., 1997) (Figure 5.).

Acetylation has a significant role in the nuclear function of NF-κB since it determines whether it exists in an active or inactive state (Greene and Chen, 2004). Acetylated NF-κB is active and is resistant to the inhibitory effects of IκB. However, when NF-κB is deacetylated, IκB readily binds to NF-κB leading to its translocation into the cytoplasm (Arenzana-Seisdedos et al., 1997, Chen et al., 2001). Moreover, acetylation of transcription factors has been shown to affect many cellular functions such as transcriptional activity, interactions with cofactors and subcellular localization of other proteins.

The transcriptional response of intra-nuclear NF-κB is also tightly regulated, not only by post-translational modifications of NF-κB itself but also by modification of histones (Boekhoudt et al., 2003, Graham and Gibson, 2005) . Stimulation by LPS can lead to increased acetylation of the amino-terminals of the histone tails surrounding NF-κB target genes (Chen and Greene, 2004). These hyperacetylated histones interact with NF-κB via coactivators to promote its transcription machinery through chromatin remodelling, whereas impaired NF-κB activity can be achieved by deacetylation of these histone residues. When activated, the NF-κB proteins interact with the same co-activator and co-repressor molecules as histones (Ashburner, Westerheide and Baldwin, 2001). Together with the dynamic changes occurring in the NF-κB - histone gateway, these molecules act as the molecular switches that control gene transcription.

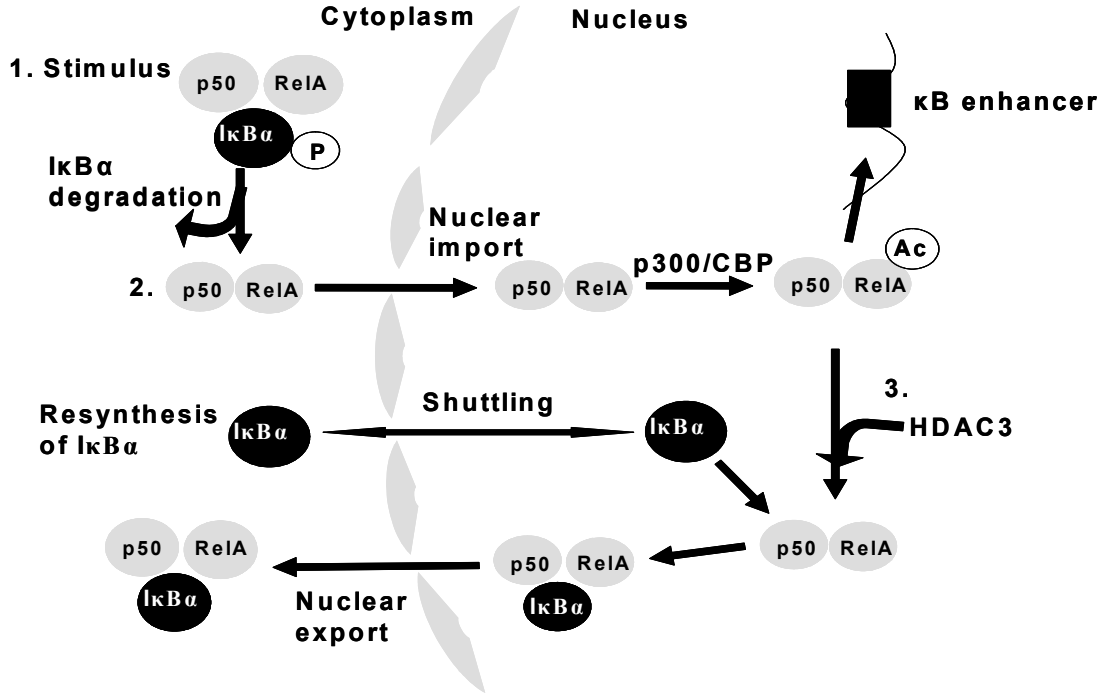

Figure 5.

1. Upon stimulation, IκBα is phosphorylated by the IκB kinase complex, containing IKKα, IKKβ and IKKγ. 2. The liberated NF-κB (p50/RelA) enters into the nucleus where RelA is acetylated by p300/CBP. The transcription of multiple genes, including the IκBα gene, is activated. 3. Deacetylation of RelA by HDAC3 promotes the binding of RelA to IκBα The newly synthesized IκBα protein enters the nucleus, removes NF-κB from its target genes, and takes it back to the cytoplasm to terminate the phase of NF-κB activation. (Adapted and modified from Chen and Greene, 2003 and Quivy and Van Lint, 2004).

2.4 Histone Post-translation

Normally genes are wrapped up in a complex, ordered structure of proteins and DNA. These proteins that are packed into the nucleosomes and complexed with DNA in chromatin are called the histones (Ramakrishnan, 1997). The structural subunit of chromatin, the nucleosome, consists of 146 base pairs of DNA wrapped around an octamer of highly conserved proteins. Each nucleosome core consists of two copies of each of the histones: H2A, H2B, H3 and H4. They are found in the nuclei of all eukaryotic cells, one of the most important features of histones is that they are susceptible to chemical modification (Grunstein, 1997). These post-translational modifications include acetylation, phosphorylation, methylation and ubiquitination and these changes occur on the N-terminal tails of core histones (Figure 6.a).

In general, increased transcriptional activity of inflammatory genes is linked together with increased levels of histone acetylation (hyperacetylation) and conversely repression of gene expression is associated with decreased levels of acetylation (hypoacetylation). For example, activation of inflammatory genes involves opening up of the chromatin structure as a result of acetylation of core histones. This unfolding of chromatin becomes possible through the action of another group of proteins called "co-activators" and the interplay between these co-activator molecules allows transcription factors to bind to a variety of DNA sequences, which in turn leads to gene induction (Barnes P., Adcock I. and Ito, 2005).

The mechanisms of how other modifications of core histones, such as phosphorylation, methylation and ubiquitination, interact with acetylation and with other transcription factors are largely unknown (Marmorstein and Roth, 2001) but recent studies have highlighted the importance of this research area (for a review see (Emre and Berger, 2006, Santos-Rosa and Caldas, 2005)). Further studies may lead to a better understanding of how genes are differentially regulated and how more specific gene manipulation might be produced in the future.

HATs and HDACs

The dynamic switches between activity states of chromatin are affected by the interplay of two competing enzymatic activities, histone acetyltransferases (abbreviated as HATs) and histone deacetylases (HDACs), which convert the acetylation state of histone proteins. Acetylation of histones (neutralization of positive charges of the histone tails) is catalyzed by HATs and it typically enhances transcription by reducing the affinity between DNA and histones (relaxation of chromatin structure) whereas removal of acetyl groups from the lysine residues by HDACs results in chromatin condensation and this process usually leads to repression of the transcriptional activity of many genes (Figure 6.b). However, a number of studies have emphasized that acetylation dependent gene regulation is anything but straightforward. There are indications that HATs can also act as repressors and HDACs can also function as (co)activators (de Ruijter et al., 2003), implying that the effects of protein (de)acetylation can be bi-directional. Futhermore, it has been observed that HATs and HDACs may be involved in the modification of other proteins in addition to histones (Hubbert et al., 2002, Juan et al., 2000). These proteins include transcription factors, such as the NF-κB (see previous chapter) and other key regulatory proteins involved in inflammation.

Several different forms of HATs and HDACs have been characterized and at least five different protein families of HATs (Roth, Denu and Allis, 2001) and eighteen different human HDACs (Verdin, Dequiedt and Kasler, 2003) have been identified. Based on their intracellular localization, histone acetyltransferases are separated into two types. Type A HATs are localized in the nucleus where they function as transcriptional co-activators. Type B HAT (Hat1p) acetylates nascent core histones in the cytoplasm and is suggested to be involved in chromatin assembly (Qin and Parthun, 2006). HDACs fall into three classes of protein families: the classical HDAC family of two different phylogenetic classes, namely class I and class II, and the recently discovered SIR2 family (SIRT 1-7) of NAD+-dependent class III HDACs.

The prevalent substrates of HDACs are found in the nuclei of eukaryotic cells. In order to exert their function, HDACs co-localize into the nucleus together with other proteins or HDACs or in response to a nuclear localization signal (NLS). Some HDACs possess a nuclear export signal (NES), suggesting that they can also move to the cytoplasm. It has been also shown that class II HDACs are able to shuttle between the nucleus and cytoplasm in response to certain signals emitted from the neighbouring cells (Verdin, Dequiedt and Kasler, 2003).

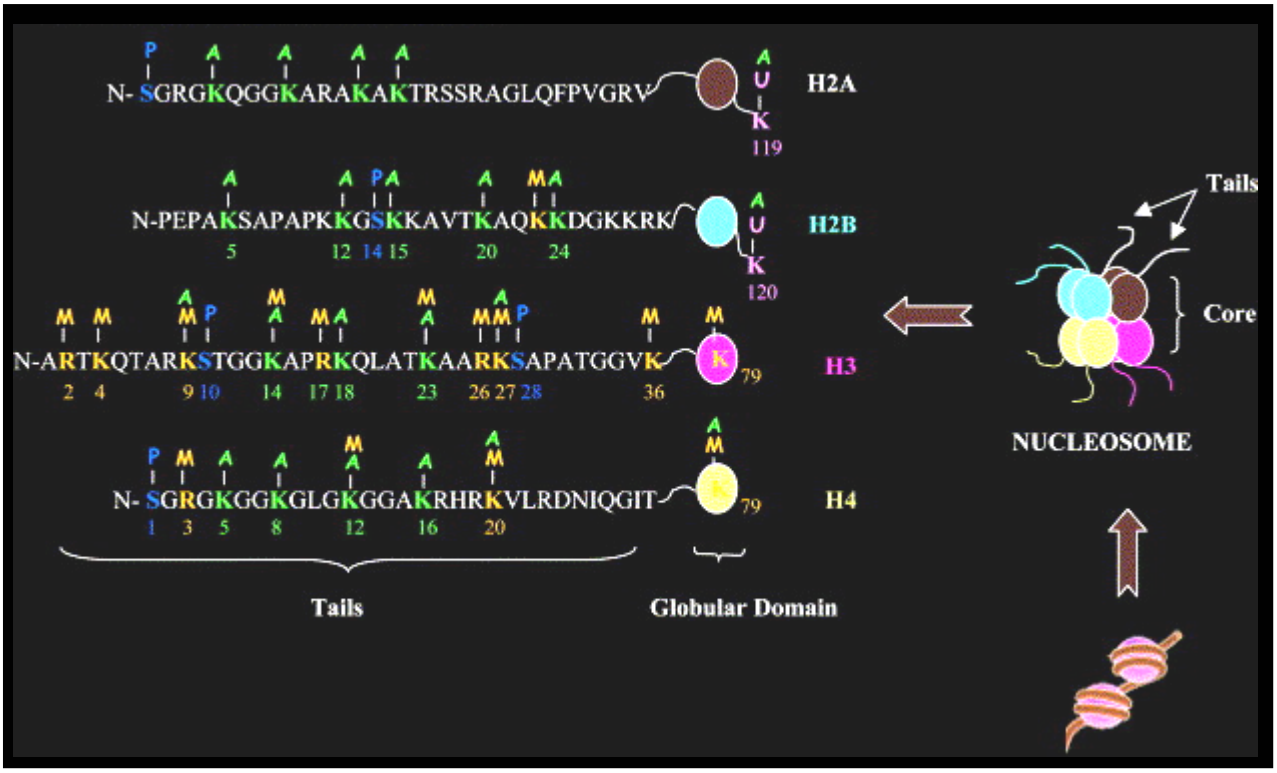

Figure 6.a

Post-translational modifications on the histone tails. Each nucleosome core consists of two copies of each of the histones: H2A, H2B, H3 and H4. These evolutionarily conserved proteins have a globular C-terminal domain critical to nucleosome formation and a flexible N-terminal tail that protrudes from the nucleosome core. A=Acetylation: M=methylation: P=phosphorylation: U=ubiquitination (Santos-Rosa and Caldas, 2005)

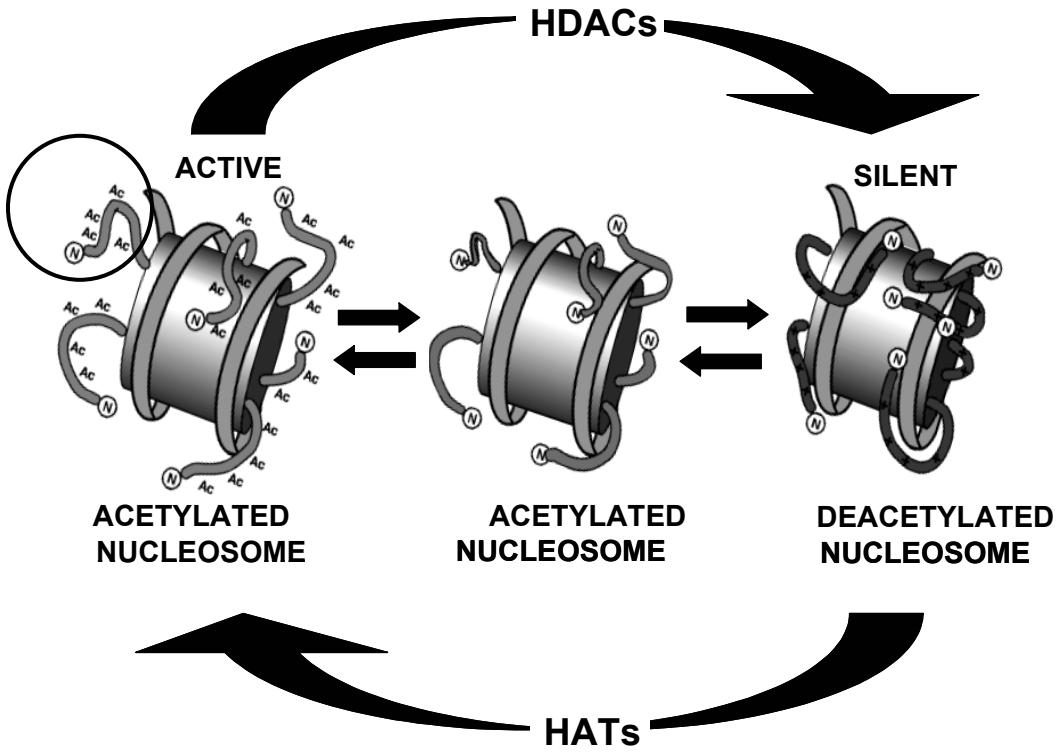

Figure 6.b

A schematic drawing to illustrate the interplay between HATs and HDACs. HATs catalyze the acetylation by adding acetyl groups to lysines in the N-terminal tails of histones (encircled). The dynamic activity of HAT-modified proteins is counteracted by HDACs. (Modified from: http://www.broad.mit.edu/chembiolab_schreiber/anims/index.htm)

Inhibition of HDACs

One well-founded view today is that many histone deacetylase inhibitors (HDACi) operate by blocking access of acetylated substrate to the active site (reversible or irreversible) of HDAC (Drummond et al., 2005), though the specific routes, i.e. whether HDACi cause direct histone hyperacetylation within the promoter/enhancer regions of cytokine genes, remain to be clarified. Generally, HDACi are known to be able to induce growth arrest, differentiation or apoptosis of cancer cells *in vitro* and *in vivo* (de Ruijter et al., 2003). HDACi are capable of inhibiting HDACs with varying efficiencies (from the nanomolar to millimolar

concentrations). Inhibition of HDACs can result in a general hyperacetylation of histones, which in turn leads to relaxing of chromatin. This "decompacting" of condensed chromatin allows access so that the transcription factors can bind to DNA and therefore increased transcriptional activation becomes possible.

Over the years, many different types of HDAC inhibitors have been developed. There are many inhibitors known but the most potent discovered so far is trichostatin A (TSA) (Eickhoff et al., 2000, Herold et al., 2002, Mishra et al., 2001, Moreira, Scheipers and Sorensen, 2003). TSA is a fermentation product of the fungi *Streptomyces* and it was originally used as an anti-fungal agent, but was later discovered to have potent proliferation-inhibitory properties against cancer cells. TSA belongs to the group of hydroxamic acids, and it has been shown to be effective at nanomolar concentrations *in vitro.* Another well known group of HDACi are the short-chain fatty acids (SCFAs) (Saemann, Bohmig and Zlabinger, 2002) such as butyrate, propionate and valerate. These compounds have been shown to be less efficient in their HDAC-inhibiting capability than TSA (act in the millimolar compared with nanomolar range), but they are also less toxic. Some cyclic tetrapeptide antibiotics form the third group of inhibitors, and these compounds are characterized by their complicated chemical structure and their high HDAC-inhibitory potential. Most of these compounds are products of bacteria or fungi, but some are chemically engineered combinations of hydroxamic acids and cyclic tetrapeptides. Benzamides comprise the fourth group of the established histone deacetylase inhibitors.

Some of the compounds mentioned above have entered into clinical trials and an increase in histone acetylation has been observed (Chen, Faller and Spanjaard, 2003). Although little is known about the specific functions of the individual HDACs and the specific consequences of using HDACi in normal cells, the results achieved in these clinical trials might contribute greatly to enhancing our understanding of HDAC function and the therapeutic possibilities offered by these enzyme systems. Therefore studies investigating the mechanisms of actions of all HDACs and the effects of using HDACi are of clinical importance. A summary of the HDACi classes and their characteristics is presented in table 3.

Table 3. Molecular characteristics and clinical trial status of HDACi

Class	Compound	effect. (range)	HDAC specificity	Clinical trials; reference
Short-chain fatty acids	Butyrate	mM	Class I, IIa	Phase I, II; Bhalla, 2005
	Valproic acid (VPA)	mM	Class I, IIa	Phase I, II; Bhalla, 2005
Hydroxamic acids	Trichostatin A (TSA)	nM	Class I, II	N/A; Bhalla, 2005
	Suberoylanilide hydroxamic acid (SAHA)	μM	Class I, II	Phase I, II, III (pre - registration) ; Bhalla, 2005
	PXD101	μM	Class I, II	Phase I; Bhalla, 2005
	LAQ824	nM	Class I, II	Phase I; Bhalla, 2005
	LBH589	nM	Class I, II	Phase I; Bhalla, 2005
	Pyroxamide	μM	Class I, unknown effect on class II	Phase I; Butler et al., 2001
	SK-7041	nM	HDACs 1 and 2	N/A; Park et al., 2004
	SK-7068	nM	HDACs 1 and 2	N/A
	CG-1521	μM	N/A	N/A
	Tubacin	μM	Class IIb	N/A; Haggarty et.al., 2003
Benzamides	MS-275	μM	HDACs 1, 2,3, 8 (marginally)	Phase I, II; Hu et al., 2003
Cyclic tetrapeptides	Depsipeptide	nM	Class I	Phase I, II; Furumai et at., 2002
	Trapoxin A	nM	Class I, IIa	N/A; Bhalla, 2005
	Apicidin	nM	HDACs 1 and 3, not HDAC8	N/A; Vannini et al., 2004

N/A; not available. effect.; effectiveness. Table modified from Bolden et al., 2006

HDACs and NF-κB mediated inflammation

The level of the inflammatory response is clearly dependent on the activation state of numerous pathways, one of the major pathways being the transcription factor NF-κB. By counteracting the effects of HATs at the site of inflammatory gene transcription, HDACs play a significant role in the regulation of NF-κB-dependent inflammatory response. Generally, HDACs, together with co-repressors, repress the expression of NF-κB-dependent inflammatory genes by influencing the repacking of the uncoiled DNA/chromatin structure. This repression process is caused by the deacetylation of NF-κB at multiple levels, including its subcellular localization, transcriptional activation, DNA-binding and IκBα assembly (Chen and Greene, 2004). The HDACs known to be involved in the modulation of NF-κB transcriptional activity are the class I members HDAC1, HDAC2 and HDAC3 (Ashburner, Westerheide and Baldwin, 2001, Kiernan et al., 2003). Recent studies have indicated that also class III HDAC SIRT1 inhibits NF-κB by affecting the transactivation potential of NF-κB subunit (Chen et al., 2005b, Yeung et al., 2004).

Both of the subunits of the NF-κB heterodimer, p50/p65 are subjected to acetylation/deacetylation (Chen et al., 2001, Chen and Greene, 2004). Deacetylation by HDAC3 apparently promotes RelA/p65 binding to nuclear IκBα, which in turn leads to activation control of NF-κB by influencing the translocation of RelA/p65 from nucleus to the cytoplasm (Chen et al., 2001, Kiernan et al., 2003). In contrast, the intranuclear p50 subunit has been shown to be complexed with HDAC1 and thus can suppress gene expression by inhibiting the NF-κB activation (Zhong et al., 2002). HDAC1 has also been shown to recruit the p65 subunit directly (Ashburner, Westerheide and Baldwin, 2001), resulting in repression of NF-κB activation. HDACs may also associate with the p50/p50 homodimer, which serves mainly as a negative regulator of NF-κB activity through competing with p50/p65 for NF-κB response elements on DNA and through its association with the co-repressor histone deacetylase. HDAC1 and HDAC2 are also capable of interacting with other co-repressors to orchestrate the suppression of gene expression .

Corticosteroids, which are widely used anti-inflammatory agents, reverse histone acetylation at the site of inflammatory gene transcription, either by direct binding of the activated glucocorticoid receptor to NF-κB-associated co-activators or by recruitment of histone deacetylases to the activated transcription complex. HDAC2, which can interact with the p65 subunit (Rahman, Marwick and Kirkham, 2004), has been shown to be recruited to the site of transcription by corticosteroids to switch off the genes which have been activated by inflammation (Barnes, 2006).

2.5 Signal integration - the role CBP/p300

All signal transduction pathways are regulated, at some level, by phosphorylation, making this covalent modification relevant to most, if not all, areas of cell signaling (reviewed in (Pawson and Scott, 2005)). Post-translational phosphorylation is a dynamic, reversible regulatory mechanism catalyzed by enzymes that add (kinases) or remove (phosphatases) phosphate groups from proteins by acting on amino acid residues, especially on serine/threonine or tyrosine. Furthermore, (de)phosphorylation switches "on" or "off" many enzymes and receptors involved in signal transduction. Kinases and phosphatases themselves may be regulated by phosphorylation and these enzymes are also able to activate many substrate molecules, which in turn can lead to amplification of the initial signal. This network of pathways usually involves several proteins phosphorylating each other either directly or indirectly.

In addition to acetylation of histones, the intensity and duration of the inflammatory response is regulated by phosphorylation, and, sequentially/subsequently by acetylation of transcription factors, and non-histone proteins. Though the basic mechanisms of the interplay(s) between many inflammatory pathways are yet to be resolved, it has been assumed that phosphorylation facilitates these interactions by inducing binding of transcriptional co-activators, such as histone acetyltransferases (Cheung et al., 2000, Lo et al., 2000).

One of the key elements controlling the proinflammatory gene transcription is the transcriptional co-activator cyclic adenosine monophosphate-response element (CRE)-binding protein, and its structural homologue, p300, also known as CBP/p300. This co-activator has been shown to possess intrinsic HAT activity and its role in chromatin remodeling has proven to be very crucial (Blobel, 2002). Moreover, this protein has an important role in the initiation of transcription by RNA polymerase II and the transcriptional activity of inducible

transcription factors, including NF-κB, appears to depend on interactions with the CBP/p300 (Sheppard et al., 1999, Janknecht and Hunter, 1996).

The role of the physical association of NF-κB subunit RelA/p65 and CBP/p300 for proinflammatory gene transcription has been studied in different cell types (Gerritsen et al., 1997, Shenkar et al., 2001) and it has been shown that phosphorylation of p65 by protein kinase A (PKA) stimulates NF-κB-dependent gene expression by enhancing p65 association with CBP (Zhong et al., 1997, 1998). Furthermore, this phosphorylation governs the recruitment of HAT activity of CBP/p300 by RelA/p65 and thus facilitates the DNA binding and full transcriptional activity of NF- κB (Duran et al., 2003, Vermeulen et al., 2003, Zhong et al., 2002). Moreover, RelA/p65 activity has been shown to be repressed by histone deacetylases 1, 2 and 3, SIRT1 and some corepressors (Ashburner et al., 2001, Chen et al., 2001, Lee et al., 2000, Yeung et al., 2004).

Mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI-3K) cascade pathways have also been shown to contribute to the transmission of extracellular inflammatory signals to NF-κB-driven transcription (Rao, 2001, Darieva et al., 2004). Although the exact mechanisms of NF-κB activation via these kinases remain to be clarified, it has been suggested that the phosphorylation of the transactivation domain of p65 is mediated via functional interaction with the transcriptional coactivator CBP/p300 (Madrid et al., 2001, Darieva et al., 2004).

Phosphorylation can be inhibited by chemical compounds that directly inhibit the enzymatic activation of some pathway's regulator (Lee et al., 2005, Liang et al., 2005). For example, inhibition of PI-3 kinase, a member in one of the major pathways of intracellular signal transduction, prevents the phosphorylation and activation of the next kinase downstream (AKT, a serine/threonine kinase residing within the cytosol) in its cascade. Deactivated AKT in turn effects the phosphorylation status of downstream target molecules and in that way modulates the expression of nuclear proteins. (Martin et al., 2003, Strassheim et al., 2004)

Instead of focusing on simply HATs/HDACs and/or phosphorylation governing the activity of a single pathway, a number of recent studies have addressed the question of cross-talk between acetylation and phosphorylation and subsequent co-regulation of two or more signaling routes. (Kouzarides, 2000, Nowak and Corces, 2004). Today there is evidence that co-regulation of signaling pathways - such as NF-κB and PI-3K - does indeed exist (Goodman and Smolik, 2000, Chen and Greene, 2004, Huang and Chen, 2005). Also, a coupling of histone H3 phosphorylation and acetylation has been observed during cytokine-induced gene expression mediated by IκB kinase α (Anest et al., 2003) and in response to epidermal growth

factor stimulus (Cheung et al., 2000). However, although the interplay between phosphorylation and acetylation is well supported by a number of studies, the extent to which one is dependent on the other to initiate a full activation of inflammatory genes is still unclear (Loury and Sassone-Corsi, 2004, Mahadevan et al., 2004). The concurrent or sequential synergistic actions of acetylation and phosphorylation nevertheless greatly expands the repertoire of the signaling systems and the absolute requirement for the transcriptional coactivators in the actions of many (or even most) transcription factors indicates that they will continue to provide a fruitful target for studies aimed at understanding the complex interactions that underlie the control of cell growth and differentiation. Thus, it will be no surprise if future studies reveal multiple interactions linked to modification of histones and transcription factors.

2.6 Regulation of IL-6 and iNOS

Interleukin 6 is a multifunctional, proinflammatory cytokine secreted by macrophages in response to infection. Thus, this cytokine can be induced in response to LPS and viral infection, and it has been shown that several signaling pathways mediate its expression (Matsuda and Hirano, 2000). The promoter region of IL-6 gene contains three transcription start sites (Yasukawa et al., 1987), including NF-κB -binding site (Libermann and Baltimore, 1990). Furthermore, IL-6 has been shown to be a target gene for transcription factors such as activator protein-1, cAMP response element (CRE)-binding protein (CREB) and nuclear factor for IL-6 expression (NF-IL6) (Akira et al., 1990, Alberts et al., 1993, Dendorfer et al., 1994), which are capable of interacting with the IL-6 promoter in order to initiate mRNA synthesis.

In vitro studies with primary human microglia culture, as well as with cultures of murine origin, have shown that microglia express mRNA transcripts of receptor for IL-6 (Sawada et al., 1993, Lee et al., 2002). Moreover, very recently both the transcriptional and protein level of IL-6 have been shown to be upregulated in microglial cells in response to LPS under in vitro and in vivo conditions (Lund et al., 2006). The activation program of signaling cascades that eventually lead to expression of IL-6 includes intracellular cross-talking signal transduction pathways MAPK, NF-κB, and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways.

The action of IL-6 is initiated by binding to its functional receptor complex that is composed of two subunits, the IL-6 binding protein and a transmembrane signal transducing glycoprotein (gp130) (Taga et al., 1989). Though the gp130 has no binding capability by itself, it associates with the IL-6/IL-6R complex and allows the activation of signal transduction (Murakami et al., 1993). The next activation step involves phosphorylation of the members of the JAK family, followed by phosphorylation of STAT(3), which is a transcription factor mediating the cellular response to IL-6 (Akira S et al., 1994, Zhong et al., 1994). Furthermore, upon dimerization of the IL-6 receptor complex, hyperphosphorylation of MAPK and upregulation of its kinase activity, leading to induction of the cascade, has also been observed (Daeipour et al., 1993). As a summary, the key events in the regulation of IL-6 are the phosphorylation steps of different signaling pathways.

Nitric oxide (NO) is a free radical that is synthesized by nitric oxide synthases from the amino acid L-arginine and oxygen (Moncada et al., 1989). As a signaling molecule, NO is unusual since it has no cell surface receptor. The inducible isoform of nitric-oxide synthase (iNOS), which catalyzes the oxidation of one of the equivalent guanidinium nitrogens of arginine to form NO and citrulline (Stuehr, 1996), is widely expressed by microglia (Zielasek et al., 1992). iNOS regulates the level of NO production primarily at the transcriptional level via NF-κB and AP-1 (Chartrain et al., 1994). The promoter region of iNOS gene contains oligonucleotide motifs for the binding of several cytokine-induced, as well as LPS-induced, transcription factors (Lowenstein et al., 1993), and thus the silent gene is capable of responding to bacterial stimuli. The LPS induced induction of iNOS expression has been shown to involve some of the same pathways involved in the regulation of IL-6. Namely, the aforementioned signaling molecules like JAK/STAT proteins, NF-κB/IκB pathway as well as MAPK have been shown to be targeted by NO. For example, NO is known to inhibit the DNA binding activity of NF-κB (Matthews et al., 1996, Moorman et al., 1996). There is also experimental support from knockout mice studies, that the activation of both STAT3 and NF-κB are iNOS dependent (Hieholzer et al., 1998). Furthermore, NO is also known to affect the production of several cytokines, including IL-6 (Bogdan, 2000). All these findings support the argument that the signaling between inflammatory cells and NO is mainly indirect through iNOS derived NO.

2.7 In vitro models of brain inflammation

Background

Though rapidly expanding, our understanding of the biochemical processes involved in inflammation is still limited. Since an invasive approach to study these interactions with human subjects is not possible, most of our knowledge has been gained through application of basic research methods. Over the years, *in vitro* systems have proven to be well suited for studying the multitude of biological processes in a more isolated context. They have been successfully used to elucidate mechanisms of brain inflammation, such as the involvement of different signaling pathways (van Noort, 2006). Many target cells of inflammation have been identified and the intricate cellular changes induced by inflammatory stimuli have been delineated. For example, *in vitro* systems have been useful for understanding the effects of certain structurally defined compounds and the mechanisms of inflammation, for which target cells and the biochemical processes involved in the inflammation are well known. The physicochemical environment of cells is easily controlled *in vitro*. Culture conditions can be manipulated by adding or removing substances from the culture medium, permitting precise analysis of sequences of events. The concentrations of the different test chemicals can be controlled in terms of the amount being delivered to the entire cell population or to an individual tissue slice.

By using *in vitro* systems it is possible to study separate areas of the nervous system isolated from normal *in vivo* homeostatic environments and mechanisms. The use of *in vitro* techniques allows for exact and highly specific measurement of many fundamental biological processes, such as production or secretion of inflammatory cytokines, chemokines etc. Since they represent a defined and controlled system, *in vitro* techniques offer many advantages in characterising multiple inflammatory mechanisms; but one should never overlook the fact that they are an artificial system. The *in vitro* cell culture systems are limited in that they represent tissues or cells taken out of the body. They may not behave normally when removed from the blood, immune system, nervous system or neighbouring tissues that help make them what they are. Therefore, *in vitro* tests hold their greatest potential in providing information on basic biological processes. This information may also be useful in refining specific experimental questions to be addressed in the whole animal but effects seen *in vitro* invariably have to be confirmed or substantiated *in vivo*. In addition, one must bear in mind that the multiple functions of individual immune cells and the overall complexity of the nervous system lead to the fact that it becomes very difficult to define how well any single *in vitro* system replicates the *in vivo* system.

Various types of *in vitro* approaches, including culturing of different cell lines, primary cell cultures and slice cultures, have been applied to produce data for evaluating potential and known inflammatory substances that affect the brain (van Noort, 2006). The use of animal - including human - cell culture methods has expanded enormously during the last 25 years, with new applications and revised methods appearing all the time.

Cell lines

A neural cell line may be obtained from the **primary culture** (see later paragraphs) by enzymatically detaching the cultured cell layers. This subcultivation process is repeated normally every three to four days, and after several steps, the cell line is established. Established (also referred to as continuous) cell lines can be obtained from donors of various ages and are comprised of a single cell type. In culture, these types of cells can be serially propagated for a limited number of cell divisions. If the cell line has transformed into tumour cells, it can be propagated indefinitely. Transformed tumour cell lines are often derived from actual clinical tumours, but it is also possible to induce transformation by chemical or viral treatments. Immortalized cell lines are also generated from established cell lines with limited life span that undergo crisis after which their growth potential changes and the life span become unlimited.

Clonal cultures are derived from the progeny of a single cell, either from established or transformed cultures. Once the cell line phenotype is established, it does not change; however, with increasing passages it slowly drifts with regard to its physiological responsiveness (Banker and Goslin, 1998)

The major advantage of using cell lines is the consistency and reproducibility of the results that can be obtained from using a batch of clonal cells (Freshney, 2000). If one wishes to study inflammation, then cell lines provide a way to learn how cells regulate certain mediatory processes such as signaling and transcription. This method also allows for new drug target identification and provides researchers an easy access to a source of unlimited numbers of cells from one individual that are all genetically identical.

Primary cell cultures

Primary cultures, which are obtained directly from the nervous tissue of an animal can maintain their differentiated state for a short period (days to weeks). Functionally differentiated primary cell cultures have a limited life span, and although maintenance of the differentiated properties can be improved by additives to the culture medium or components of the extracellular matrix, cell specific functions will eventually decline.

In primary cultures the initial cell population is extremely heterogeneous, and it is impossible to obtain a group of cells in culture that is completely representative of those in vivo. For some specific questions it may be desirable to have cultures of only one cell type, such as astrocytes or microglia. These cultures can be obtained from distinct brain regions by selection of donor optimal age. Once growing in culture, the cell type of interest can be selected by a variety of methods. (Banker and Goslin, 1998)

The benefit of primary cultures is that the cells have not been "modified" in any way (other than enzymatic or physical dissociation). Similarly to cell lines, primary cultures can be used to test the effect of various chemical compounds or drugs on specific cell types. The ability to provide a cell type-enriched culture offers a primary advantage over dissociated cell cultures, i.e. the possibility to make biochemical measurements on large numbers of cells rather than on a single cell.

The disadvantage of primary cultures is that, after a period of continuous growth, the cell characteristics can change and may become quite different from those found in the starting population (Freshney, 2000). Cells can also adapt to different culture environments (e.g. different nutrients, temperatures, salt concentrations etc.) by varying the activities of their enzymes. The mixed nature of each preparation, the limited lifespan of the culture and potential contamination problems are some of the other disadvantages associated with using primary cultures.

Co-cultures

Mixtures of two or more different kinds of cell types that are grown together are referred to as co-cultures. Co-cultures are used to study the interactions of different cell types and the utility of these systems has been well documented in the literature. One of the major advantages of co-culture system is the ability to investigate the molecular changes that occur as a result of cell-cell interactions (Freshney, 2000). For example, direct cell-cell contacts can be avoided by culturing two different cell types in different compartments of a culture well. In this kind of system, cells are grown on separate permeable supports but in the same culture medium interface, allowing study of functions such as transport and secretion. Although the "cross-talk" between cell types might be bidirectional and different from *in vivo*, this system provides extra information when cell based assays are applied. Similar to cell lines and primary cultures, co-cultures can also be easily manipulated by adding the compounds of interest into the culture medium. Modern assay techniques provide the ability to determine how potential compounds affect cell proliferation, cytokine production, cellular responses and to interpret and predict many biological properties of potential drug compounds.

Tissue slices

Slice cultures are normally prepared from infant animals. In these cultures, the age of the donor is a critical parameter determining the degree of organotypic organization achieved in the final preparation. Usually, tissue is obtained from rodents within the first week of life. By that time, a fair degree of tissue-specific cytoarchitecture has already been established and the peak of neuronal migration has passed. Within 2 to 3 weeks in culture, an original slice will thin to form a pseudomonolayer of cells and the degree of maturation and differentiation can be determined prior to the experimental manipulation. For *ex vivo* -studies, tissue can be obtained from any age of the animal; however, the maturation of the region of interest prior to sampling and interpretation of data must be considered. (Banker and Goslin, 1998).

Slice cultures prepared from the hippocampal region are perhaps the most widely used *in vitro* -method of organotypic tissue culture (Figure 5.). Although the hippocampus has significant longitudinal connections and inputs from various areas of the brain, which are disrupted during the transverse sectioning, the resulting slices still possess a complex laminar circuitry that remains functional during the culture time (Freshney, 2000). When compared to cell lines and co-cultures, this phenomenon provides possibility of studying cellular interactions in living tissue.

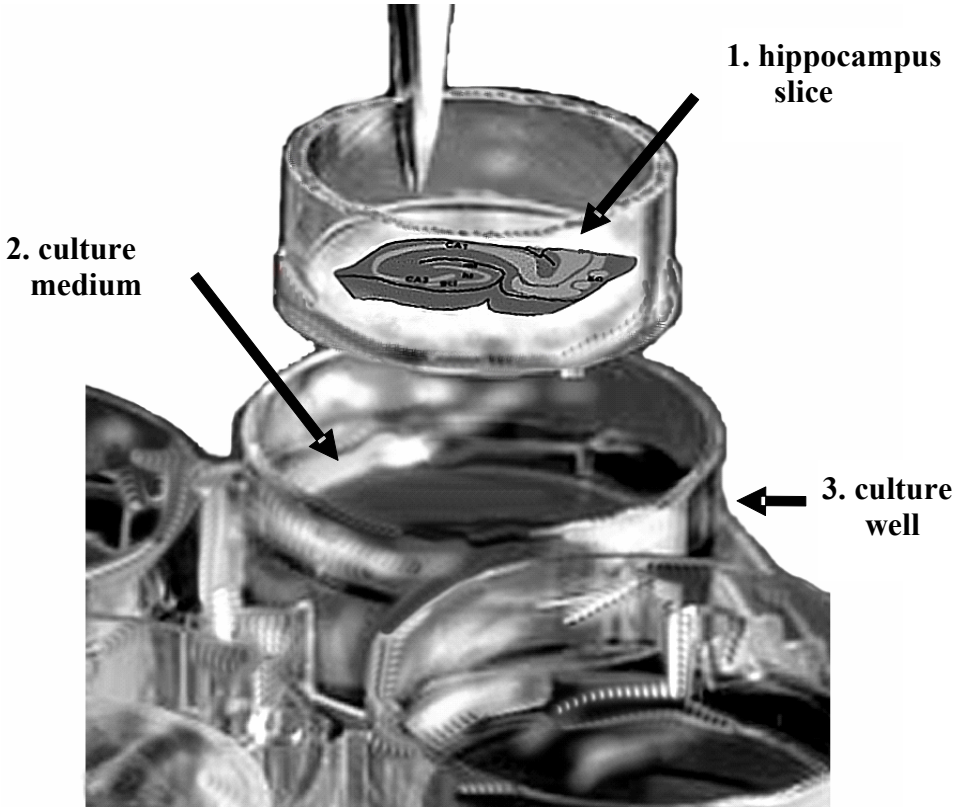

Figure 5.

A schematic drawing showing the principle of the organotypic slice culture system using the so called interface method;

1. Hippocampus slice is placed on a membrane of a culture insert.

2. Culture medium is added into the culture well to reach the level of the insert membrane.

3. Insert is placed inside the well.

3. AIMS OF THE STUDY

Inflammation in the brain attracts an unremitting stream of immune cells towards the injured area. To lessen the direct and/or bystander damage, microglia cells appear to pave the way for repair by releasing a complex array of chemicals and subsequently converting themselves into phagocytic macrophages that engulf dead cells. On the other hand, it seems that excessive inflammation can lead to a sequence of events where overactivated microglia become cytotoxic and thus worsen the outcome of inflammatory response.

The intracellular innate immune response involves post-translational modification of histones and transcription factors by histone acetylases and deacetylases. Since HDACs have been identified as playing a key role in the regulation of not only a number of inflammatory related diseases but also cancer, controlling the activity of these enzymes with specific inhibitors has fascinated scientists working in the field of drug development.

Different *in vitro* brain slice techniques have been introduced as representing "intermediate" models between cell cultures and *in vivo* -models to help to disentangle the complexity of neuroinflammation, but the efficacy of these models has seldom been reported.

Against this background we wanted to explore whether the extent of the inflammatory response could be affected by HDACs in different neural inflammation models. Moreover, we wanted to gain a better insight into the applicability of using the slice culture model in inflammation studies.

The specific aims were:

1. To refine and characterize the organotypic hippocampal slice culture model to suit the needs of neuroinflammation studies, and furthermore, to use the model together with other cell culture methods to study the impact of pro- and anti-inflammatory compounds, especially histone deacetylase inhibitors.

2. To investigate the effects of histone deacetylase inhibitors on inflammatory responses of microglia cells using the models mentioned above.

4. MATERIAL AND METHODS

4.1 Animals

The primary astrocytes and microglial cells used in Studies II and III were isolated from 1- to 2-day-old Wistar rat pups. Hippocampal cells used in Study II were isolated from 17-day-old Wistar rat embryos. Cerebellar granule cells were isolated from the cerebella of 7-day-old Wistar rats and postnatal day 7-8 (P7-P8) Wistar rat pups for organotypic slice cultures were used in Studies I-IV. All the animals were obtained from National Laboratory Animal Center (NLAC), University of Kuopio, Finland. The studies were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

4.2 Chemicals

To induce inflammation in Study I, the hippocampal slices were exposed to lipopolysaccharide (LPS from *E. coli* 055:B5, L6529, Sigma, St Louis, USA). To test whether the slices would respond to regulators of inflammation, a pro-inflammatory stimulus together with LPS was introduced by using a histone deacetylase inhibitor trichostatin A (Sigma, St Louis, USA). To test the response of an anti-inflammatory stimuli, we used helenalin (BIOMOL Research Laboratories, Plymouth Meeting, PA, USA), a known NF-κB inhibitor.

In addition to LPS and TSA, HDAC inhibitors suberoylanilide hydroxamic acid (SAHA, Alexis Biochemicals, Lausen, Switzerland) and 4-dimethylamino-*N*-(6-hydroxycarbamoyl-hexyl)-benzamide (M344, Alexis Biochemicals, Lausen, Switzerland) were used as proinflammatory stimuli in Study II and in addition to helenalin, another NF-κB inhibitor, caffeic acid phenethyl ester (CAPE, Sigma, St Louis, USA) was used. Pro- and anti-inflammatory compounds were tested in different cell and slice culture models.

In Study III we expanded our inflammation research and tested whether different short-chain fatty acids (SCFAs, purchased from Sigma), especially sodium butyrate, could regulate the inflammatory responses in different neural models. The involvement of protein acetylation and NF- κB binding activities were also studied.

Study IV supplemented the previous studies, a number of different Toll-receptor ligands and different inhibitors of signaling pathways together with TSA, okadaic acid and/or LPS/LPS Ultra Pure treatments were analyzed using N9 cell line and hippocampal slices.

Table 1. Summary of the compounds and their properties used in **Studies I-IV**. (For references, see text).

Compound	Property	Study I	Study II	Study III	Study IV
TSA	HDACi	x	x		x
SAHA	HDACi		x		
M344	HDACi		x		
Helenalin	NF-κB inhibitor	x	x		
CAPE	NF-κB inhibitor		x		
Butyrate	SCFA, HDACi			x	
Dexamethasone	Glucocorticoid, immunosuppressant				x
Valproate	SCFA			x	
Propionate	SCFA			x	
Valerate	SCFA			x	
Caproate	SCFA			x	
LY294002	PI3K inhibitor				x
Wortmannin	PI3K inhibitor				x
Pam3CSK4	TLR2 ligand				x
SLTA	TLR2 ligand				x
Zymosan	TLR2 ligand				x
Flagellin	TLR5 ligand				x
ODN1826	TLR9 ligand				x
IKK-2 IV	inhibitor of IKK-2 in NF-κB signaling				x
LPS (E.coli 055:B5)	endotoxin, induces inflammation, TLR2 and TLR4 ligand	x	x	x	x
Ultra Pure LPS (E. coli 0111:B4)	purified LPS, TLR4 ligand				x
Okadaic acid	inhibitor of PP1 and PP2A				x

4.3 Cell culture

Murine N9 microglia

The murine N9 microglial cell line was kindly provided by Dr. Paola Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy). N9 cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco) with 5% heat-inactivated fetal bovine serum (Gibco) on Nunc (Nalgene) dishes and 12-well clusters.

Rat primary astrocytes

Primary astrocytes were isolated from 1- to 2-day old Wistar rat as described earlier by Kerokoski et al. (2001). The cerebral cortices and midbrain were excised and the tissue without meninges and blood vessels was minced into small pieces. Cells were separated by trypsinization (0.025 % trypsin, 15 min, 37°C). After centrifugation and trituration, the tissue was filtered through 230 μm strainer. The cells were cultured in tissue culture flask in Dulbecco's modified eagle media (DMEM, Sigma) supplemented with 10 % heat-inactivated FBS, 2 mM glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Flasks for pure astrocyte cultures were shaken vigorously before each medium change, which was done first after two days and then after every four days. The astrocytes were detached by trypsinization after the culture bottles were confluent and used for both astrocyte and co-cultures.

Rat primary microglia

Primary microglial cells were isolated in the same way as astrocytes and they matured on the top of astrocytes. The culture flasks were not shaken until two weeks after the isolation, when the floating microglia were harvested from confluent astroglial layers by rotating the flasks on an orbital shaker for a few hours. Microglia were harvested from the same flasks 4-5 times every seventh day. After 1-2 hours of plating, the medium was changed to remove nonadherent cells. Cells were used for co-culture or pure microglia culture. Experiments were initiated 24 h after plating. The purity of the microglial cultures (over 95 %) was confirmed using antisera to CD11b (OX-42, Serotec, Oxford, UK).

Rat hippocampal cells

Hippocampal cells were isolated from 17-day-old Wistar rat embryos and cultured as described by Brewer et al. (1993). After preparing the hippocampus from embryonic brain, the cells were separated by papain treatment (0.5 mg/mL, 10 min, 37°C) followed by centrifugation and trituration. The cells were plated onto poly-D-lysine-coated (Sigma) plates in Neurobasal medium (Gibco) supplemented with 0.5 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 5% inactivated FBS. On the following day, the FBS-containing medium was substituted with B27-supplemented (Gibco) medium.

Rat cerebellar granule cells

Cerebellar granule cells were isolated from the cerebella of 7-day-old Wistar rats and cultured as described by Schousboe et al. (1989). After dissecting the cerebella out of the brains, the cells were separated by trypsin treatment (0.025 % trypsin, 15 min, 37°C) followed by centrifugation and trituration. The cells were plated onto poly-D-lysine-coated (Sigma) plates in the DMEM media supplemented with 10 % heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 25 mM potassium chloride (KCl). After two days in culture, the medium was supplemented with 10 μM 1-β-D-cytosine-arabinofuranoside (Ara-C, Sigma).

4.4 Co-cultures

In Study II, the hippocampal cells, the astrocytes and the microglial cells were co-cultured in Neurobasal medium using 24-well plates. Astrocytes were added four days after isolation and culturing of hippocampal cells. Microglia were added 3 days after the astrocyte-hippocampal culture. The co-cultures were developed 24 hours before experiments were initiated.

In Study III, we used a co-culture of cerebellar cells, astrocytes and microglial cells. Similarly as in Study II, astrocytes were added 4 days after isolation of cerebellar cells. Microglia were added 3 days after astrocytes and the experiments were initiated 24 h after addition of microglia. DMEM media supplemented with 10 % heat-inactivated dialysed FBS (Sigma), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 25 mM KCl without Ara-C was used as culture media and the cells were plated onto 24-well plates.

4.5 Slice culture

Organotypically cultured hippocampal slices used in Studies I-IV were isolated from P7-P8 rats and prepared using the modified interface culture method described by Stoppini et al., 1991. Wistar pups were decapitated, and the brains were rapidly dissected and placed in a petri dish in ice-cold 1x Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (Biowhittaker™, Belgium). The hippocampi of both sides were isolated and sectioned into 400-µm transverse slices with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Goose Green, UK). The slices were then carefully separated and transferred on to porous membrane inserts (one slice per insert) of 12-well culture plates (Transwell TR 3462; Costar, Corning, NY, USA). To reach the level of insert membrane, some 600 µL culture medium, consisting of Neurobasal medium with 1x B27-supplement (from Gibco), 1 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, was added to the lower compartment of each well and the culture plates were then placed in a 37°C humified incubator enriched with 5% CO_2. On the first day of culture, inactivated fetal bovine serum (Hyclone) was added to the culture medium at a concentration of 10%. On the next day, the culture medium was replaced with fresh medium without serum and from that time serum-free media was changed twice a week.

Table 2. Summary of the different culture methods applied in each study

Method	Study I	Study II	Study III	Study IV
Slice culture	x	x	x	x
Primary microglia		x	x	
Primary astrocytess		x	x	
Primary hippocampal cells		x		
Murine N9 microglia		x	x	x
Cerebellar granule cells			x	
Co-cultures		x	x	

4.6 Characterization of hippocampal slice culture model

Monitoring biochemical recovery and responses

First, some hippocampal slices were cultured for 7 days *in vitro* (7 DIV) and the culture medium was collected at 24h intervals during the culture period. LDH leakage to the culture medium was measured with a CytoTox 96 nonradioactive cytotoxicity assay kit obtained from Promega (Madison, WI, USA) and the nitrite concentration in the medium was measured by the Griess reaction. The concentrations of cytokines IL-6 and tumour necrosis factor (TNF)-α released into the medium were measured by an ELISA using OptEIA^{TM} kits or sets obtained from Pharmingen (BD Biosciences, San Diego, CA, USA).

Subsequently, samples of culture medium were collected at appropriate time points to further monitor the biochemical status of cultures at the one month time period.

Monitoring morphological recovery and responses

In Study I, some hippocampi were also rapidly sliced at 400 μm and immediately fixed with 4% paraformaldehyde for 1-2 h and rinsed three times with 0.05 M TBS-T, pH 7.6 (Tris-buffered saline + 0.1% Triton X-100) prior to staining to reveal the morphological status at the beginning of the culture. For microglia, hippocampal slices were stained with Alexa Fluor 488 conjugated fluorescent *Griffonia simplicifolia* isolectin IB_4 (Molecular Probes, Eugene, OR). IB_4 was applied at a concentration of 0.5 μg/ml in TBS-T and slices were incubated overnight at 4°C on a shaker. Before visualization, samples were rinsed with 0.1 M phosphate buffer and mounted on slides. We also performed immunocytochemical staining with a microglia marker OX-42, an antibody that recognizes type 3 complement receptors CR3 on mononuclear phagocytes. Primary antibody was diluted 1:20 000 with 0.05 M TBS-T, pH 7.6, slices were incubated for 1 week at 4°C on a shaker, rinsed thoroughly, followed by overnight incubation (4°C) with biotinylated secondary Ab (sheep anti-mouse, 1:1000, Serotec) and 2h incubation (room temperature) with avidin peroxidase (1:1000 ExtrAvidin E-2886, Sigma). The immunoreactive product was visualised with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB, 0.5 mg/ml, Sigma) in a nickel solution containing hydrogen peroxidase (25 μg/ml).

For epifluorescence immunodetection, primary antibodies to glial fibrillary acidic protein for astrocytes (GFAP: dilution 1:10 000; DAKO, Denmark) and doublecortin for neurons (DCX C-18: dilution 1:2500; Santa Cruz Laboratories, Santa Cruz, CA, USA) were used. After an overnight incubation with primary antibody at 4°C and thorough rinsing, 1:1000

diluted goat anti-rabbit Alexa Fluor 488 secondary antibody (Molecular Probes) to GFAP and 1:500 diluted biotinylated rabbit anti-goat secondary (Vector BA-5000; Burlingame, CA, USA) followed by tertiary antibody (goat anti-rabbit Alexa Fluor 488; 1:1000 dilution) to DCX were applied, respectively, also overnight at 4°C. All the antibodies were diluted with 0.05 M TBS-T, pH 7.6.

Subsequently, samples of slices were collected and stained at appropriate time points to further monitor the morphological status of cultures at the one month time period. To visualize the morphological integrity and both dead or dying cells and living cells, we used standard Nissl staining and the Live/dead-cytotoxicity kit (L-3224, Molecular Probes), respectively, according to the manufacturer's protocol.

4.7 Microscopy

Pictures from stained slices were taken with an Olympus DP50 microscope digital camera system connected to an Olympus BX40 microscope (Olympus Optical Co, Ltd, Japan) with appropriate filters. Except for adjustment to the contrast and brightness levels, no other manipulations were done to any of the images.

The purity of the cultures was checked with a Nikon Eclipse TE 300 Inverted microscope (Nikon Co, Tokyo, Japan)

4.8 Molecular biology

Electrophoretic mobility shift assays (EMSA) in Studies II and III were performed as described earlier in detail by Helenius et al. (1996). Protein-DNA binding assays were done with 5 μg nuclear protein. Double-stranded consensus and mutated oligonucleotides for activator protein (AP)-1 (Study II) and NF-κB binding sites (Studies II and III) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Radioactive bands were visualized with a Storm 860 PhosphorImager and pixel volumes of specific bands were calculated with ImageQuaNT 4.2a software (Molecular Dynamics).

For Northern blot analysis (Study II), some 4 μg of total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Gene-specific fragments for riboprobes were generated by PCR, cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and verified by sequencing. After hybridization, the membranes with the ^{32}P-labelled riboprobes (generated with the Strip-EZ kit, Ambion, Austin, TX, USA) were rinsed and the signals visualized with the Storm 860 PhosphorImager. Filters

were reprobed with ^{32}P-labelled 18S riboprobe in the presence of cold 18S in vitro transcripts and pixel volumes of specific bands were calculated with ImageQuaNT 4.2a software. For normalization and standardization, the intensities of specific bands were divided by the intensities of the 18S bands corresponding lanes.

The N9 microglial cells in Study IV were transfected with pTAL-Luc and pNFκB-Luc Vectors from Clontech Lab (BD Biosciences, Palo Alto, USA). pNFκB-Luc is a reporter vector designed for monitoring the activation of NF-kB signaling pathway. The pTAL-Luc vector served as a negative control for the background. For transfection, the recommended protocol (Roche, Penzberg, Germany) and FuGENE™ 6 Transfection Reagent was used. In brief, transfection with pTAL-Luc and pNFκB-Luc vectors (0.6 μg/ml) was done for 12h in culture medium after which LPS and drugs were added for 24h. Luciferase activity of the cells was assayed with Luciferase kit from Promega (Madison, USA).

4.10 Statistics

In Studies I-IV, the statistical analyses were conducted with SPSS software (program version 11.5.1 for Windows, SPSS Inc., Chicago, IL). The differences between control and treated groups were analyzed using non-parametric, 2-independent samples Mann-Whitney U-test with the p-values of 0.001, 0.01 and 0.05 as thresholds. All values are expressed as means ± SD.

Estimation of optimal sample size for slice cultures

To determine the minimum number of animals, yet appropriate sample sizes for slice culture experiments, we undertook power analysis calculations. First, based on our previous results, we estimated the effect size among different experimental units. Thereafter, the sample size was set to six per group and the significance level to 5% to determine the effect on statistical power. In order to reduce the within-group variation, we used a lognormal distribution and carried out the statistical power analysis calculations using nQuery Advisor® , version 5.0, (Statistical Solutions, Saugus, MA) computer program for Wilcoxon (Mann-Whitney) rank-sum test.

5. RESULTS

5.1 Characteristics of hippocampal slice culture model

Organotypic slice cultures from different brain regions have been introduced as an "intermediate" model between cell lines and *in vivo* models. One of the purposes of Study I was to gain insight into the behaviour of hippocampus tissue slices in refined, serum-free culture protocol.

Biochemical changes after explantation

The isolation procedure of rat hippocampal slices from postnatal brain causes trauma and acute inflammatory reaction due to removal of afferents and efferents and the transversal slicing of hippocampus.

In order to characterize how well the slices had recovered from the isolation, we collected the culture medium after every 24 h during the 7 DIV culture period and measured the secretion of IL-6, TNF-α and LDH leakage. The secreted level of IL-6 was highest at 48 h after explantation and returned to the basal level by 4 DIV. The peak levels of LDH and TNF-α were recorded at 1 DIV and reverted to control levels by 3 DIV. From that time, the levels of cytokines and LDH exhibited some variability but remained at rather low levels, implying that after 4-5 days of culture the hippocampal slices had largely recovered from the explantation procedure as assessed by these biochemical assays. (Study I, fig.1)

Morphological changes during culture period

Immediately after sectioning, microglia displayed an idiotypical "resting", ramified morphology with small cell bodies and numerous branching processes (Study I, fig. 7A). After 1 DIV, the microglia started to revert gradually into an intermediate, "reactive" form with larger cell bodies and several thicker branches (Study I, fig. 7B) and by 4-5 DIV a number of the IB4-positive cells appeared with their characteristic rounded, "amoeboid" phenotype, though also pleomorphic cells exhibiting projections were visible (Study I, fig. 7C-D). As the culture time extended, the morphological polymorphism of the microglial-cell population continued, e.g., all shapes of microglia from "amoeboid" like to "resting", ramified were found in all of the IB_4 labeled slices. The same phenomenon was seen when the slices were stained with OX-42 (Study I, fig. 8).

GFAP staining revealed the characteristic star-shaped morphology of astrocytes immediately after the explantation. In addition, the expansions of astrocytic processes, the "end-feet", enveloping the microvessels were clearly visible. (Study I, fig. 9A). After 2 DIV, GFAP-positive cells started to transform into fibrous cells with long processes (Study I, fig. 9B), eventually extending throughout the whole slice by 7 DIV.

The dentate area remained prominently covered with cells expressing doublecortin, showing the typical morphology of neurons (Study I, fig. 11A and B; inserts). DCX-positive cells were found, though in lesser numbers, also in the CA-areas.

5.2 Inflammatory responses in different culture models

LPS, the bacterial endotoxin, is a well characterized inducer of inflammation. We examined the basic inflammatory responses in our different models to determine which would best suit our purpose in the subsequent studies in which we wanted to regulate these responses.

Effect of LPS induced inflammation on slice cultures

To investigate whether the concentration of LPS had any effect on the slices during the 24h exposure, we pre-exposed the cultures to 0.1, 0.5, 1, 5 and 10 μg/ml of LPS at day 4. The LDH leakage did not differ significantly at any of these LPS-concentration levels and the control slices showed only a minimally lower level of LDH than the LPS treated slices (Study I, fig. 3A). The NO and IL-6 levels were clearly higher compared to control slices but LPS did not induce any prominent concentration-dependent effect in any treatment group (Study I, fig. 3B-C).

To address the question of whether the slice cultures would respond to LPS differently during the culture time, we added LPS to the medium at days 7, 14 and 21 *in vitro*. LPS evoked extensive secretion of IL-6 at all of the time points (Study I, fig. 4) in a similar manner as at 4 DIV (Study I, fig. 3C).

We also determined the temporal profile of LPS response by measuring the levels of IL-6, TNF-α, NO and LDH at different time points both at DIV 4 and 7. The secreted level of IL-6 was clearly increased after 6 hours and the highest levels were found after 48 hours both at 4 DIV and 7 DIV (Study I, fig. 5A). TNF-α secretion was already prominently elevated at 3 hours and was highest after 12 hours of exposure at 4 DIV. The pattern of TNF-α secretion at 7 DIV was similar to that seen at 4 DIV, but the overall levels were significantly lower and the value after 48 hours was higher than the value at 24 hour exposure time point. (Study I, fig. 5B). The NO levels at 7 DIV increased by degrees at the different time points but at 4 DIV

ascended more evenly (Study I, fig. 5D). The effect of LPS on LDH leakage was highest after 12 hours both at 4 and 7 DIV. The levels of LDH at 7 DIV were higher than the 4 DIV levels, but the overall values at both days remained rather low (Study I, fig. 5C). The DNA-binding activity of nuclear NF-kB complex was increased by LPS treatment (Study III, fig. 8).

Effect of LPS induced inflammation on cell culture models

Generally, in primary microglia, co-cultures and N9 cells, LPS induced a prominent, time and concentration dependent secretion of IL-6, TNF-α and NO (see e.g. Study II, fig.1-3) . It also upregulated the DNA-binding activity of the NF-kB complex in N9 nuclear extract (Study II, fig. 7-8 and Study III, fig. 4).

LPS treatment also induced AP-1 binding activity but to a lesser extent than that of NF-kB binding (Study II, fig. 9 and Study IV, fig. 5). Moreover, the effect of LPS could be detected also at the RNA level i.e. elevation of the levels of IL-6 and inducible nitric oxide (iNOS) mRNA expression (Study II, fig. 6)

5.3 Modulation of inflammatory responses

Acetylation and phosphorylation of proteins have been shown to diversely alter inflammatory responses. We wanted to elucidate whether different agonists and antagonists would affect the extent of LPS induced inflammation in different neural inflammation models.

HDAC inhibitors

Histone deacetylase inhibitors are recognized as inducers of protein hyperacetylation. We examined the effect of well-known HDACi against LPS induced inflammation and whether neurone-glia interactions were involved in this scheme.

Trichostatin A , a potent, reversible inhibitor of histone deacetylase, potentiated the LPS induced inflammatory response time dependently in N9 cells. This potentiation increased exponentially from 12 h to 24 h (Study II, fig. 1 and Study IV, fig. 2). These results indicated that the proinflammatory response to TSA was a late rather than an early event.

Similarly, a clear potentiation was observed in primary microglia (Study II, fig. 2), neural co-cultures (Study II, fig. 3A) and slice cultures (Study II, fig. 4, Study I, fig. 6A), demonstrating that the effect of TSA was not restricted to transformed N9 cells and that neurone-glia interactions did not significantly affect this response.

TSA also enhanced the expression levels of iNOS and IL-6 mRNAs of LPS-stimulated N9 microglia, but alone did not affect the expression levels of these cells (Study II, fig. 6). Also

the DNA binding activity of the NF- κB complex was not enhanced by TSA treatment (Study II, fig. 7-9).

Two other inhibitors of HDACs, SAHA and M344, were also able to induce pro-inflammatory potentiation of LPS-stimulated secretion of IL-6 and NO, but their effective concentrations were at the micromolar level (Study II, fig. 5.)

OKA

Okadaic acid, a naturally occurring polyether toxin, stimulates intracellular phosphorylation and protein translocation. It selectively inhibits protein phosphatases PP2A and PP1 and its hydrophobic backbone enables it to enter cells, thus making it a useful tool to study cellular processes that are regulated by phosphorylation (Shanley et al., 2001).

When we compared the okadaic acid and TSA induced proinflammatory responses we observed that OKA potentiated the LPS-induced IL-6 secretion in N9 cells already after 6 hours (Study IV, fig. 2), indicating that the response obtained via phosphorylation mediated signaling pathway was faster than that via acetylation mediated pathway.

Okadaic acid also potentiated the LPS induced IL-6 secretion in hippocampal slice cultures. In contrast to TSA treatment, the secretion of NO in N9 cells was clearly downregulated by okadaic acid treatment together with LPS exposure when compared to LPS alone but this downregulation could not be observed in slice cultures (Study IV, Fig. 2 b and supplementary material 1.).

Altogether these observations demonstrate that the signaling mechanisms behind hyperacetylation and hyperphosphorylation are different.

SCFAs

The anti-inflammatory effect of short-chain fatty acids, especially butyrate, have been suggested to be mediated via suppression of NF-kB activation. On the other hand, SCFAs have the ability to function as HDAC inhibitors.

When we examined the role of butyrate in our models, we observed that pretreatment with sodium butyrate induced a significant protection against LPS-induced inflammatory response in rat primary microglial cells and in co-cultures (Study III, fig. 5A and fig. 6). Instead, transformed N9 cells responded to butyrate treatment in exactly the opposite way. Whether exposed simultaneously to LPS or pretreated, butyrate potentiated the cytokine and nitric oxide secretion of these cells (Study III, fig. 1). Moreover, both its anti- and pro-inflammatory effects were concentration dependent.

Irrespective of whether hippocampal slice cultures were exposed to butyrate, either simultaneously with LPS or in pretreatment, the concentration of butyrate had a clear effect on the secreted levels of IL-6, NO and LDH leakage (Study III, fig. 7A). At the 5 mM concentration level, butyrate was clearly anti-inflammatory as judged by the expression of IL-6.

This diversity in results may be related to different properties of butyrate, i.e. either inducing protein hyperacetylation or inhibiting proteasome activity.

The expression of IL-6 protein as well as expression of IL-6 and iNOS mRNAs were also enhanced by butyrate in N9 cells. On the basis of LDH leakage, this response was not toxic to LPS-stimulated N9 cells.

Other short-chain fatty acids, propionate, valerate and caproate, enhanced the LPS-induced inflammatory response of N9 cells but neither a protective nor a proinflammatory response could be observed when hippocampal slices were exposed to these compounds.

5.4 Regulation of signaling pathways

Inflammation is highly regulated by different signaling pathways where acetylation and phosphorylation are key players of protein modifications. These reversible chemical modifications switch "on" and "off" the enzymes that control the activity of genes that are responsible for the production of pro-and anti-inflammatory end-products. In Study IV we tried to shed some light on some of the presently poorly understood mechanisms of the multitude of signaling regulators.

Toll-like receptors

LPS is recognized by TLR4 on the cell membrane of microglial cells but also TLR2 is activated (Laflamme et al., 2001, Olson and Miller, 2004) . To determine whether other receptors of the Toll family might be involved in the regulation of inflammation, we treated N9 cells with different Toll ligands in Study IV.

Treatment of N9 microglia with specific TLR2 ligands evoked no responses as judged by the secretion of IL-6. Flagellin, a specific ligand for TLR5 and a substance that can be found as an impurity in LPS products, increased the secretion of IL-6. This secretion was also strongly potentiated by TSA treatment. A specific ligand for TLR9, ODN1826 (Schluesener et al., 2001), also increased the secretion of IL-6 and TSA treatment potentiated this response. (Study IV, fig. 1).

When we compared the responses of Ultra-pure LPS and "normal" LPS, the IL-6 cytokine response was clearly lower with Ultra-pure LPS, though the potentiation obtained with TSA was very similar (Study IV, fig. 1).

These results suggest that N9 cells perhaps do not contain TLR2 and the effect of LPS is mediated through TLR4. Furthermore, it seems that protein acetylation affects the inflammatory response down-stream to the level of Toll-like receptor.

NF-κB

The NF-kB signaling pathway, which is connected upstream to Toll-like and cytokine receptors (Medzhitov and Janeway, 2001), is probably the best characterized key player in inflammatory responses. Recent studies have also shown that HDACs can acetylate also non-histone proteins, such as signaling proteins and transcription factors (Hubbert et al., 2002, Juan et al., 2000). In Studies I, II, III and IV, we addressed the question of how NF-kB might be involved in the regulation of inflammatory responses by treating cells and slices with several NF-kB- related compounds.

IKK-2 inhibitor IV, a specific inhibitor of IKK-2 (Podolin et al., 2005) and a compound able to inhibit the IKK-2 mediated, proinflammatory NF-kB signaling pathway, potently inhibited the un-potentiated LPS response as well as potentiated IL-6 responses, induced either with TSA or okadaic acid. IKK-2 inhibitor also inhibited the NO secretion in all treatments (Study IV, fig. 3).

Dexamethasone, a glucocorticoid that inhibits NF-kB signaling (Scheinman et al., 1995) and induces an anti-inflammatory response, totally blocked both the LPS-induced and TSA-potentiated responses both in N9 microglia and in hippocampal slice cultures (Study IV, fig. 3 and fig. 4)). Dexamethasone also inhibited the okadaic acid –induced secretion of IL-6 and NO from hippocampal slices (see e.g. supplementary material 1.).

The NF-κB inhibitors, CAPE and helenalin (Natarajan et al., 1996, Lyss et al., 1998) inhibited the nuclear DNA binding of the NF-κB complex and blocked the TSA induced potentiation of cytokine and NO responses of N9 cells (Study II, fig. 7 and fig. 8). The response of hippocampal slices to TSA and helenalin was similar to that seen in N9 cells, i.e. both IL-6 and NO levels were downregulated when helenalin was added to LPS/TSA-stimulated slices (Study I, fig. 6).

The LPS-stimulated NF-κB-binding capacity was downregulated when both N9 cells and hippocampal slices were treated simultaneously with butyrate (Study III, fig. 4 and fig. 8). This downregulation was observed also when N9 cells were pretreated with butyrate before LPS exposure.

TSA or okadaic acid treatments did not enhance the nuclear NF-kB DNA-binding activity although they induced a prominent potentiation of IL-6 secretion by LPS.

When N9 microglia were transiently transfected with the pNF-kB-Luc vector, the control pTal-Luc vector showed a minimal Luc reaction with all treatments but the NF-kB sites at the enhancer of the Luc vector potentiated the responses (Study IV, fig. 9B). Furthermore, TSA treatment also enhanced the expression of vector in control samples but especially in LPS treated N9 microglia, implying that the extent of the proinflammatory response attained by hyperacetylation is attributable to the enhancement of transactivation of the NF-kB driven inflammatory genes.

These results reveal that indeed NF-kB is involved in various stages of inflammatory responses. Since NF-kB inhibitors strongly inhibited the TSA induced potentiation, it can be concluded that NF-kB signaling plays a role in the HDACi induced inflammatory response. However, as TSA did not affect the DNA-binding activity but a potentiation of IL-6 and iNOS mRNA expression could be observed, it seems that the proinflammatory responses enhanced by HDAC inhibitors may be under the regulation of transcriptional activity.

PI-3K

The PI-3-kinase pathway has been shown to be involved in TLR-signaling and in the networks regulating NF-kB activation (Hazeki et al., 2006). In particular, this pathway plays a role in the NF-kB mediated anti-inflammatory responses induced by glucocorticoids. In Study IV, we examined the effects of some well-known PI-3-kinase inhibitors, especially LY294002, in N9 cells and hippocampal slices.

The PI-3-kinase inhibitor, LY294002 (Jung et al., 1999), inhibited the LPS-induced IL-6 secretion and blocked the TSA-induced potentiation of IL-6 secretion (Study IV, fig. 7A). The inhibitory effect of LY294002 was not restricted to N9 microglia but occurred also in rat primary hippocampal slice cultures. It strongly inhibited the LPS-induced increase in IL-6 and NO secretion and blocked the TSA-induced potentiation of IL-6 secretion (Study IV, fig. 8).

The DNA binding of the NF-κB complex in N9 microglia was not inhibited by LY294002 (Study IV, fig. 9A), but the LPS-induced as well as the TSA-potentiated transactivation of NF-kB-driven Luc reporter gene were both inhibited (Study IV, fig. 6). Moreover, okadaic acid induced potentiation of IL-6 secretion in N9 microglia was not affected by LY294002 (Study IV, fig. 7A).

Based on these findings it seems that PI-3K is involved in the regulation of "hyperacetylation" via NF-κB but the okadaic acid induced phosphorylation response is mediated by some other signaling system.

Other signaling

To search for other regulatory pathways and inhibitors for the pro-inflammatory potentiation induced by hyperacetylation, we also tested some additional well-known inhibitors of signal transduction.

We examined several inhibitors, such as H7 (10 μM, final conc.), H89 (1.0 μM), PMA (200 nM), JAK-3 inhibitor (1.0 μM), herbimycin (100 nM) and protein kinase Cζ pseudosubstrate inhibitor (1.0 μM) but none of these compounds significantly ($p<0.01$) altered the TSA-induced potentiation at non-toxic concentrations.

Remarks about the results:

There are some variations in the concentrations of different compounds tested with the different models. Some of the results show mild variance, most probably due to slight modifications in study designs occurring along the way. Nevertheless, all the results are in line to support the findings that the biological response to HDAC inhibitors was potentiation of LPS induced inflammation. Furthermore, hyperphosphorylation also potentiated this response, but the phenomenon occurred earlier. In addition, the observed potentiated response could be downregulated with specific blockers to NF-kB and PI-3K-mediated signaling .

The number of cells in N9 cultures were affected by the different treatments, especially when okadaic acid was introduced to cells. On the other hand, the treatments did not have any effect on the total protein levels in cultures (see e.g. supplementary material 2.).

6. DISCUSSION

6.1 Preface

What is the role of inflammation in neurodegenerative diseases? Or conjointly, what is the role of microglia in neuroinflammation? These kinds of questions are definitely of great scientific interest in the field of neuroinflammation, but at the same time it must be stated that the answers are far from clear, being largely unresolved and a topic of intense debate.

Terms like "a friend or foe", " the enemy within" and "double-edged sword" have been used when the role of microglia has been debated (Kempermann and Neumann, 2003, Lippoldt, Reichel and Moenning, 2005, Schenk and Yednock, 2002). The general assumption has been that the brain is in fact in an inflamed condition due to the overactive microglia which have been activated in the course of many neurological diseases. However, this view has been questioned and a different opinion proposed, i.e. the reason why microglia lose their ability to support neuronal functions is due to the fact that the senescence of these cells has occurred (Flanary, 2005). Nevertheless, much progress has taken place since the discovery of immunity by Mechnikov and his collaborators. Today the enigmatic nature of microglia as part of the brain's innate immune system is widely recognized. There is major interest on intracellular pathways e.g. down-stream regulators of the microglial inflammatory response. These studies have produced new information and many pieces have fitted into the jigsaw puzzle over the recent years. It may well be reasonable to state that the overall picture of microglial dynamics is beginning to take shape.

We used different *in vitro* culture models, to examine whether manipulation of protein acetylation and phosphorylation status could change the microglial inflammatory responses and, if so, which players might be involved in transmitting these signals. The results strengthen the view that the outcome of both pro- and anti-inflammatory effects is tightly regulated and that the feedback responses are dependent on both the transcriptional activity of the inflammatory genes and the receptor system complexes that activate the signals of innate immunity. Furthermore, modification of proteins seems to be an important mechanism regulating inflammation.

This discussion will focus on the interesting findings that inflammation can indeed be manipulated at different levels of a variety of signaling pathways, but before we will be able to design better anti-inflammatory drugs, a deeper understanding on the regulation of the intracellular signaling is needed.

6.2 Modulation of microglial response

Treating neurodegenerative disorders with anti-inflammatory drugs may ameliorate the disease progression by altering the microglial response, as has been shown in animal models (Sastre, Klockgether and Heneka, 2006). Modulation of microglial activity by altering the regulation of these cells' intracellular signaling may be beneficial in a number of ways, for example by enhancing their ability to remove apoptotic cells or to release proteins that protect cells and promote cell growth. However, microglia may also contribute to inflammation by releasing cytokines or attracting and recruiting immune-system cells that lead to inflammation in the long term.

The different neural inflammatory models revealed that an increase in protein acetylation leads to a proinflammatory response in transformed microglia as well as in cultured primary microglia. Since the potentiation of the inflammatory response after HDACi induction was observed also in neural co-cultures and hippocampal slice cultures, we conclude that the effect of the inhibitor (TSA) was not restricted to transformed cells and glial-neurone interactions did not, at least significantly, limit the response. This conclusion is supported by the recent findings of some other groups, for example, TSA has been shown to enhance LPS-induced expression of the Cox-2 gene in murine bone marrow-derived macrophages (Aung et al., 2006).

Similarly, enhancement of protein phosphorylation evoked a proinflammatory reaction but the response appeared as soon as 6 hours after exposure whereas at the same time TSA actually inhibited the LPS-induced response in IL-6 secretion. Up to the time point of 12 h, TSA caused a significant inhibition of IL-6 secretion but subsequently it induced an exponential increase in cytokine and nitric oxide production. When both acetylation and phosphorylation were enhanced simultaneously with compounds affecting the separate pathways, the inflammatory response showed synergistic potentiation. These findings clearly indicate that the overall acetylation and phosphorylation mediated immune responses are governed by different signaling pathways. Furthermore, we propose that the aforementioned potentiation of the inflammatory response by either "hyperacetylation" or "hyperphosphorylation" is attributable to the enhancement of transactivation of NF-kB driven inflammatory genes. Moreover, our studies imply that increase in the DNA-binding activity of NF-kB is necessary for the inflammatory response to occur, but the acetylation specifically seems to regulate the extent of the inflammatory response.

The proinflammatory responses could be inhibited by using blockers targeted against the different signaling pathways. For example, dexamethasone proved to be profoundly anti-

inflammatory against both TSA (acetylation) and okadaic acid (phosphorylation) induced potentiation, as well as against the LPS response itself. Furthermore, the TSA induced proinflammatory response could be blocked with the PI-3K inhibitor LY294002. Also NF-kB inhibitors such as helenalin and CAPE, and also butyrate, a SCFA, exerted anti-inflammatory properties by downregulating cytokine expression.

It has been previously shown that the site-specific acetylation of p65 component increases the transactivation capacity of NF-κB complex (Chen and Greene, 2003, Quivy and Van Lint, 2004, Rahman et al., 2004). Also, Natoli et al. (2005) have shown that even the recruitment of NF-κB complex to the promoters of selected inflammatory genes is highly regulated and dependent on MAP kinases and histone acetylation (enhanced by TSA exposure). In addition, Avdi et al. (2002) have proposed a model where PP2A has a central role in the regulation of the c-Jun NH2-terminal kinase pathway. Hence, okadaic acid induced inflammatory potentiation might be linked to the priming of chromatin to the NF-κB complex binding, since this response was inhibited by NF-κB inhibitor and dexamethasone.

6.3 Evaluation of models

The foundation for fundamental understanding of how cells communicate lies in the deconvolution of the signaling pathways. As the treatment of diseases can be implemented at various levels of signal transduction, it is essential for targeted drug discovery to understand the overall pathways and to have the right cell based models in which these pathways can be dissected.

In the present studies, the murine N9 microglial cell line represented a model with "quick'n easy" access to preliminary answers, i.e. the aim was to prioritize for further evaluation synthetic compounds showing selective inhibition or potentiation of the inflammatory response. The N9 cell line has been derived by retroviral immortalization of day 13 embryonic mouse brain cultures carrying an activated v-myc oncogene (Corradin et al., 1993). Such immortalized microglia can be maintained as a stable, substantially homogeneous cell line *in vitro* and they represent a good model for preliminary screening of new drug molecules aimed at treating inflammation. The advantages of these cells are that they express typical markers of resting mouse microglia and they may be stimulated, in a similar manner to primary microglia, to produce proinflammatory cytokines (Righi et al., 1989) and nitric oxide (Corradin et al., 1993). However, immortalized cell lines often have abnormal characteristics compared to non-transformed cells. For this reason, they are not good models of normal tissue.

In addition to the N9 cell line, we used different primary cultures and co-cultures of rat origin to further delineate the effects of the HDAC- and NF-κB-inhibitors. These intact cells provide a platform for measuring complex biological processes and screening for the effects of our drug candidates. By applying modern assay techniques, it is possible to monitor functions and analyze multiple-step pathways. Moreover, primary cultures and co-cultures can be used to determine regulation of gene expression in response to biological and pharmacological stimuli and to permit identification of new targets for drug discovery.

There is one potential limitation of utilizing these cells in serial experiments and long-term research. As a result of replicative senescence, these cells undergo progressive changes in morphology, proliferation and biological activity. Because of this limitation, many research laboratories prefer to use transformed cell lines for their studies.

To translate the findings from basic cellular research more feasibly, cell-based models need to mimic both the multicellular complexity and the 3-D organization of an organ. Therefore we extended our models to the tissue-specific 3-D organotypic slice culture of postnatal rat hippocampus. This model carries a significant advantage over 2-D models, i.e. since the laminar circuitry remains functional and the cytoarchitecture is fairly well preserved, these types of cultures offer the advantage of accommodating systematic experimental intervention in living tissue. Moreover, these tissue-specific organotypic models offer great potential for understanding the networks of cell-cell interactions involved in signal transduction. Thus, organotypic cultures allow for the design and development of drug therapies in an environment that mimics the situation *in vivo* better than other cell-based models.

In my opinion, the strength of this thesis is that we used all the aforementioned models in sequence in order to delineate the overall microglial responses to different modulations of inflammatory stimuli. As our findings demonstrate, the models correlate well, in that they provided results that are comparable and in line with each other. Therefore I postulate that these models, used in combination, are well suited for revealing different biochemical immune responses in the area of basic neuroinflammation research..

Despite the advantages of the different culture models, it is clear that *in vivo* studies and species extrapolation still remain a necessity. Animal model studies provide the only opportunity to evaluate the pharmacodynamic effects of a candidate anti-inflammatory drug in the whole-animal setting. Specifically, it is important to determine prior to clinical trials what the overall effect the drug has on the recovery when given during various stages of inflammation.

Nevertheless, to assist in answering these questions, validated cell-based models have a role to play in preliminary screening and only by using all of the available methods can accurate conclusions be drawn.

6.4 Aging and inflammation

Although many consider life extension as the desired outcome of longevity research, the pharmacologically-mediated prolongation of life is currently science fiction, thus making aging the ultimate challenge to quality of human life. Senescence is an irreversible state or process of aging characterized by a declining ability to combat infection and because of this, inflammation is the major risk factor for death in the elderly. Today, despite the fact that vaccines save the lives of more than 10 million people worldwide every year, bacterial infections are still responsible for nearly 20 % of all deaths. According to estimates by the World Health Organization WHO (The World Health Report 2004), nearly 11 million people worldwide still die each year from infections.

Some researchers regard aging itself as a disease but it could be described also as a "rusting path" we all experience. One common pathway involved in aging is inflammation. As we grow older, inflammation starts to "rust" our bodies and systemic inflammation can evoke devastating consequences throughout the body (Caruso et al., 2004, Clowes, Riggs and Khosla, 2005, Griffin,2006, McEwen et al., 2005, McGeer, Klegeris and McGeer, 2005).

Cellular senescence is a phenomenon where isolated cells demonstrate a limited ability to divide, yet remaining metabolically active, in the culture dish. Furthermore, they exhibit changes in morphology and function which may lead to age-related changes. As an example one only has to consider the difference between the supple skin of a child and the wrinkled skin of an old man.

Organismal senescence is the aging of organisms that is generally characterized by a declining ability to respond to stress, increasing homeostatic imbalance and increased risk of disease. Because of this, death is the ultimate consequence of aging.

Aging itself results in an increase in the levels of inflammatory cytokines and a decline in the ability to fight against infections (Krabbe, Pedersen and Bruunsgaard, 2004, Plowden et al., 2004). When the cytokine blood profile is examined in the aged in a weakened condition, an excess level of one or more of the inflammatory cytokines is usually found. A growing consensus among scientists is that many common neurological disorders, including Parkinson's and Alzheimer's diseases, are all caused in part by a chronic inflammatory

syndrome. Also people with multiple degenerative disorders exhibit excess levels of pro-inflammatory markers, e.g., TNF-a, IL-6, IL-1(b), or IL-8, in their blood.

Furthermore, high levels of several inflammation-related markers are associated with diseases/syndromes such as insulin resistance, type 2 diabetes and obesity (Ahmad et al., 2006, Gogia and Agarwal, 2006). These levels have been shown to decline in association with increased levels of anti-inflammatory molecules after consumption of a low calorie diet (Clement et al., 2004). Interestingly, caloric restriction (CR) has been shown to be one of the most effective means of slowing the pace of aging and extending lifespan in different laboratory animals, ranging from worms and flies to rats and fish, possibly even non-human primates (Barrows and Kokkonen, 1982, Weindruch and Walford, 1988, Kemnitz et al., 1993, Bodkin et al., 1995, Lane et al., 1998). However, whether long-term calorie restriction with adequate nutrition slows aging in humans remains to be seen.

The signaling pathways suggested to be the responsible mediators of the "anti-aging" effects induced by CR are the same that regulate inflammation. For example, upregulation of NF-κB is suppressed by CR via inhibition of IκB kinase activation (Chung et al., 2002). The beneficial effects of CR have also been linked to PI-3/AKT kinase and MAPK pathways (Gan et al., 2005, McCurdy, Davidson and Cartee, 2005). Although the mechanisms of actions are poorly understood, it seems possible that CR exerts these effects by preserving anti-oxidative and other defense mechanisms through these mediators.

Based on animal models, many candidate genes have been proposed as having distinct influences on lifespan. Most of these genes are involved in immune-system regulation or metabolic processes, for example the age-1 gene (encoding the PI-3K), identified from the nematode *Caenorhabditis elegans*, shares homology with the mammalian insulin-IGF1-pathway which eventually targets a transcription factor that regulates innate immunity. Two other genes, called methuselah (*Drosophila melanogaster*) and Indy (I'm not dead yet, *Drosophila melanogaster* and *Xenopus oocytes*) are also under intensive investigation. Mutations in these genes have been shown to result in an increase in the average lifespan of fruit flies, possibly as a consequence of altered energy metabolism (Knauf et al., 2006, Lin et al., 1998). Furthermore, results from a population-based genetic study of Swedish elderly indicate that a common polymorphisms in apolipoprotein E (APOE) gene have an influence on lifespan (Corder et al., 1996).

The recently described "longevity genes", sirtuins (the class III HDACs) (Guarente and Kenyon, 2000), have raised hopes of finding new ways to fight against aging related diseases linked to inflammation. These are enzymes that are found in almost all life forms from yeasts to mammals and they apparently play critical roles in a number of life processes. Some studies have indicated that low calorie diets increase sirtuin activity (Chen et al., 2005a, Guarente, 2005), thus linking metabolism and aging. Increased activity of these enzymes is thought to induce defensive, survival programs in cells to allow the organism to battle against a stressful environment. Indeed, it has been shown in mixed cortical cultures containing neurons, astrocytes and microglia, that the overexpression of class III HDAC, SIRT1, inhibits NF-κB signaling and suppresses amyloid-β mediated toxicity (Chen et al., 2005). Furthermore, the same study revealed that addition of the SIRT1 activator, resveratrol, a polyphenol compound found in red wine, to the amyloid-β treated cultures increased the survival of neurons in the presence of microglia. Interestingly, resveratrol has been found to increase the lifespan in *C. elegans* by 14% and in *D. melanogaster* by 29% (Howitz et al., 2003, Wood et al., 2004).

The idea behind the quest for finding new therapies using sirtuins seems to be the same as with other HDACs - by specifically manipulating the activity of these enzymes one could either "switch on" or "shut down" the target genes and restore the appropriate levels of the specific protein involved.

6.5 Therapeutic perspectives

It has become increasingly clear that abnormalities in the HAT-HDAC balance, phosphorylation status and/or in the regulation of NF-κB pathway are involved in the pathogenesis of several inflammatory related diseases. Thus, in general, the therapeutic potential lies behind the intervention and the consequent restoration of the impaired homeostasis caused by these mechanisms.

Currently ongoing phase I/II clinical trials of several different "first generation" HDAC inhibitors, particularly in the field of oncology, have shown some promise but also adverse effects, such as unwanted side effects have been detected (Minucci and Pelicci, 2006). Since they are relatively unselective in their abilities to block the different classes of HDACs, their benefit is probably derived from general repression in gene activity followed by hyperacetylation. On the other hand, the outcome of using HDAC inhibitors might vary, e.g. depending for example on the different cell types or target tissue. Indeed, as we observed in Study III, butyrate (pre)treatment induced a proinflammatory response in transformed N9

cells, but in contrast was anti-inflammatory in primary microglial cells and in hippocampal slice cultures.

Trichostatin A has been shown to provoke anti-inflammatory responses by splenocytes in a knockout mouse model of systemic lupus erythematosus (Mishra et al., 2003) and in cultured murine mesangial cells (Yu et al., 2002), as judged by the production of IL-6 and NO. There is also *in vivo* evidence that SAHA can reduce LPS induced levels of circulating IL-6 and TNF-α (Leoni et al., 2002). Instead, in our models both TSA and SAHA were clearly proinflammatory. This might seem to preclude the use of these inhibitors for the treatment of neural inflammation. On the other hand, in N9 cells, TSA seemed to be anti-inflammatory up to 12 h before "reverting" to being proinflammatory. Furthermore, we have also observed an anti-inflammatory feedback response when pretreating microglial cells with a low dose of TSA before LPS exposure (unpublished results). These inducing or inhibitory effects of TSA therefore seem to depend on the treatment protocol.

A further prospect for possible therapeutic intervention is the cross-talk between phosphorylation and modulation of HDAC activity. Even though these interactions are not yet well understood, the research in this field represents a fascinating area of drug research and development. Thus, one of the true future challenges will be to learn the nature of the combinatorial HDAC-protein-DNA interactions, and furthermore, to understand how these interactions vary in different tissues and cell types. In fact, during our studies we found that the simultaneous treatments with TSA and okadaic acid induced an additive response in IL-6 secretion in LPS-treated hippocampal slice cultures (unpublished results). Furthermore, both the TSA and okadaic acid induced responses were blocked with dexamethasone, implying that there is a possibility to modulate the synergistic effect of the inducers. Hence, a better understanding of the regulation of the prolonged activation of microglial NF-κB pathway may lead to the discovery of new types of blockers to treat chronic inflammatory conditions. On the other hand, suppression of NF-κB activity for long periods may turn out to be a ill-advised strategy since microglia play such a crucial role in the host defense responses.

Nevertheless, the research with these "first generation" HDAC inhibitors has not been in vain. Expanding understanding gained from previous studies has paved the way for the development of the "second generation" inhibitors that will target specific HDAC isoenzymes (Ward et al., 2003). These selective inhibitor candidates hopefully will target the transcription of inflammatory related genes with improved specificity and, safety.

Though still in its infancy, identification of new molecules that affect the HDAC complexes is likely to lead to new therapies to treat a variety of inflammatory related diseases.

6.6 Concluding remarks

The Good Side of Inflammation

The inflammatory response to tissue damage is of enormous value. By isolating the damaged area, mobilizing effector cells and molecules to the infection site, and — in the late stages — by promoting healing, inflammation acts to protect the body. In fact, our immune system has evolved to control pathogens, so proinflammatory responses are likely to be evolutionarily programmed to resist fatal infections.

The Bad Side of Inflammation

Often the inflammatory response is out of proportion to the threat it faces. In that case, the result can be more damage to the body than the agent itself would have produced. The many types of allergies as well of the autoimmune diseases are typical examples of maladaptive inflammation in response to what should have been a harmless, or at least a noninfectious, agent.

Treating Inflammation

Today, inappropriate inflammation is treated with steroids like the glucocorticoid cortisol and with nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin or ibuprofen. While these NSAIDs may slow the progression of some diseases, the feedback loops in the brain are clearly too complicated to allow for such simplistic approaches e.g. as needed in the treatment of such multi-factorial diseases like Alzheimer's disease.

Studying the mechanisms of histone deacetylase inhibitors may offer the potential to develop better targeted drugs to treat different inflammatory diseases in the future. Perhaps most importantly, however, these drug candidates may be pioneers in the development of inhibitor therapies with synergistic efficacy, and highlight the need for obtaining a comprehensive perspective of the potential therapeutic drug target before effective treatments can be developed.

7. CONCLUSIONS

Protein acetylation and phosphorylation, two major intracellular modification systems, are clearly important factors contributing to the regulation inflammatory responses also in the brain. Moreover, the brain can no longer be considered as an immunoprivileged organ and innate immunity, the first line of defence, serves and protects the brain, this being mediated through the resident immune cells of CNS, the microglia.

The following conclusions can be drawn from this series of studies:

1. The axis from cell membrane Toll-receptors to the production of a number of inflammation-related proteins involves an intracellular signaling machinery that is time and dose dependently regulated by phosphorylation and acetylation.

2. Microglial cells, the resident immune cells of the CNS, seem to be intimately involved in the regulation of the inflammatory responses. These responses could be modulated by using compounds that affect the acetylation and phosphorylation status of proteins.

3. The methods and models applied in these studies are suitable for the preclinical studies of potential drug candidates aimed at repressing excessive inflammation

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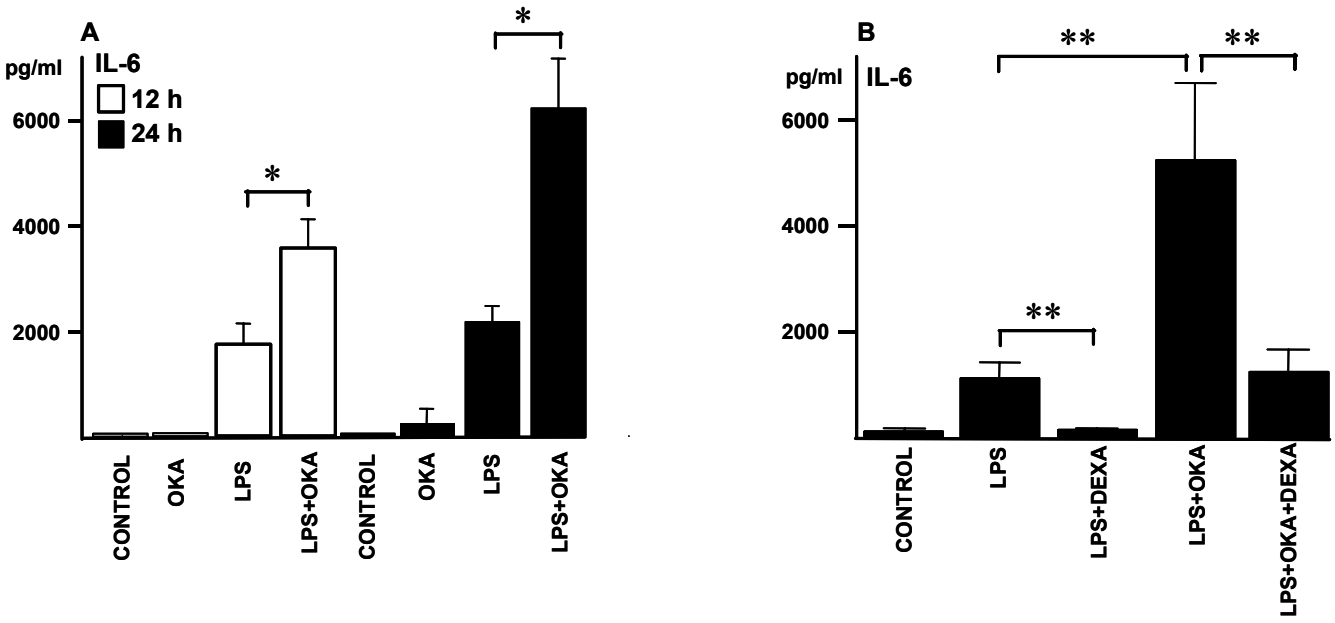
APPENDIX I: Supplementary material to results

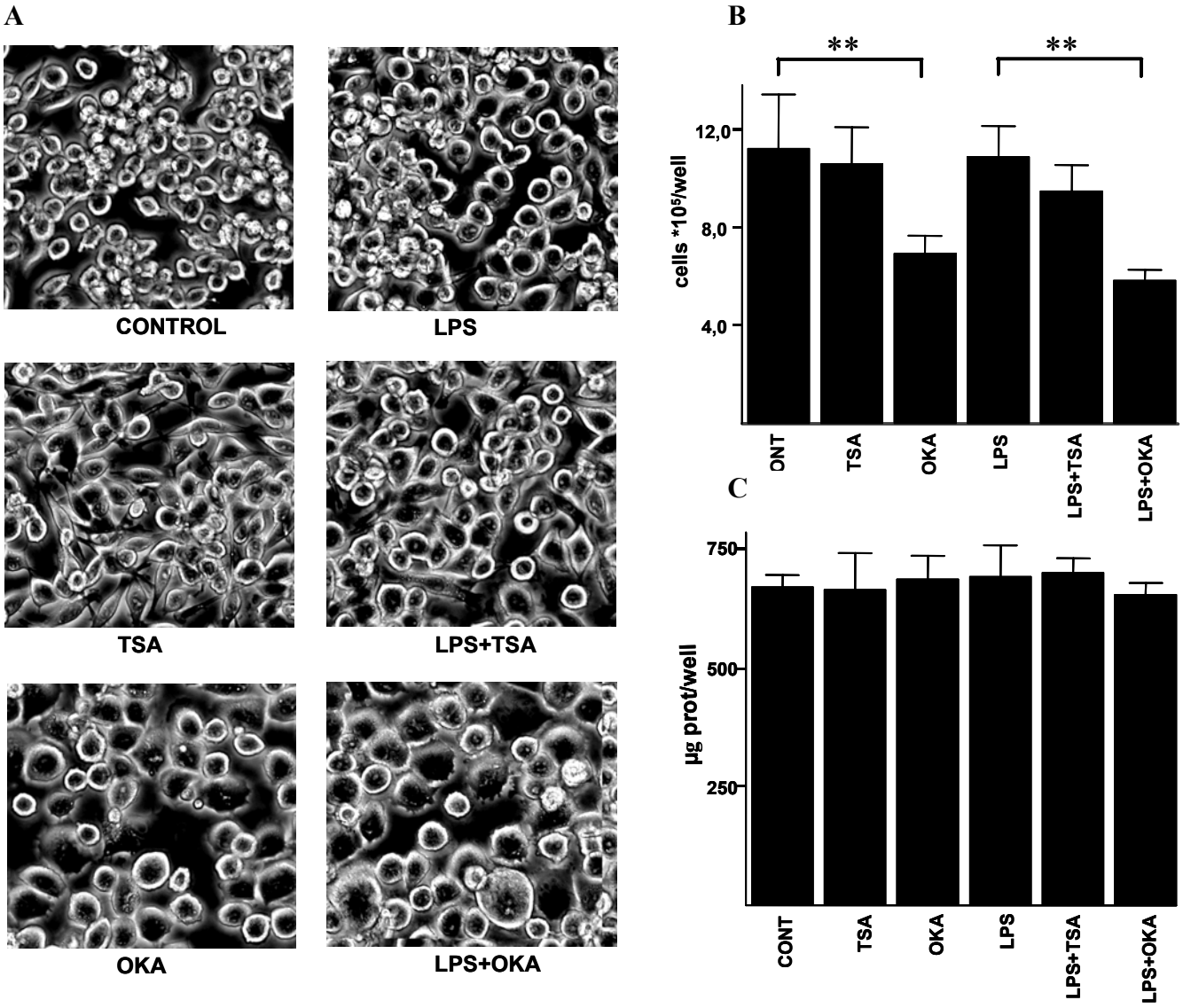
Supplementary material 1.



A. Okadaic acid potentiation of LPS induced IL-6 expression on hippocampal slices at 4 DIV after 12h and 24h of exposure. LPS concentration was 5 µg/ml and that of okadaic acid 20 nM. * $p < 0.05$

B. The effect of dexamethasone on LPS alone and LPS+okadaic acid induced IL-6 expression on hippocampal slices at 4 DIV after 24h of exposure. LPS concentration was 5 µg/ml and that of okadaic acid 30 nM. ** $p < 0.01$

Supplementary material 2.



The effect of different treatments on the (A) morphology, (B) number of cells and (C) the amount of protein in N9 cells. After the introduction of okadaic acid, the cells arrested their proliferation and cell size increased, which in turn seemed to affect the number of cells/well. Instead, the protein levels remained similar despite the different treatments. The treatment time was 24 h and concentrations were 40 nM for okadaic acid, 20 nM for TSA and 5 µg/ml for LPS.

APPENDIX II: ORIGINAL PUBLICATIONS (I-IV)

I

A refined in vitro model to study inflammatory responses in organotypic membrane culture of postnatal rat hippocampal slices.

Huuskonen J, Suuronen T, Miettinen R, van Groen T, Salminen A.

J Neuroinflammation. 2005;2:25

II

Regulation of microglial inflammatory response by histone deacetylase inhibitors.

Suuronen T, Huuskonen J, Pihlaja R, Kyrylenko S, Salminen A.

J Neurochem. 2003;87:407-16.

III

Regulation of microglial inflammatory response by sodium butyrate and short-chain fatty acids.

Huuskonen J, Suuronen T, Nuutinen T, Kyrylenko S, Salminen A.

Br J Pharmacol. 2004;141:874-80.

IV

Characterization of the pro-inflammatory signaling induced by protein acetylation in microglia.

Suuronen T*, Huuskonen J*, Nuutinen T, Salminen A.

Neurochem Int. 2006; 49:610-618.

PUBLICATIONS

SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

1. **Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
2. **Eeva Leino (1981):** Clinical and biochemical studies on progressive myoclonus epilepsy.
3. **Hilkka Soininen (1981):** Senile dementia. A clinical, neurochemical and etiological study.
4. **Rolf Danner (1982):** Adverse effects of anticonvulsive treatment on peripheral nerve conduction and posterior dominant EEG rhythm.
5. **Markku Saksa (1982):** The autonomic nervous system in experimental allergic neuritis. A functional, morphological and biochemical study.
6. **Juhani Sivenius (1982):** Studies on the rehabilitation, epidemiology and clinical features of stroke in East Central Finland.
7. **Asla Pitkänen (1987):** Somatostatin in epilepsy. An experimental and clinical study.
8. **Esa Mervaala (1987):** Evoked potential in human epilepsy. A neurophysiological study.
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