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UNDERSTANDING EPIGENETIC MECHANISM IN CNS DISORDERS: HISTONE MODIFICATION REGULATES LPS-INDUCED INFLAMMATORY TRANSCRIPTIONAL RESPONSE

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Emerging evidence suggests that features of the environment modify gene expression through epigenetic processes independently of the primary DNA sequence. Accordingly, dysfunction in epigenetic mechanisms offers a plausible mechanism by which neurons adapt their transcriptional response to environmental cues, such as immune stimuli or stress (Toyokawa et al., 2012). Epigenetic mechanisms represent a form of cellular memory that contributes to either short- or long-term changes in neuronal function in response to a variety of behavioral experiences, environmental factors, and pharmacological stimuli. Thus, providing a unique mechanism for the molecular tagging of individual cells or sets of cells at the level of the epigenome and the possibility to identify experience-dependent, cell-specific plasticity. Genetic polymorphisms in a large number of genes quantitatively and qualitatively affect immune and inflammatory responses altering the susceptibility to or course of a large number of central nervous system (CNS) diseases with an inflammatory component (Wilson, 2008). For example, in the presence of a peripheral infection, the innate immune system produces inflammatory cytokines that act on the brain and cause sickness behavior. When activation of the peripheral immune system continues, such as during systemic infections, cancer or autoimmune diseases, the ensuing immune signaling to the brain moves from causing sickness behavior to the development of symptoms of depression in vulnerable individuals (Dantzer et al., 2008).

Our research is focused on the epigenetic mechanisms occurring in such pathophysiological processes as neuroinflammation. In particular, this event was modeled by administering systemic bacterial lipopolysaccharide (LPS) which induces sickness behavior, but more importantly, LPS induces a massive transcriptional CNS response, mediated by different pathways, responsible for changes in the allostasis of the brain. These changes then may ultimately be at the onset of various CNS disorders induced by epigenetic changes in immune system activation.

1. INTRODUCTION

1. EPIGENETICS

The term *epigenetics* was used for the first time by Waddington to describe the conceptual solution to a puzzle that arises as a fundamental consideration in development biology (Waddington et al., 1957). All the different cells in the body have exactly the same genome, that is, the same DNA nucleotide sequence, with only a few exceptions in the reproductive and the immune system; however, these types of cells clearly are vastly different in term of the gene products that they generate (Sweatt, 2009). In his paper, Sweatt described a mechanism that was "beyond" the level of genes encoded by a DNA sequence that controlled the DNA readout.

The term 'epigenetic' refers to the information contained in chromatin, that defines a stable and heritable, but yet dynamic and reversible, specific gene expression pattern and is responsible for the complex epigenetic network that controls gene expression programs in higher eukaryote. The total sum of all potentially heritable changes in gene expression that do not involve changes in the DNA sequence is termed the 'epigenome' (Jaenisch et al., 2003). The epigenome is the result of a complex interplay between enzymes that modify DNA and histones, proteins that can recognize these modifications, sequence-specific and nonspecific DNA binding factors, scaffold proteins, non-coding RNAs, the chromatin structure and the organization of the genome in the nuclear space. The epigenome plays an essential role in the regulatory mechanisms that define the transcriptome; hence, the analysis of the epigenome and the transcriptome can define cell type, its physiological state, and pathological stage in a disease (Talese et al., 2013). Unlike the genome, the epigenome is highly variable between cells and fluctuates in time according to conditions even within a single cell. There are, therefore, at least as many epigenomes as there are cell types. Epigenetic mechanisms assure precise transcriptional responses to intrinsic and extrinsic signals and enable the storage of regulatory information in the genome even as the signals have subsided. Perturbation of the epigenetic balance may lead to alterations in gene expression, for instance, resulting in cellular transformation and malignant growth (Bird, 2007; Santos-Rosa et al., 2005). New concepts have recently emerged about chromatin remodeling and gene regulation. Transcription is not occurring on naked DNA but rather in the context of chromatin, providing an additional level of complexity yet to be fully understood.

In eukaryotic cells, changes in chromatin structure are achieved mainly by three distinct mechanisms: DNA methylation, histone post-translational modifications and ATP-dependent chromatin remodeling.

1.1 Histone modifications

In the early 1960s, evidence accumulated that both histone and globular domains were subject to a vast array of post-translational modifications (PTMs) that represent enormous potential for functional responses and may play a role in RNA synthesis (Allfrey et al., 1964). Histone proteins are subjected to a diverse array of covalent modifications that occurs primarly, but not exclusively, at amino (N-) and carboxy (C-) terminals (i.e., tails). The tail regions of core histones contain flexible and highly basic amino-acid sequences that are generally conserved across eukaryiotic organisms, and it is well established that histone tails act as substrates for several types of post-translational modifications, including methylation of arginine (R) residues; methylation (Zhang et al., 2006), acetylation (Sterner et al., 2000), ubiquitination (Shilatifard, 2006; Davie et al., 1990), ADP-ribosylation (Hassa et al., 2006; Huletsky et al., 1985), sumoylation of lysines (K) (Nathan et al., 2003); and phosphorylation of serines (S) and threonines (Thr) (Nowak et al., 2004; Kouzarides, 2007; Cosgrove et al., 2004) (Figure 1). There are over sixty different residues on histones where modifications have been detected either by specific antibodies or by mass spectrometry. Such modifications have long been correlated with various nuclear functions including replication, chromatin assembly and transcription.

Histone modifications regard histone N-terminal tails and the globular nucleosome core (Sarma et al., 2005; Berger, 2001). This manipulation of chromatin via the addition of functional groups to histone tails serves two main purposes: (i) to provide recruitment signals for non-histone proteins involved in transcriptional activation and silencing (Kouzarides, 2007; Taverna et al., 2007) and (ii) to relax chromatin by disrupting contacts between nucleosomes as well as interactions between histone tails and genomic DNA (Kouzarides, 2007).

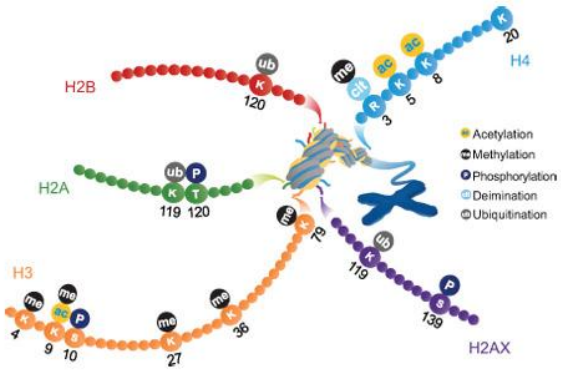


Figure. 1: Schematic of the most common histone modifications. The modifications on human histones include acetylation, methylation, phosphorylation and ubiquitination.

Lysine (K) is a key substrate residue in histone biochemistry, because it undergoes many exclusive modifications, including acetylation, methylation, ubiquitylation and sumoylation. Acetylation and methylation involve small chemical groups, whereas ubiquitination and sumoylation add large moieties, two-thirds the size of the histone proteins themselves, which may lead to more profound changes in chromatin structure. Another degree of complexity is that methylation can occur several times (mono-, di-, trimethylation for lysines and mono- or di- (asymmetric or symmetric) methylation for arginines) and each type of modification has different biological outcomes. For example, there is abundant evidence that acetylation is activating transcription, whereas sumoylation seems to be repressing transcription, and these two types of modifications may interfere reciprocally. By contrast, methylation and ubiquitination have variable effects, depending on the precise residues and contexts. For example, trimethylation of lysine 4 in histone H3 (H3K4me3) typically displayes peak levels at the 5' region of actively transcribed genes, shows strong positive correlations with transcription rates, activated RNA polymerase II occupancy, and histone acetylation level; on the contrary, H3K9me3 occurs in compact pericentromeric heterochromatin, which is transcriptionally inert. Two ubiquitination sites in the C termini of H2B and H2A correlate with active and repressed transcription, respectively. In H3 and H4, arginine residues can also be mono- or di-methylated, and in the latter case the methyl groups are placed symmetrically or asymmetrically on the side chain. Arginine methylation seems to be strictly activating transcription. Serine/threonine phosphorylation is also involved in transcription: phosphorylation of Serine 10 on histone H3 correlates with both activated transcription and mitotic chromosome condensation, thus, by both opening and closing chromatin, which illustrates the importance of genomic context (Berger, 2007). Extensive literature documents an elaborate collection of PTMs within the N-terminal tails of histone proteins: since their identification decades ago, histone modifications have been proposed to have a number of different functions. First, with the exception of methylation, histone modifications result in a change in the net charge of nucleosomes, which could loosen inter- or intranucleosomal DNA-histone interactions.

Second, it is well accepted that protein modifications can be recognized by other proteins (Seet et al., 2006). Given the diversity of covalent modifications, it has been proposed that individual histone modifications or modification patterns might be read by other proteins (called "readers", see Ruthenburg et al., 2007b) that influence chromatin dynamics and function (Jenuwein et al., 2001; Strahl et al., 2000; Turner, 2000).

Third, some modifications directly influence higher-order chromatin structure: for instance, acetylation of H4K16 inhibits the formation of compact 30 nm fibers (Shogren-Knaak et al., 2006).

The abundance of modifications on histone tail makes ‘‘crosstalk’’ very likely (Fischle et al., 2003 and 2008).

Comunication can occur between modifications on the same histone tail: in gene activation, phosphorylation of histone H3 on S10 facilitates acetylation of K14 and methylation of K4, resulting in an open chromatin conformation (Lo et al., 2001; Cheung et al., 2000b). Phosphorylation of S10 also facilitates acetylation of K9, thereby preventing the repressive K9 methylation marks (Rea et al., 2000). Moreover, K4 methylation on histone H3 facilitates acetylation by creating a specific binding site for the chromodomain containing protein Chd1, component of an HAT complex (Pray-Grangt et al., 2005).

Cross-talk can take place even between modifications on different histones: for example, ubiquitination of histone H2B on K123 is required for an efficient methylation of K4 and K79 on histone H3, both involved in transcriptional activation (Sun et al., 2002). Mechanistically, such communication between modifications may occur at several different levels.

The binding of a protein could be disrupted by an adjacent modification: phosphorylation of H3S10 affects the binding of HP1 to methylated H3K9 (Fischle et al., 2005). The catalytic activity of an enzyme could be compromised by modification of its substrate recognition site and, finally, an enzyme could recognize its substrate more effectively in the context of a second modification: the GCN5 acetyltransferase recognizes H3 more effectively when it is phosphorylated at H3S10 (Clements et al., 2003).

1.1.1 Histone methylation

Histone methylation mainly occurs on the side chains of two amino-acids: lysine and arginine. Unlike other histone modifications, methylation does not alter the charge of histone protein.

Lysine residues can be differently methylated: the ‘‘degree’’ of histone lysine methylation matters, with significant differences in large-scale patterns of mono-, di-, and trimethylation at specific lysine residues, whether activating or repressing (Barski et al., 2007). Different lysine positions can be methylated in the N-terminals of histones: on H3 (K4, K9, K27, K36) and H4 (K20), while another one occurs in the histone-fold domain of histone H3 (K79) (Lan et al., 2009).

Lysine methyltransferases have very high specificity compared to acetyltransferases: they usually modify one single lysine on a single histone and their output can be either activation or repression of transcription (Kouzarides, 2002; Hublitz et al., 2009; Lachner et al., 2002 and 2003; Martin et

al., 2005; Sims III et al., 2003; Beaujean, 2002). Histone lysine methylation has been shown to induce transcriptional activation. The main sites of lysine methylation that have been associated with gene activity include K4 and K36 on histone H3. Interestingly, the methylation of both sites seems to be directly linked to the transcriptional process. The location and timing of H3K4 methylation during gene activation led to the prediction that complexes recruited by this histone modification are involved in transcriptional activation and gene induction (Table 1).

Histone residue	Transcription
H3-lysine 4 (di- and tri-methyl)	Activation (peak levels aroud transcription start sites)
H3-lysine 4 (mono-methyl)	Activation (peak levels mostly at enhancer sequences)
H3-lysine 9 (di- and tri-methyl)	Repression (peak levels in heterochromatin, DNA repeats, but found at promoters and other sequences)
H3-lysine 9 (mono-methyl)	Activation
H3-lysine 27 (di- and tri-methyl)	Repression (peak levels aroud transcription start sites)
H3-lysine 27 (mono-methyl)	Activation
H3-lysine 36 (tri-methyl)	Activation (peak levels within gene coding and non-coding sequences)
H3-lysine 79 (tri-methyl)	Repression (?)
H3-lysine 20 (di- and tri-methyl)	Repression (peak levels in heterochromatin, DNA repeats, but found at promoters and other sequences)
H3-lysine 20 (mono-methyl)	Activation

Table 1. Overview of histone lysine forms, including association with transcription. The role of H3-lysine 79 methylation is not yet completely understood (from Akbarian et al., 2009).

Regarding the existence of demethylases was controversial hypothesis and histone methylation was considered static and enzymatically irreversible.

Arginine is a positively charged amino acid known to mediate hydrogen bonding and amino-aromatic interactions. Arginine residues within histones can be post-translationally modified to contain methyl groups, a process termed arginine methylation. There are two classes of arginine methyltransferase (PRMTs), the type-I and type-II enzymes.

These methylating proteins proved to be involved in diverse cellular processes such as RNA processing transcriptional regulation, signal transduction, and DNA repair, highlighting the role played by PRMTs in various pathways (Bedford et al., 2009; Pal et al., 2001).

Histone arginine methylation correlates with transcriptional activation of a variety of genes. Several laboratories have shown functional synergy between arginine methylation and histone acetylation in transcriptional activation events (Stallcup et al., 2000; Daujat et al., 2002).

Arginine methylation was recently shown to be reversibile: the enzyme peptidyl arginine deaminase 4 (PADI4/PAD4) is the only enzyme that antagonizes histone arginine methylation by

converting either unmethylated arginine or monomethyl-arginine to citrulline. This enzyme, however, cannot remove methyl groups from symmetrically or asymmetrically dimethylated arginine residues, suggesting that PAD4 exerts its effect on transcription by preventing dimethylation of histone arginines (Cuthbert et al., 2004).

1.1.2 Histone acetylation

Allfrey and collaborators first reported histone acetylation in 1964 (Allfrey et al., 1964). Histone modification is a highly dynamic reversibile modification that was originally linked to transcriptional activation (Roth et al., 2001). It modulates transcription in multiple ways: acetylation of lysine residues within histone tails neutralizes their positive charge, thereby relaxing chromatin structure and weakening interactions between the histone tail and DNA (Wolffe et al, 1999; Mizzen et al, 1998; Clark et al, 1993). This interferes with the generation of higher-order chromatin structures and increases the accessibility of transcription factors to their target genes (Annunziato et al., 2000; Shahbazian et al., 2007).

The acetylation of lysines is regulated by the contrasting action of two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs).

Histone acetyltranferases are among the first histone-modifying enzymes to be identified and functionally characterized (Brownell et al, 1996). HATs catalyze the acetyl-coenzyme A (acetyl-CoA)-dependent acetylation of the ε-amine groups of specific lysines side chains in histones, as well as in transcription factors and other nuclear proteins. In doing so, they neutralize the positive charge of lysine and this action has the potential to weaken the interactions between histone and DNA, and regulate transcription, histone deposition during nucleosome assembly, DNA repair and other genomic processes (Peterson et al., 2004; Carrozza et al., 2003).

There are two major classes of HATs: type-A and type-B. The type-B HATs are predominantly cytoplasmic, acetylating free histones but not deposited into chromatin; moreover, this class of HATs is highly conserved and all type-B HATs share sequence homology with scHat1, the founder of this type of HAT. Type-B HATs acetylate newly synthesized histone H4 at K5 and K12 and this pattern of acetylation is important for deposition of the histones, after which these marks are removed (Parthun, 2007). The type-A HATs are a more diverse family of enzymes. They can be classified into three separate groups depending on amino-acid sequence homology and conformational structure: Gcn5-related N-acetyltransferases (GNATs), CBP/p300 (cAMP response

element binding protein (CREB)-binding protein/E1A binding protein protein acetyltransferase of 300 kD), and MYST proteins (MOZ/Ybf2/Sas2,3/Tip60) (Allis et al., 2007; Lee et al., 2007; Marmorstein, 2001; Yang et al., 2008; Sterner et al, 2000). In general, these enzymes modify more than one lysine but some limited specificity exists for some enzymes.

The most prominent members of the GNAT family are GCN5 and PCAF (p300/CBP-associated factor), both of which acetylate histone H3 and H4 and are related by sequence similarity but have many diverse functions. The MYST family of HATs acetylates histone H3, H4, and H2A. CBP was firstly identified by its physical interaction with the transcription factor CREB: they interact via the Ser133 phosphorylated kinase-inducible domain of CREB and a specific domain of CBP (Goodman et al., 2000). CBP and p300 are distinct proteins but are often referred to as being interchangeably due to their high degree of similarity: they both have HAT activity and function as transcriptional co-activators (Bannister et al., 1996). CBP participates in the regulation of gene expression through its HAT activity, promoting the decondensation of chromatin at specific sites and the transcription of those specific genes. Additionally, CBP stimulates transcription by recruiting other components of the general transcriptional machinery (Kalkhoven, 2004). Moreover, CBP/p300 can acetylate H2A, H2B, H3, H4 and non-histone proteins, such as CREB, NF-κB and p53 (Barrett et al., 2008).

Importantly, each of these classes of HATs can acetylate non-histone proteins as well, which are known to play an important role in a variety of cell signaling pathways (Marmorstein et al., 2001): α-tubulin, cortactin, and hsp90 can be substrates of HAT activity (Yang et al, 2008), allowing the HATs to act not only as chromatin remodeling enzymes, but also as mediators of most of the cellular signaling processes that affect chromatin. In common with many histone-modifying enzymes, the type-A HATs are often found associated in large multiprotein complexes (Yang and Seto, 2007).

While chromatin regions engaged in transcription are associated with dynamically acetylated histone (Turner et al., 2000; Grunstein et al., 1997), the recruitment of HDACs, leading to hypoacetylation, favours transcriptional repression and silencing by inducing chromatin to compact (Jenuwein et al., 2001; Narlikar et al., 2002; Kuo et al., 1998). HDACs reverse lysine acetylation, an action that restores the positive charge of the lysine. This potentially stabilizes the local chromatin architecture and is consistent with HDACs being predominantly transcriptional repressors. There are four classes of HDAC: classes I and II contain enzymes that are most closely related to yeast scRpd3 and scHda1, respectively, class IV has only a single member, HDAC11,

while class III are homologous to yeast scSir2. This latter class, in contrast to the other three classes, requires a specific cofactor for its activity, NAD^+ (Yang and Seto, 2007).

Class I HDACs (HDAC 1, 2, 3, 8) are ubiquitously expressed and are far more active against histone substrates than class II HDACs (HDAC 4, 5, 6, 7, 9). Both class I and class II HDACs share a conserved catalytic domain, which requires zinc for activity, but class II HDACs are much larger proteins with a N-terminal regulatory region that controls subcellular localization and interaction partners. Sirtuins are distinct from the other classes structurally and mechanistically; they require NAD^+ as a cofactor instead of zinc to catalyze histone deacetylation.

Moreover, HDACs are highly regulated through post-translational modifications that control their activity, subcellular localization, or stability (Sengupta et al., 2004). Together, the large families of HATs and HDACs integrate a wide range of extracellular signals to determine the appropriate balance of acetylation/deacetylation on diverse protein substrates. In the case of histone substrates, the balance of acetylation plays a critical role in the activation or repression of specific gene programs (Figure 2).

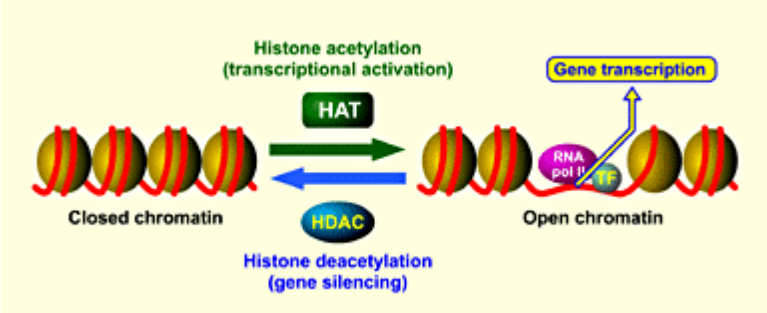


Figure 2. Acetylation and deacetylation of nucleosomal histones play an important role in the modulation of chromatin structure, chromatin function and in the regulation of gene expression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two opposing classes of enzymes, which tightly control the equilibrium of histone acetylation (from Graul et al, 2006).

1.1.3 Histone phosphorylation

Among all PTMs that occur on histone tails, phosphorylation is the one that establishes a direct link between chromatin remodeling and intracellular signaling pathways.

The phosphorylation of histone is highly dynamic; it takes place on serines, threonines and tyrosines, predominantly, but not exclusively, in the N-terminal histone tails. The levels of the modification are controlled by kinases and phosphatases that add and remove the modification respectively (Xhemalce et al., 2011). All of the identified histone kinases transfer a phosphate group from ATP to the hydroxyl group of the target amino-acid side chain; in doing so, the modification adds significant negative charge to the histone that undoubtedly influences the chromatin structure.

Phosphorylation of serine residues on histone tails induces changes in chromatin structure from condensed heterochromatin to more open euchromatin: phosphorylation alters the electrostatic interactions between the N-terminal domain of H3 and DNA due to the additional, negatively charged, phosphate group and, thus, favours the accessibility to transcription factors (Cheung et al., 2000b) and other enzymes involved in further chromatin remodeling such as HATs (Roth et al., 1992). Phosphorylation of histones H1 and H3 on specific, conserved serine residues on the N-terminal tails was first observed more than 30 years ago in the context of chromosome condensation during mitosis: H3 was the first histone whose phosphorylation was characterized in response to the activation of mitogenic signaling pathways (Gurley et al., 1973).

In the case of histone H3, phosphorylation on serine residues may lead to either activated gene expression or chromatin condensation/segregation during mitosis: Ser10/Ser28 phosphorylations are two events inducing opposite alterations in the degree of chromatin compacted in interphase, phosphorylation of H3 correlates with chromatin relaxation and gene expression, whereas in mitosis it correlates with chromosome condensation (Jenuwein et al., 2001; Cheung et al., 2000a; Nowak et al., 2004; Prigent et al, 2003). However, the phosphorylation of histone H3 on serine 10 plays a crucial role in chromosome condensation at prophase during mitosis (Wei et al., 1998; Prigent and Dimitrov, 2003) and is associated with the mitotic spindle through anaphase remaining within the midzone and midbody until completion of cytokinesis (Li et al., 2005).

Phosphorylation of Serine 10 on H3 is mediated by mitogen- and stress-activated protein kinase 1 (MSK 1), which is downstream from extracellular signal-regulated kinase (ERK) and has been associated with immediate early gene (IEG) activation in response to growth factor treatment (Mahadevan et al., 1991; Sassone-Corsi et al., 1999; Clayton et al., 2003).

In addition, phosphorylation at specific sites is coupled to other distinct modifications, such as acetylation (K9/K14 acetylation) that could serve to amplify the readout of upstream signaling pathways causing greater changes in the overall charge density of tails that lead to greater changes in the chromatin structure of target genes (Rea et al., 2000; Cheung et al., 2000b; Thomson et al., 2001; Nowak et al., 2000). Evidence supports synergism between histone acetylation and phosphorylation in the induction of IEGs after mitogenic stimulation (Cheung et al., 2000b; Clayton et al., 2000). Phosphorylation on histone tails is enzymatically reversible and reversed by phosphatases. So far, the protein phosphatases PP-1 and PP-2A have been shown to regulate levels of phosphorylation on H3 (Chadee et al., 1999; Nowak et al., 2003). This enzyme-based reversibility makes sense because it provides the cell with a means to respond quickly to changes through a rapid alteration in its gene expression programs.

2. EPIGENETICS IN CENTRAL NERVOUS SYSTEM

During development, multi-cellular organisms acquire adaptive change to the environment by the development of complex physiological and behavioral systems coordinated by the central nervous system (CNS). The nervous system permits rapid adaptation to changing environmental conditions, without genetic mutation (Kandel, 1984), by epigenetic modifications that translate inputs from the internal and external environment in functional and morphological responses directed to the adaptation to the environment , the maintenance of homeostasis and well as the management of allostatic load.

In the cell type diversity of the nervous system, as in the immune system, it is more likely that permanent changes in gene expression patterns are achieved through permanent changes in chromatin remodeling without changes in DNA sequence. Such epigenetic mechanisms are likely used to maintain both cell memory (how the cell maintains its differentiated characteristics) as well as preserve and strengthen synaptic connections that sustain long-term changes in behavior. Thus, the concept of chromatin remodeling addresses a key challenge in neurobiology of how stable changes in gene expression are induced in neurons and glia to produce long-lasting changes in behavior (Colvis et al., 2012).

The nervous system responds to complex metabolic signals, diverse cellular processes and environmental changes by gene transcription responses and synthesis of new proteins, mediated by the dynamic remodeling of chromatin architecture transacted by an ever expanding array of enzymes and associated signal transduction pathways (Citri et al., 2007; Colvis et al., 2005). Despite the fully differentiated state of post-mitotic neurons, their chromatin retains a surprising level of plasticity, thus extending the functional flexibility of the cells.

Epigenetics is a type of molecular and cellular “memory” that results in stable changes in gene expression without alterations to the DNA sequence itself (Santos et al., 2006).

Although adult neurons are terminally differentiated and no longer divide, recent studies indicate that epigenetic mechanisms may come into play with respect to information storage in the adult nervous system (Levenson et al., 2004a; Chwang et al., 2006 and 2007). This regulation is achieved through the action of epigenetic factors and chromatin-modifying enzymes that can be divided into three distinct categories: (i) histone-modifying enzymes which covalently acetylate, phosphorylate, ubiquitinate, or methylate histones, like HATs and HDACs as well as HMTs and HDMs (Bhaumik et al, 2007); (ii) DNA-modifying enzymes which methylate CpG-rich sequence (DNMTs)(Ooi et al., 2008) and (iii) ATP-dependent chromatin-remodeling complexes which can

disrupt nucleosome structure and increase accessibility to DNA and histones, using the energy from ATP hydrolysis to move histone octamers along DNA molecules (Becker et al., 2002; Gregory et al., 2001; Narlikar et al., 2002) (Figure 3).

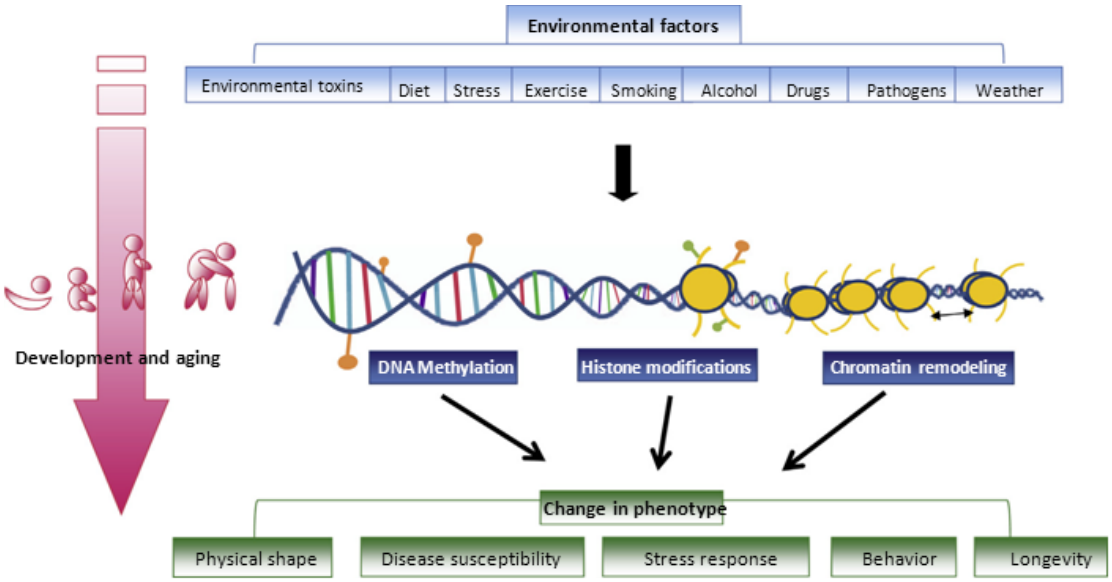


Figure 3. Epigenetic mechanisms provide the link between environmental factors and phenotypical changes during the whole lifetime (from Tammena et al., 2013).

It is becoming increasingly clear that changes in the chromatin architecture are important factors in gene regulation and understanding these molecular processes and their functional outcomes may provide new insight into normal neural function and disease (Borrelli et al., 2008; Jiang et al., 2008). Epigenetic alterations are present in brain processes and play a key role in a diverse set of functions including learning and memory processes, drug addiction (Crepaldi, 2009), neurodegeneration, and circadian rhythms. Epigenetic mechanisms have been implicated in specific human disorders including neuropsychiatric disorders, like major depression, Alzheimer's disease, Rett syndrome, Rubinstein-Taby syndrome, Fragile X syndrome, Huntington's disease, schizophrenia, bipolar disorder and epilepsy (Graaf et al., 2009; Tsankova et al., 2007; Shahbazian et al.., 2002; Kim et al., 2008). Understanding the molecular components and environmental conditions that cause or result in epigenetic changes may provide unique opportunities to develop novel interventions and therapies to treat a variety of neurological and psychiatric conditions.

2.1 Transcriptional regulation in central nervous system

The main process underlying the CNS activity is the neuroplasticity. It is a multifaceted and dynamic process involving gene-environment that results in both short- and long-term changes in gene expression, cellular function, circuit formation, neuronal morphology, and behavior. As the brain matures, such connections remain malleable permitting alterations in the synaptic strength of specific circuits required of various form of experience dependent plasticity. Throughout adulthood, environmental stimuli are continuously encoded at the level of the synapse in an unparalleled process in other tissues (Ian Maze et al., 2012).

Literature indicates that environmental stimuli experienced during early stage of neurodevelopment result in altered patterns of transcription in the brain that are essential for the establishment and maintenance of synaptic connections (Greer and Greenberg, 2008).

The mammalian brain depends on numerous complex and highly regulated mechanisms to appropriately activate or silence gene programs in response to environmental input and developmental cues. At the molecular level, these events are controlled by activity-dependent signaling pathways that mediate gene expression by modifying the activity, localization, and/or expression of transcriptional-regulatory enzymes in combination with alterations in chromatin structure in the nucleus (McClung and Nestler, 2008). Neural activity can modulate chromatin acetylation by regulating the intracellular distribution of HDAC; the shuttling of class II HDACs, in and out of the nucleus, can be regulated by extracellular cues. In hippocampal neurons, for example, nuclear export of HDAC4 is induced by spontaneous electrical activity, whereas HDAC5 export depends on Ca^{2+} influx mediated by the activation of NMDA receptors (Chawla et al., 2003). Synaptic activity can also influences changes in chromatin structure by inducing other histone modifications, such as histone H3 phosphorylation (H3S10 phosphorylation). Interestingly, H3S10 phosphorylation induced by synaptic activity is associate with a change in nuclear structure (Wittmann et al., 2009), which might suggest that nuclear geometry and transcriptional response are functionally linked.

Other signaling pathway, particularly the mitogen-activated protein kinase (MAPK) cascade, is known acting directly on chromatin proteins, such as histone H3, to modify chromatin concomitant with gene induction (Clayton et al., 2003). In brain, the dopamine and cAMP regulated protein phosphatase inhibitor, DARPP-32, as well as the MAP kinase, MSK1, provide excellent examples of enzymes regulating H3S10 phosphorylation in response to environmental

stimuli, indicating histone phosphorylation as an important regulator of adult neuronal function (Brami-Cherrier et al., 2005; Stipanovich et al., 2008).

Although H3S10 phosphorylation was first characterized as a mitotic signature in proliferating cells, it is now known to exist at gene promoters in post-mitotic cells and has been linked to transcriptional activation. Specially, stimulation of grow factors and neurotransmitter-mediated kinase pathways induce rapid and transient phosphorylation of H3S10 at promoters and throughout coding regions of immediate early genes (IEGs) (Thomson et al., 1999; Clayton et al., 2000; Crosio et al., 2003). In cultured striatal neurons, for example, cAMP or glutamate stimulations have been shown to increase H3S10 phosphorylation enrichment at the c-Fos promoter (Li et al., 2004; Brami-Cherrier et al., 2007). In the adult brain, neuronal stimulation resulting in the activated release of neurotransmitters, such as dopamine, acetylcholine and glutamate, induce H3S10 phosphorylation in the dentate gyrus of the mouse hippocampus (Crosio et al., 2003). Furthermore, numerous other environmental stimuli have been found to induce H3S10 phosphorylation in brain, including administration of drugs of abuse (Brami-Cherrier et al., 2005) and kainic acid-induced seizures (Sng et al., 2006).

At IEG, c-Fos and c-jun promoters, H3S10 phosporylation is often associated with acetylation at H3K14 (Lo et al., 2000), a modification that has been shown to be regulated by a variety of stimuli in the adult brain (eg, social stress, Covington et al., 2009) and is coupled to transcriptional activation.

Histone phospho-acetylation can occur as two indipendent modifications (Thomson et al., 2001) or in concert (Cheung et al., 2000b): H3S10 phosphorylation provides a docking site for the HAT GCN5, on chromatin. GCN5 then maintains a hyperacetylated state, which promotes gene activation as described above. Moreover, acetylation of lysine K9 on histone H3 prevents the methylation of this same residue, which is a mark of repressed chromatin, and the subsequent recruitment of HP1. These effects of lysine K9 acetylation further promote active transcription.

At the same time, H3 phospho-acetylation was observed to promote c-Fos early gene induction after an immune challenge in a neuronal system (Ottaviani et al., 2013) (Figure 4).

Given that neurons are highly responsive to environmental cues and are capable of rapidly responding to cellular activity through the initiation of numerous phospho-dependent signaling cascades, it is likely that histone phosphorylation state will have important roles in the integration of receptor-mediated signaling and influence transcriptional outputs necessary for proper neuronal function.

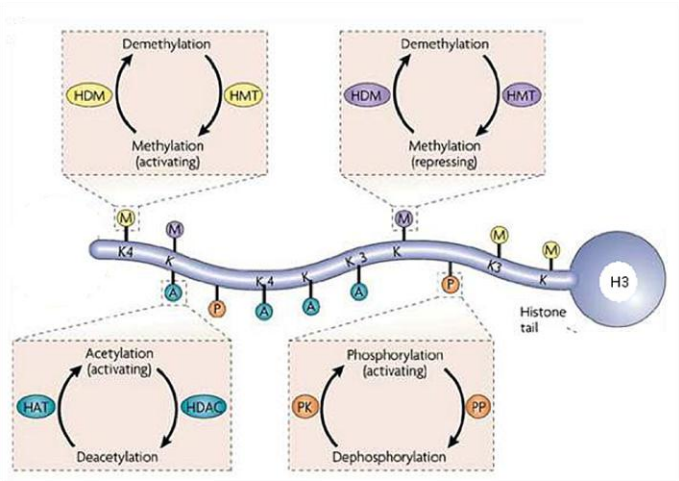


Figure 4. Representation of the possible histone H3 modifications.

In addition to modifications to histones, DNA methylation could be an important mechanism that contributes to neuronal plasticity as well. DNA methylation is a crucial mechanism for controlling chromatin remodeling in the adult mammalian nervous system (Levenson et al., 2006; Miller et al., 2007): dysregulation of DNA methylation has been implicated in mental illnesses such as schizophrenia, depression, bipolar disorder, Rett syndrome, and fragile X mental retardation (Grayson et al., 2005; Amir et al., 1999; Sutcliffe et al., 1992; Veldic et al., 2004).
Recent studies show that changes in DNA methylation may be more transient and underlie certain types of long-term plasticity: both DNA methylation and demethylation might be involved in long-term memory consolidation as *de novo* DNMT gene expression is upregulated in the adult rat hippocampus after contextual fear conditioning (a hippocampus-dependent learning paradigm whereby an animal learns to associate a novel context with an aversive stimulus) and DNMT inhibition blocks memory formation in this same paradigm (Miller et al., 2008 and 2007; Levenson et al., 2006).

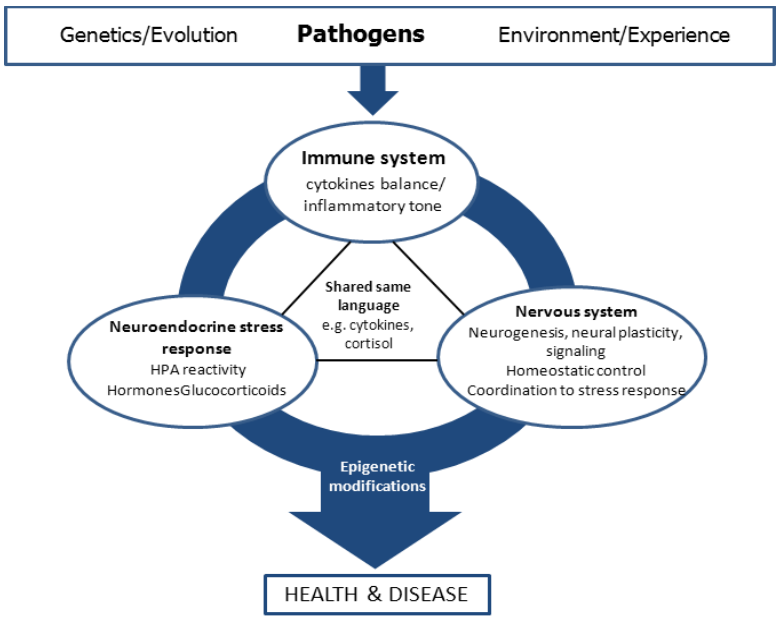
Signal transduction mechanisms are integral components of the neuronal information processing machinery. During synaptic transmission, neurons respond to neurotransmitters by receptor-mediated intracellular signal transduction events, which, among other actions, activate or inhibit transcription factors. The regulation of transcriptional activity by transcription factor binding to DNA depends on interactions of the transcription factors with many co-activators or co-repressors and the underlying structure of chromatin. All these mechanisms regulate the expression of specific sets of neuronal genes that are important for neural activity, survival, morphology and ultimately the integrated regulation of complex behavior.

At present the identity of the neuronal populations, the neurocircuitry and the molecular mechanisms steering plasticity processes in neurons are largely not resolved and the signal transduction processes controlling histone acetylation and phosphorylation in the mature CNS are just beginning to be investigated and undoubtedly, many important additional mechanisms await discovery.

Chromatin remodeling is thus intimately linked to activation or repression of genes by synaptic activity: one of the most important signaling cascades that have been implicated in controlling histone acetylation and phosphorylation and chromatin structure in the mature CNS thus far is the MAPK cascade - exemplified by the ERK/MSK pathway. In this pathway ERK activates its downstream target MSK, which in turn phosphorylates CREB at Ser133 (Swank et al., 2001; Chwang et al., 2006 and 2007; Brami-Cherrier et al., 2005). Phosphorylation of transcription factors is a key trigger for transactivation (the process by which genes are activated by means of a transactivating domain that is contained in a transcription factor): in the nucleus, CREB, once phosphorylated, binds to its consensus sequence and activates cAMP response element (CRE)-mediated gene transcription that plays a crucial role in associating synaptic activity with long-term changes in synaptic circuitry in many kinds of neuronal systems.

Moreover, phosphorylation and activation of CREB recruits CBP, a transcriptional co-activator with intrinsic HAT activity that regulates local chromatin structure as part of CREB-dependent activation of nuclear gene transcription (Ogryzko et al., 1996; Sweatt et al., 2008; Bito et al., 2003; Johannessen et al., 2004; McClung et al., 2008).

Given that, the nervous system can rapidly adapt response to changing environmental conditions by epigenetic modifications that translate inputs from the internal and external environment in functional and morphological response determining maintenance of homeostasis or the onset of a disease state, unraveling the identity of the neuronal populations, the neurocircuitry and the molecular mechanisms steering plasticity processes in neurons. The signal transduction processes controlling histone acetylation and phosphorylation in the mature CNS are of great importance as, undoubtedly, many important additional mechanisms await discovery. Indeed, new emerging evidences indicated that the CNS, endocrine and immune systems communicate through multiple anatomical and hormonal-neuropeptide routes and controlled interactions between these systems are believed to be critical for the maintenance of a homeostatic balance within good health. An imbalance or alterations in these systems in response to disease, stress, injury and/or metabolic alterations can lead to significant changes in immune responsiveness and susceptibility to infections and autoimmune disease states.



A greater understanding of the interplay between these systems may provide valuable insights into how disruption within one or more of these compartments may influence the ability of the host to regulate inflammation or disease development (Taub, 2008). The regulation of inflammatory response also depends on other factors including a genetic predisposition and an individual vulnerability besides the experience and environment influences. In this contest epigenetic mechanisms constitute the means by which this complex system implements adaptive changes in the allostasis of the brain.

3. INFLAMMATION IN CENTRAL NERVOUS SYSTEM

Inflammation represents the pathophysiological process of host defense to injury, tissue damage, autoimmune disease or infectious agents. Inflammation elicits a generalized sequence of events known as the acute phase response that consists of the production of pro-inflammatory mediators (lipid mediators, peptides and amines, cytokines), activation of the sympathetic nervous system, changes in cardiovascular function, altered neuroendocrine status, behavioral changes which lead to energy conservation such as increased sleep, lethargy, reduced appetite, and the most common feature of infection, fever, which can limit bacterial proliferation. When these responses persist in an inappropriate and excessive way, the inflammation becomes a chronic state that can cause numerous diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease. Moreover, inflammation is a major component of the damage caused by autoimmune diseases, and a fundamental contributor to diseases like cancer, diabetes and a risk factor for cardiovascular diseases.

Until just over a decade ago, the brain was not regarded as a susceptible organ to inflammation or immune activation and was thought to be unaffected by systemic inflammatory and immune responses. Now it is accepted that it coordinates and regulates many aspects of the acute phase, which may begin to explain the behavioral response to disease as fatigue and depression, and how psychological state can influence susceptibility to disease and recovery. In most organ systems, inflammation causes bystander injury that is typically reversible due to the inherent regenerative capacity of the cellular elements of that tissue. However, in the CNS, the stakes are higher. The common consequence of bystander injury in the CNS is irreversible neuronal loss and atrophy due to regenerative failure.

The most intense interest in inflammation in the CNS has arisen from its potential role in chronic degenerative diseases with a more and more increasing incidence and social impact, including acute brain injury, stroke, epilepsy, neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis), and more recently some psychiatric disorders such as depression, anxiety and schizophrenia (Lucas et al., 2006).

The active involvement of inflammatory processes in these diseases are sustained by a substantial number of data, much of which have been obtained from experimental studies in rodents using appropriate models of the clinical conditions. The resulting evidence demonstrates the activation and invasion of inflammatory cells and increased expression of transcription factors which

coordinate inflammatory responses to injury; nevertheless, this does not necessarily indicate a causal role and it is important to investigate the functional consequence.
Moreover, the hypothesis that CNS output can modulate immune function is becoming more and more accredited (Correa, 2007) as the fact that the immune system in turn affects the nervous system thus providing the basis for neuro-immune communication.

3.1 Central nervous system regulation of inflammation

Contrarily to what was previously believed, nowadays increasingly emerging data show the brain as an "immunological" active site. While the knowledge that the CNS regulates thermoregulatory and neuroendocrine responses to disease and injury it is not surprising we now also know that the CNS affects peripheral immune function.
Pro-inflammatory cytokines play an essential role in CNS inflammation through the induction of chemokines and adhesion molecules, recruitment of immune cells into parenchyma and activation of immune cells and endogenous glial cells (Rothwell & Luheshi, 2000). All brain cells (neurons, astrocytes, oligodendrocytes and microglia) can produce inflammatory mediators and cytokine receptors are expressed constitutively throughout the CNS, but the brain also contains specific cells with immune activity, such as macrophages and dendritic cells, which are present in the choroid plexus and meninges, and microglia cells, brain parenchimal macrophages, which are activated rapidly in response to inflammatory insults, and take on the morphology of activated macrophages (Streit, 2002). They are important phagocytic cells and release cytokines and prostaglandins, besides inducible factors like nitric oxide and neurotrophins (Hanish, 2002).
The contribution of astrocytes is more complex. These cells play key physiological roles in supporting neurons, regulating ion and transmitter concentrations and in electrical transmission, but are also an important source of neuroprotective (e.g. a number of neurotrophins) and inflammatory/potentially neurotoxic molecules (Chen & Swanson, 2003).

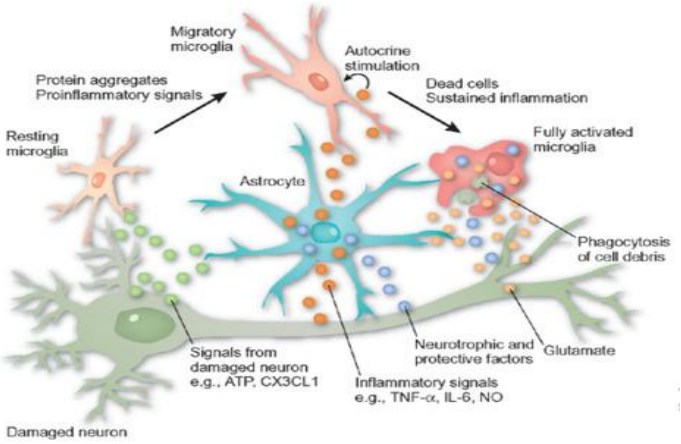


Figure 5. CNS cells involved in inflammation. Atrocytes contribute to inflammatory response with neurotrophins and pro-inflammatory molecules production. Microglia cells present 3 states: 1) where they scan their immediate environment for toxins or signals from neurons which indicate injury/infection, 2) an active state where they seek out the site of insult and secrete inflammatory signals, 3) a phagocytic state where they ingest cellular debris and clean up dead material.

The afferent signals from inflamed, injured or infected tissues to the CNS include several means that act in parallel. One way involves afferent nerves: locally produced cytokines activate primary afferent nerves, such as the vagal nerve during abnormal visceral infections and the trigeminal nerve during oro-lingual infections (Bluthè, 1994). In a second, humoral pathway, Toll-like receptors (TLRs) on macrophage-like cells residing in the circumventricular organs and the choroid plexus respond to circulating pathogen-associated molecular networks by producing pro-inflammatory cytokines (Quan, Whiteside, Herkenham, 1998) that can enter the brain by volume diffusion (Vitkovic, 2000).

A third pathway comprises cytokine transporters at the blood-brain barrier: pro-inflammatory cytokines overflowing in the systemic circulation can gain access to the brain through these saturable transport systems (Banks, 2006). Finally, a fourth pathway involves the pro-inflammatory cytokine interleukine-1 (IL-1) receptors that are located on perivascular macrophages and endothelial cells of brain venules (Konsman, 2004; Schiltz, 2002). Activation of these IL-1 receptors by cytokines results in the local production of prostaglandin E2 (PGE2).

3.2 Neuro-immune crosstalk

Research has shown a tightly link between immune and nervous system by specialized and homeostatic pathways (Haddad, 2002). Current data indicate that the CNS is both immune competent and actively involved in the interaction with the peripheral immune system (Ransohoff, 2003). There is now growing evidence that neural-immune crosstalk may even occur in non-disease conditions in the healthy brain. For instance, cytokines have subsequently been found to mediate a diverse array of functions in non-immune tissues, including the CNS. Cytokines are also normally produced in the healthy brain in particular in critical phases such as cell fate decisions, neuronal development and differentiation, neuronal and glial cell migration and synaptic plasticity required for learning and memory (Deverman and Patterson, 2009; Carpentier and Palmer, 2009; Bilbo and Schwarz, 2009).

On the other hand, alterations in immune response can be conditioned and regulated by neuronal activity, for example electrical stimulation or lesions of specific brain sites can modulate immune functions; so, activation of the immune system is correlated with altered neurophysiological, neurochemical and neuroendocrine activities of brain tissue (Carson et al., 2006).

In the CNS, immune cells employ shared mediators to promote crosstalk with neuronal cells but the effect of this crosstalk depends on the contest of the interaction. It has long been established that inflammatory reactions in the CNS can cause or contribute to increase tissue injury; however, emerging evidence suggests that in some paradigms inflammatory cells can favor neuroprotection and repair. This dual role of CNS inflammation is also suggested at the molecular level where it is increasingly clear that immune cells can release both neurodestructive and neuroprotective molecules in the presence of CNS lesions.

The interaction of immune and nervous cells is complex and a growing body of studies advances the hypothesis that inflammation in the CNS can, in addition to well-documented neurotoxic effect, also convey neuroprotection (Rapalino et al., 1998; Moalem et al., 1999; Hammarberg et al., 2000). Immune and nervous system produce mediators (i.e. cytokines and chemokines for the immune system and neurotrophic factors for the CNS) that modulate cell growth and differentiation.

3.3 Peripherally cytokine production act in the brain to induce sickness-behavior

The immune, physiological, metabolic and behavioral responses of the organism to fight pathogenic micro-organism infections, are an highly organized strategy mediated by the activation of innate immune cells. These phagocytic cells express pattern-recognition receptors in the form of Toll-like receptors. A typical pathogen-associated molecular pattern is represented by lipopolysaccharide (LPS), an endotoxin that constitutes a major component of the outer membranes of Gram negative bacteria. To exert its pathogenic effects, LPS requires Toll-like receptor-4 (TLR-4), that is present in circulating monocytes/macrophages and other immune cells which activates complex intracellular signaling pathways including docking proteins and phosphorylation cascade, resulting in activation of transcription factors, in particular the nuclear factor kappaB (NF-κB). The activation and translocation of NF-κB to the nucleus results in the transcription of pro-inflammatory cytokines, including interleukin-1α and β (IL-1α and IL-1β), tumor necrosis factor-α (TNF-α), adhesion molecules and certain enzymes such as cyclooxygenase-2 (COX-2), as well as different isozymes of nitric oxide synthase (NOS). IL-1 then is able to induce its own synthesis and the synthesis of other cytokines potentiating its action, as TNF-α and interleukin-6 (IL-6), or antagonizing it and are therefore called "anti-inflammatory cytokines", such as interleukin-10 (IL-10) and the specific antagonist of IL-1 receptors (IL-1Ra). Given this, pro-inflammatory cytokines that are produced as a result of the activation of the NF-κB signaling pathway are part of a network that includes cytokines opposing the production and action of pro-inflammatory cytokines (Dantzer, 2004). Pro-inflammatory cytokines do not act as hormones, because aside from IL-6, they are not transported in the circulation to distant cell targets. They act in an autocrine manner, on the same cells that have manufactured them, or in a paracrine manner, on adjacent cells within the same tissue. Cytokines usually are produced only when needed. Once released, cytokines are biologically active at nano- to pico-molar concentrations, and they act on a limited number of receptors per cell that amplify their action via the activation of a large number of genes. In the brain, both neural and non-neural cells express receptors for these mediators: brain cytokine receptors are equivalent structurally and functionally to those characterized on peripheral immune and non-immune cells. The brain monitors peripheral and systemic innate immune responses to pathogens by several means that act in parallel (Dantzer, 2007).

Peripheral administration of a cytokine inducer, such as LPS, or of recombinant cytokines, such as IL-1β or TNF-α, mimics all nonspecific symptoms of sickness, including fever, activation of the hypothalamic-pituitary-adrenal (HPA) axis, reduction of food intake and other behavioral activities, as well as withdrawal from the physical and social environment (Dantzer, 2008).

Typical symptoms of sickness include weakness, malaise, lethargy and inability to concentrate. Sick individuals are often somewhat depressed and lethargic. They show little interest in their surroundings and stop eating and drinking. This constellation of non-specific symptoms is collectively referred to as "sickness behavior" (Hart, 1988). Sickness behavior usually is assessed by reduction in food intake and decreased social investigation and is very often accompanied by pain. It has been proposed that this response is an integral part of sickness behavior if not the main determinant of it (Watkins and Maier, 2000).

Conversely, administration of cytokine antagonists abrogates the physiologic and behavioral effects of the cytokine inducer, LPS. Experimental findings indicate that pro-inflammatory cytokines mediate the clinical signs of the host response to infection. The physiologic and behavioral changes that are characteristic of sickness are mediated in the CNS. Fever, for instance, represents a regulated rise in body temperature resulting from increased production of heat (thermogenesis) and decreased thermal loss (thermolysis) in response to an elevated set point for the regulation of body temperature. Given that the body temperature set point is controlled by temperature-sensitive neurons in the pre-optic region of the hypothalamus, pyrogenic cytokines, such as IL-1β and IL-6, need to act in the CNS to induce fever (Romanovsky et al., 2005). In the same manner, IL-1β acts on the paraventricular nucleus of the hypothalamus where the neurons that contain corticotropin releasing hormone (CRH) are located (Berkenbosch et al., 1987; Ericsson et al., 1994). CRH is released in the portal blood, which leads to the release of corticotropin from the pituitary, which, in turn, increases the release and secretion of glucocorticoids (GR) by the adrenal cortex.

Whereas cytokines are considered short-range communication molecules that act predominantly in an autocrine or paracrine manner rather than a hormonal manner, several pathways of immune-to-brain communication are proposed for the action of these cytokines on the nervous system, from the induction of prostaglandins in those brain areas that are devoid of a functional blood-brain barrier to the existence of specific saturable transporters (Konsman et al., 2002).

The hypothesis that cytokines act indirectly on the CNS by activating afferent nerves is based on the recognition that two of the cardinal signs of inflammation, *calor* (heat) and *dolor* (pain), require sensory processing, which implies that inflammatory mediators released at the site of injury or infection are able to signal the brain. When LPS or cytokines are injected into the

abdominal cavity, they induce inflammation of the peritoneum. One of the major routes of visceral sensibility is represented by the afferent branches of the vagus nerve. These branches contain macrophages and dendritic cells in their peri-neural sheath that express membrane TLRs and produce IL-1β in response to an intraperitoneal injection of LPS (Goehler et al., 1999). Sensory neurons of the vagus nerve express IL-1 receptors, and circulating IL-1β stimulates vagal sensory activity (Ek et al., 1998). Vagal afferents are shown to mediate the activation of the brainstem, hypothalamus, and limbic structures in response to peripherally administered LPS, as demonstrated by the attenuation of the expression of the early activation gene c-Fos in the primary and secondary projection areas of the vagus nerve (Wan et al, 1994).

The importance of the neural pathway in the transmission of the immune message from the periphery to the brain is not the same for all components of sickness behavior. In particular, vagal afferents are less important for cytokine-induced fever and activation of the HPA axis than for cytokine-induced sickness behavior, because vagotomized rats do not develop the behavioral alterations that are characteristic of sickness while they still are able to mount a fever (Konsman et al., 2000). These findings indicate that other pathways of communication function in parallel with the neural pathway. Indeed, besides the relatively fast neural pathway of immune-to-brain communication, there is a slower pathway that involves the action of pathogen-associated molecular patterns (PAMPs) or circulating cytokines on macrophage-like cells in circumventricular organs and endothelial cells of brain vessels. This results in the local production of cytokines and molecular intermediates, such as prostaglandins of the E2 series (PGE2) and nitric oxide. PGE2s represent the main mediators of cytokine-induced fever and activation of the HPA axis, because pretreatment with a specific inhibitor of these prostaglandin synthesizing enzymes, cyclooxygenase 2 (COX-2), attenuates these responses (Rivest et al., 2000; Romanovsky et al., 2000).

The synthesis of PGE2 is dependent on the induction of COX-2 and the enzyme, prostaglandin E synthase, both of which are expressed in endothelial cells of cerebral blood vessels and perivascular macrophages after intravenous IL-1β administration. PGE2 diffuse into the brain parenchyma and act on neuronal EP3 or EP4 receptors in the brainstem and hypothalamic neural structures that are involved in the control of HPA axis activity and the regulation of body temperature. These brain areas include the catecholaminergic brainstem nuclei, the paraventricular nucleus of the hypothalamus, and the ventromedial preoptic area. The reduction in social behavior and the anorexia that develop in response to peripheral LPS and IL-1 are mediated by brain IL-1, because these responses are attenuated by intra-cerebroventricular administration of the IL-1 receptor antagonist (Laye et al., 2000; Kent et al., 1992). In response to

peripheral LPS, IL-1β is synthesized by macrophage-like cells in the circumventricular organs and choroid plexus, where the blood-brain barrier is deficient (Van Dam et al., 1992; Konsman et al., 1999). This certainly is the result of the action of circulating LPS on TLR4 receptors that are present on the same cells. Another possibility is that IL-1 is produced in response to circulating cytokines and PAMPs. IL-1 acts on neuronal IL-1 receptors in the *area postrema*, the circumventricular organ of the brainstem (Ericsson et al., 1995). This results in the activation of a neuronal pathway projecting to the parabrachial nucleus and from there to the central amygdala and the bed nucleus of the *stria terminalis*. There is evidence that IL-1 also spreads by volume transmission from the choroid plexus into the surrounding brain parenchyma to reach distant structures, such as the basolateral amygdala, that contains neurons expressing IL-1 receptors (Vitkovic et al., 2000). These two pathways, of which the respective importance remains to be elucidated, could be responsible for the behaviorally depressing effects of IL-1. In the same manner, diffusion of IL-1 from the median eminence to the arcuate nucleus could mediate IL-1–induced anorexia. The exact mechanisms of the depressive action of cytokines on food intake, however, are obscure, because lesions of the arcuate nucleus do not disrupt the effects of IL-1 on food intake (Reyes et al., 2002).

3.4 From sickness to depressive-like behavior

It is well-established that inflammatory cytokines released peripherally have profound effects on mood and behavior. Naturally-occurring infectious illness produce symptoms that resemble depression (Bucks et al., 2008); from the physical and social environment that is accompanied by pain, malaise, fatigue, anorexia and decreased reactivity to reward (anhedonia). Moreover, some components of sickness behavior are improved by antidepressant treatments.

Sickness behavior can become abnormal or pathologic when it occurs out of context (i.e, in the absence of any inflammatory stimulus) or when it is exaggerated in intensity or duration. Several conditions can be responsible for this situation: (1) pro-inflammatory cytokines can be produced in higher quantities and for a longer duration than normal; (2) the regulatory molecules that normally down-regulate activation of the molecular and cellular components of the sickness response are faulty; or (3) the neuronal circuits that are the targets of inflammatory mediators and organize sickness behavior become sensitized.

Data indicate that there is a functional dissociation between cytokine-induced sickness behavior and depression. LPS-induced cytokines or recombinant cytokines can induce in animal models both sickness-like and depressive-like behavior. More precisely, cytokine-induced depressive behavior has been described in different animal models of depression to develop later then the sickness behavior, although showing some overlap (Frenois et al., 2007). These data demonstrate that cytokine-induced sickness behavior develops 2-6 hours following a peripheral LPS administration with a maximum peak at 6 hours which gradually wanes, whereas the depressive-like behavior arises later and peaks at 24 hours. This difference can be explained by dissociation of the neurobiological mechanisms that underlie LPS-induced sickness from those involved in the depressive-like behavioral response to activation of the innate immune system (O'Connor et al., 2009) (Figure 6).

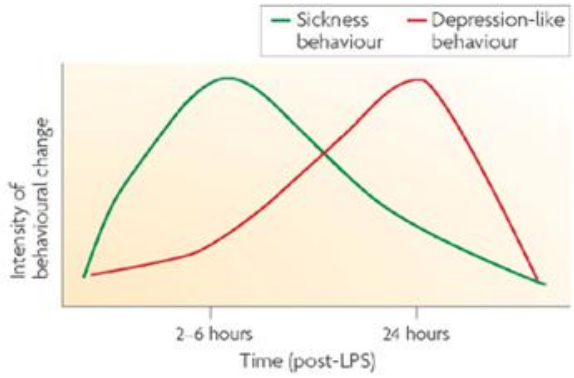


Figure 6. LPS-increased depression-like behavior in mice. Peripheral administration of LPS induces sickness behavior that peaks 2 to 6 hours later and gradually wanes. Depression-like behavior, as measured by increased immobility in the forced-swim test or the tail-suspension test and decreased preference for a sweet solution, emerges on this background. The development of sickness behavior requires activation of pro-inflammatory cytokine signalling in the brain in response to peripheral LPS (from Danzter et al, 2008).

Chronic activation of the innate immune system can precipitate the development of depressive-like disorders, as exemplified by the psychopathologic alterations that occur in patients receiving repeated injections of recombinant cytokines, mainly IL-2 or interferon (IFN)-α, for the treatment of viral infections (hepatitis C) or cancer. At later stages of treatment, up to one third of patients develop alterations in mood that are characteristic of depression, including sadness, inability to feel, depressed mood, and even suicidal ideation (Capuron et al., 2004). The onset of depressive symptoms depends on the cytokine and treatment modalities. These findings can be interpreted

to suggest that depressive disorders develop from cytokine-induced sickness behavior only in vulnerable patients. Vulnerability, in the present context, refers to an innate or acquired predisposition to develop a given pathology when causal factors are present. Dysfunction in genes controlling key proteins in cytokine production, as IL-6, and serotoninergic neurotransmission (e.g, activity of the serotonin transporter (Bull et al., 2008) or serotonin receptor subtype (Kraus et al., 2007)) are identified as vulnerability factors for cytokine induced depression. Vulnerability to cytokine-induced depression can be revealed by psychologic features.

At the clinical level, there is growing evidence that major depression is associated with significantly elevated circulating levels of pro-inflammatory cytokines, in particular IL-6 and tumor necrosis factor (TNF) (Lutgendorf et al., 1999; Kiecolt-Glaser et al., 2002; Alesci et al., 2005; Maes et al., 1995; Maes et a.l, 1997; Brambilla et al., 1998). It is difficult to establish any causal link between depression and increased blood levels of inflammatory cytokines. Some studies suggest the presence of a neuro-inflammatory process in depression, such as increased microglial density (Steiner et al., 2008), and elevated cerebrospinal fluid levels of IL-1 and IL-6 in depression and those who attempted suicide (Levine et al., 1999; Lindqvist et al., 2009). However, in the brains of suicide victims there was an increase of anti-inflammatory cytokines IL-4 and IL-13 (Tonelli et al., 2008). The link between the immune and brain pathway is still complex as well as the link between depression and the immune system, but it has been clearly established that peripherally-released cytokines have an effect on emotions, cognition and behavior. It is plausible that depressive-like symptoms, whether they occur in the context of idiopatic depression or as a consequence of inflammatory stimulus, may involve overlapping neural pathways.

4. EPIGENETICS AND INFECTIONS

Sickness behavior seems to be nothing else than the outward expression of a reversible episode of cytokine expression and action in the brain in response to peripheral immune stimulation. The expression of sickness behavior is not simply the result of the changes in internal state experienced by sick subjects but the joint function of the changes in their internal state and the environmental constraints to which they are exposed (Dantzer, 2008; Hart et al., 1988).

Epigenetic mechanisms regulate expression of the genome to generate various cell types during development or orchestrate cellular responses to external stimuli. Pathogen-induced epigenetic dysregulations may affect host cell function either to promote host defense or to allow pathogen persistence. Thus, pathogenic stimuli can be considered as potential epi-mutagens able to reshape the epigenome; their effects might generate specific, long-lasting imprints on host cells, leading to a memory of infection that influences immunity and might be at the origin of unexplained diseases (Bierne et al., 2012).

Upon a microbial attack, host cells undergo massive changes in their transcriptional program, mobilizing genes involved in key processes to trigger an appropriate response including immunity, cell death/survival, adhesion/motility (Jenner and Young, 2005). Host transcription factors are first targets to reprogram the genome and pathogens use different tricks to alter their function. For instance, bacterial factors can hijack cellular signaling pathways that activate or sequester transcriptional factors in the cytosol of targeted cells, or manipulate their half-lives via post-translational modifications (Bhavsar et al., 2007; Ribet & Cossart, 2010; Perrett et al., 2011).

However, selective activation or silencing of specific genes not only depend on transcription factors, but also on their cross talk with epigenetic modulators, which regulate DNA accessibility by controlling the chromatin structure. Epigenetic modifications of chromatin during development and in response to distinct environmental factors contribute to adult phenotypic variability and susceptibility to a number of diseases, including cancers and metabolic and autoimmune disorders (van Vliet et al., 2007; Liu et al., 2008; Wilson, 2008; Portela and Esteller, 2010).

So far, most of the reported chromatin modifications induced by bacteria are histone acetylation/deacetylation and phosphorylation/dephosphorylation events generated through activation of host cell signaling cascades by bacterial components (e.g., microbe-associated molecular patterns, metabolites, and virulence factors). The effects are complex, because they differ according to the bacterial agonist, cell type, and kinetic parameters. Among the host

signaling pathways that activate a number of bacteria, MAPKs (e.g., ERK and p38), NF-κB, and PI3K pathways are known to trigger the kinases that phosphorylate histone H3 in Ser10 in the nucle us (Yamamoto et al., 2003; Baek, 2011). Any bacterial stimulus activating these pathways has therefore the potential to induce histone H3 phosphorylation in Ser10 and associated acetylated histones. An example is reported by Ottaviani and collaborators that recently showed the induction of phospho-acetylation of histone H3 after administration of LPS in an invertebrate model (Ottaviani et al., 2013).

Aberrant inflammatory reactions in response to sustained exposure to microbes and microbial products, such as LPS, lead to tissue damage, multi-organ dysfunction, septic shock, and death. To compensate for these adverse effects, the immune system has developed post-septic immunosuppression (PSI) mechanisms that enable hematopoietic cells to become temporary hypo-responsive. This compensatory anti-inflammatory response counteracts the harmful effects of sepsis, but leaves individuals more susceptible to opportunistic infections for extended periods of time (weeks to years). Although PSI is a complex multifactorial process, the contribution of epigenetic regulations is increasingly recognized, as reviewed recently (McCall et al., 2010; Carson et al., 2011). One of the facets of PSI is LPS tolerance, in which LPS-elicited TLR4 responses are reprogrammed toward silencing of pro-inflammatory cytokine genes and expression of anti-inflammatory or antimicrobial mediators. LPS activation of TLR4 first elicits transcription of poised pro-inflammatory genes, which are rapidly depressed and then returned to basal state within hours. Opening the chromatin at target genes during this acute phase involves histone phosphorylation and acetylation. However, sustained exposure to LPS or subsequent LPS challenge activates a pathway leading to permanent gene repression, as characterized for TNF-α and IL1-β *(*El Gazzar et al., 2008;Chen et al., 2009); this relies on a change in the composition of the NF-κB transcription factor at the proximal promoters of TNF-α and IL1-β.

LPS tolerance can last for weeks in humans, but whether this memory is passed through cell division is not yet proven. Additionally, even if imprinted hematopoietic cells divide, why new cells from progenitors in the bone marrow do not restore an efficient immune system remains a question to be answered. A tempting hypothesis would be that epigenetic imprinting also occurs at the level of stem cells. This hypothesis needs to be investigated by analyzing the epigenome of stem cells isolated in animal models of sepsis. The reversal of heterochromatin to euchromatin at genes targeted for LPS-mediated repression is also a key issue to understand how “imprinted” immune cells return to homeostasis.

2. GENERAL AIM

Given the premises, the existing knowledge is that the CNS is capable to respond to environmental cues by gene transcription and synthesis of new proteins through the dynamic remodeling of chromatin architecture. Epigenetic mechanisms have been proposed as the most accredited mechanisms in altering chromatin structure and determining an active or repressive conformation to the transcription activity. In particular, our research is focused on the histone modification occurring in such pathophysiological processes as inflammation and how this process mediates the brain immune response.

In this context the general aim of this thesis was to better understand the role of epigenetic mechanisms, in particular the histone H3 phospho-acetylation, in regulating gene transcription in the cells of hypothalamus and hippocampus respectively, of a rat model in which a systemic administration of the bacterial lipopolysaccharide was used to mimic a neuro-inflammatory condition.

Consequently, in a step by step way, answering to six specific hypothesis, we tried to find explicit answers to sustain our general aim. In specific aim

1) the hypothesis was tested that LPS-challenge affects the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, the immediate early gene c-Fos, and the inducible nitric oxide synthase enzyme (iNOS) at 2 and 6 hours following LPS exposure (830 μg/kg) in the rat hippocampus and hypothalamus; in
2) that phospho-acetylation of histone H3, induced by LPS, plays a role in the regulation of the transcription of the main inflammatory mediators produced in the immune response; in
3) that LPS affects the histone H3 phospho(Ser-10)acetylation(Lys-14) protein expression at 2 and 6 hours after LPS injection in the nuclear protein fraction of rat hypothalamus and hippocampus, in
4) that the possible signaling pathways mobilized and responsible for LPS-induced histone H3 phospho(S10)-acetylation(K14) are the transcription factors CREB and NF-kB; in
5) that some specific cell populations respond to histone H3 phospho(S10)-acetylation(K14) induced by LPS stimulus in a time dependent way; and in
6) that microglia are the predominant cell population which responds to the LPS-induced histone H3 phospho(S10)-acetylation(K14), therefore, they have the capacity to regulate the transcriptional response to the immune stimulus in the brain.

3. AIM 1

In response to a pathogen, activated innate immune cells in the periphery produce pro-inflammatory cytokines such as IL-1β, IL-6 ant TNF-α and induce the production of the same molecular mediators by microglia cells and macrophage-like cells in the brain. Thus orchestrating the development of sickness behavior (Dantzer et al., 2009). Sickness behavior refers to the behavioral and physiological symptoms that develop during infection and include fatigue and malaise, depressive-like state, lethargy, decreasing sleep patterns, hyperalgesia, fever and neuroendocrine activation (Hart, 1988). This response can be induced experimentally in animal models by the administration of viral or bacterial material mimicking infection such as lipopolysaccharide (LPS), the active fragment of endotoxin from Gram-negative bacteria. LPS induces a strong activation of the immune system that causes the production of both peripheral and brain pro-inflammatory cytokines (Layè et al., 1994). These mediators seem to act at different levels in the CNS and determine profound alterations in neurological and endocrine functions, particularly in brain areas belonging to the limbic system. Considering that LPS-induced sickness behavior in rodents was previously demonstrated to occur peak level in a specific time- and dose-dependent manner (Frenois et al., 2007), we used these parameters to evaluate the LPS effects at the chromatin level and on transcriptional activity.

By means of Real Time PCR, LPS-induced molecular effects were studied in the rat hippocampus and hypothalamus, 2 and 6 hours after receiving an intraperitoneal injection with LPS (830 µg/kg). The hypothalamus and hippocampus were chosen as the two limbic areas of interest because they have been implicated in the neurobiological mechanisms underlying the behavioral, cognitive, emotional and motivational alterations of cytokine-induced sickness behavior as well as the physiological response to infection (Dantzer et al., 2009; Andrè et al., 2008; Frenois et al., 2007). Specifically, the hypothalamus participates in the control of the HPA axis activity and in the stress response and coordinates physiological functions such as sleep and feeding, altered in the cytokine-induced sickness syndrome (Gore, 2010; Harden et al., 2010). The hippocampus plays an important role in the negative feedback on the HPA axis activity as well and regulates the mechanisms such as learning and memory both strongly affected by immune challenges such as by LPS (Arai et al., 2001; Pugh et al., 1998). Notably, the hypothalamus and hippocampus are also key structures for brain cytokine expression and function (Andrè et al., 2008).

Given this, first series of experiments, that pertain general aim 1, tested the effects of an LPS-challenge on pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, the immediate early gene c-Fos, and on the expression of the inducible nitric oxide synthase enzyme (iNOS) at 2 and 6 hours following LPS administration (830 µg/kg) in the rat hippocampus and hypothalamus.

4. RESULT 1

4.1 Systemic LPS increased the expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, the enzyme iNOS and the immediate early gene c-Fos in rat hypothalamus and hippocampus.

In order to ensure the effectiveness of LPS (830 μg/Kg) in stimulating the immune system and induce an inflammatory condition in the CNS, the expression levels of major pro-inflammatory cytokines known to be produced in the brain, such as IL-1β, IL-6 and TNF-α, were measured in the rat hypothalamus and hippocampus after a peripheral LPS challenge by Real Time PCR (Dantzer, 2004).

Before testing the effect of LPS on the expression of our target genes of interest, a comparison between all the control samples, at 2 and 6 hours, was performed; controls were analyzed by one-way ANOVA analysis.

Pro-inflammatory cytokines in hypothalamus

In the hypothalamus, one-way ANOVA revealed a main effect of time [$F(1;15) = 6{,}306$, $p = 0.022$] and of treatment [$F(1;15) = 169{,}661$, $p < 0.01$] for IL-1β mRNA expression as well as for TNF-α mRNA, time [$F(3;15) = 6{,}094$, $p = 0.024$] and LPS treatment [$F(1;15) = 50{,}554$, $p < 0.01$] respectively to control group. An effect of treatment was observed for IL-6 mRNA expression [$F(3;15) = 27{,}274$, $p < 0.01$]. Interestingly, *post hoc* tests indicated that after 2 hours following LPS treatment the mRNA levels of IL-1β, IL-6 and TNF-α significantly increased with respect to the control group ($p < 0.01$) (Figure 1A,C). The increase continued also after 6 hours, especially for IL-1β and TNF-α RNA ($p < 0.01$) (Figure 1B).

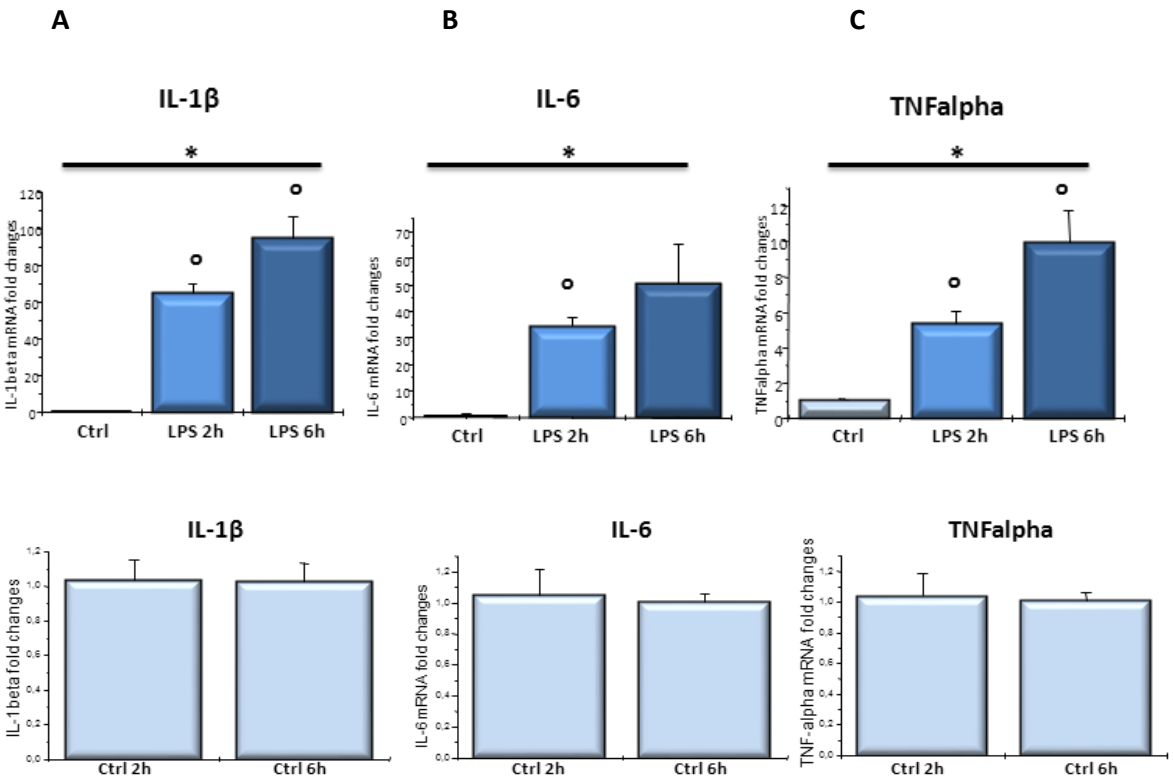


Figure 1. A peripheral LPS challenge (830 µg/Kg, i.p.) induces, in rat hypothalamus, the pro-inflammatory cytokines IL-1β (A), IL-6 (B) and TNF-α (C) 2 hours after exposure to LPS which last at least until 6 hours. Adult rats were injected i.p. with LPS (n=6) with respect of control treated with saline (n=6). The relative expression level of IL-1β, IL-6 and TNF-α were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes of treated animals above the expression of control (saline injected) animals. Bars indicate the mean ± S.E.M.; * statistical significant relative to difference between LPS-treated and control animals *p<0.05(One-way ANOVA); ° p<0.01 (*post hoc* Dunnet). No effect comparing 2 hours control group and 6 hours control group (One-way ANOVA) was observed.

Pro-inflammatory cytokines in hippocampus

A similar expression pattern was observed in the hippocampus: one-way ANOVA evidenced a significant effect of time [F (1;17) = 5,300, p = 0.034] and treatment [F (1;17) = 8,564, p < 0.01] for IL-1β mRNA and for TNF-α mRNA level of expression, time [F (1;17) =13,019, p < 0.01] and treatment [F (1;17) = 13,650, p < 0.01] respectively. With respect to IL-6 mRNA expression, only a treatment effect was observed [F (1;16) = 16,567, p < 0.01]. *Post-hoc* tests revealed that after 2 and 6 hours following LPS treatment the mRNA levels of IL-1β, IL-6 significantly increased with respect to the control group (p < 0.05) (Figure 2A,B) and continued stay up to 6 hours. In contrast, a strong increase of TNF-α mRNA was detected at 6 hours (p < 0.01) but not at 2 hours (Figure 2B).

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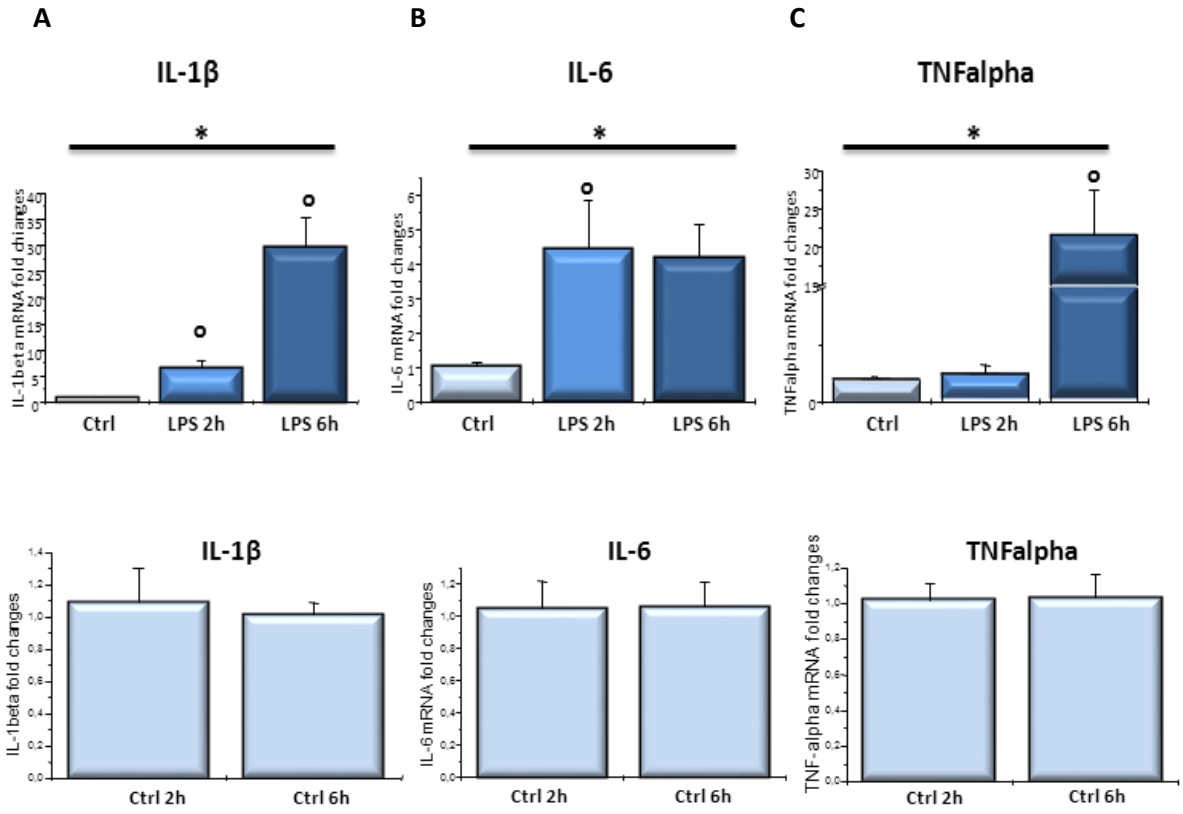

Figure 2. A peripheral LPS challenge (830 µg/Kg, i.p.) induced, in rat hippocampus, the pro-inflammatory cytokines IL-1β (A), IL-6 (B) already 2 hours after exposure to LPS which last at least until 6 hours, instead TNF-α expression (C) increased at 6 hours. Adult rats were injected i.p. with LPS (n=6) with respect of control treated with saline (n=6). The relative expression level of IL-1β, IL-6 and TNF-α were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes of treated animals above the expression of control (saline injected) animals. Bars indicate the mean ± S.E.M.; * statistical significant relative to difference between LPS-treated and control animals *p<0.05(One-way ANOVA); ° p<0.01 (*post hoc* Dunnet). No effect comparing 2 hours control group and 6 hours control group (One-way ANOVA) was observed.

The hypothesis was tested whether a peripheral LPS challenge is able to induce brain cells activation 2 and 6 hours after receiving an immune challenge in the rat hypothalamus and in the hippocampus.

iNOS in hypothalamus and in hippocampus

Subsequently, we focused on another cellular signaling molecule, nitric oxide (NO), known to be part of defense mechanisms. In particular, we investigated inducible nitric oxide synthase (iNOS) which plays an important role in the response of the body to the attack by parasites, bacterial infection, as well as tumor growth, and produces NO upon stimulation by pro-inflammatory cytokines such as IL-1 and TNF-α.

Results indicated a significant main effect of treatment [$F(1;17) = 23{,}960$; $p < 0.01$] and of time [$F(1;17) = 19{,}925$; $p < 0.01$] for iNOS mRNA expression in hypothalamus indicating a significant interaction between the two factors [$F(1;17) = 19{,}940$; $p < 0.01$]. Particularly, *post hoc* tests suggested that LPS induced a strong activation of iNOS at 6 hours ($p < 0.01$) with respect to control animals (Figure 3A).

In the hippocampus, time appeared not to have an effect on iNOS expression, unlike LPS treatment [$F(1;17) = 14{,}890$; $p < 0.01$]; here, *post hoc* tests denoted a significant increase of iNOS mRNA expression at 6 hours ($p < 0.01$) in LPS-treated animals with respect to saline-treated animals (Figure 3B).

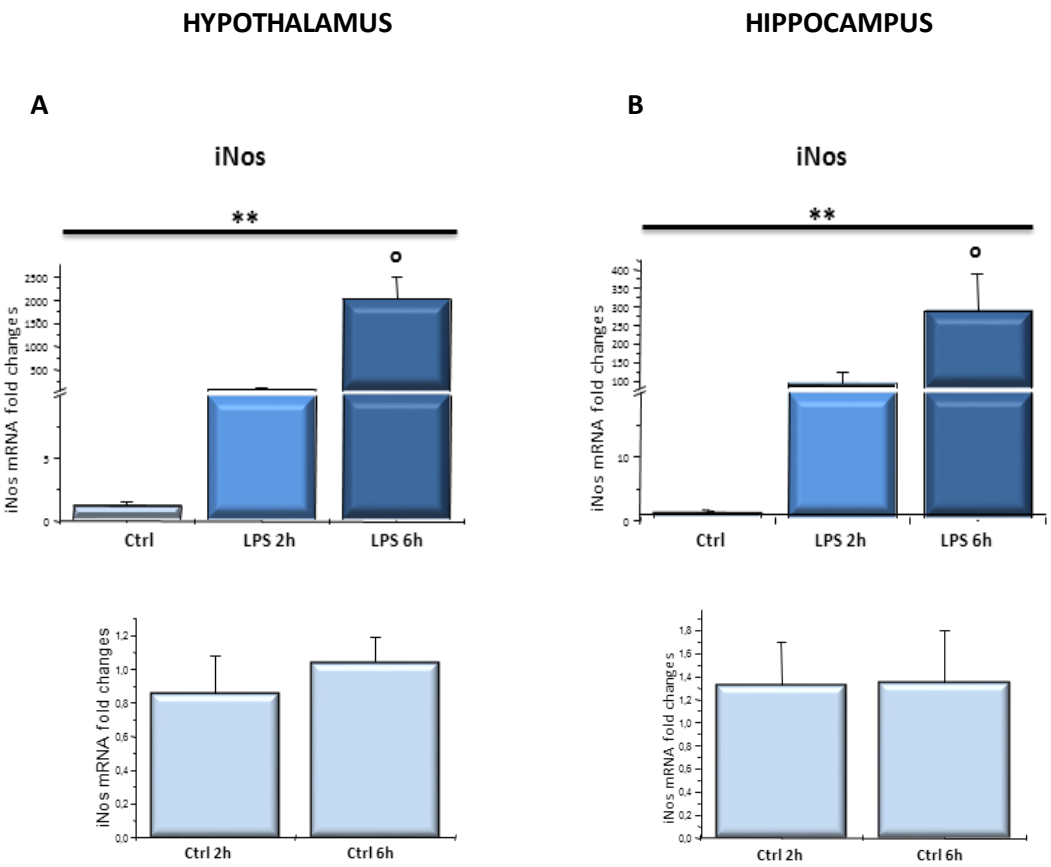

Figure 3. A peripheral LPS challenge (830 µg/Kg, i.p.) induced the expression of iNOS after 2 hours which further increased until 6 hours, in the rat hypothalamus (A) and hippocampus (B). Adult rats were injected i.p. with LPS (830 µg/Kg, n=6) or with saline (n=6). The relative expression levels of iNOS were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes of treated animals above the expression of control (saline injected) animals. Bars indicate the mean ± S.E.M.; *statistical significant relative to difference between LPS-treated and control animals **p<0.01(One-way ANOVA); ° p<0.01 (*post hoc* Dunnet). No effect comparing 2 hours control group and 6 hours control group (One-way ANOVA) was observed.

Finally, the hypothesis was tested that LPS affects the expression of the immediate early gene c-Fos, which is considered to be a marker of neuronal activity and shown to be induced after the activation of the peripheral innate immune system in a rapid and transient manner in a number of brain areas proposed to be involved in the cytokine-induced sickness response (Konsman et al., 2000).

C-Fos in hypothalamus and in hippocampus

One-way ANOVA showed a main effect of LPS in rat hypothalamus [F (1;17) =28,635; p < 0.01] and hippocampus [F (1;18) = 5,259; p = 0.034] which did not change significantly over the time. *Post hoc* tests identified an increase of c-Fos mRNA expression after 6 hours following LPS administration in the hippocampus (p < 0.05) (Figure 4A), while in the hypothalamus LPS enhanced c-Fos expression at 2 hours (p < 0.01) up to at least 6 hours after exposure (Figure 4A).

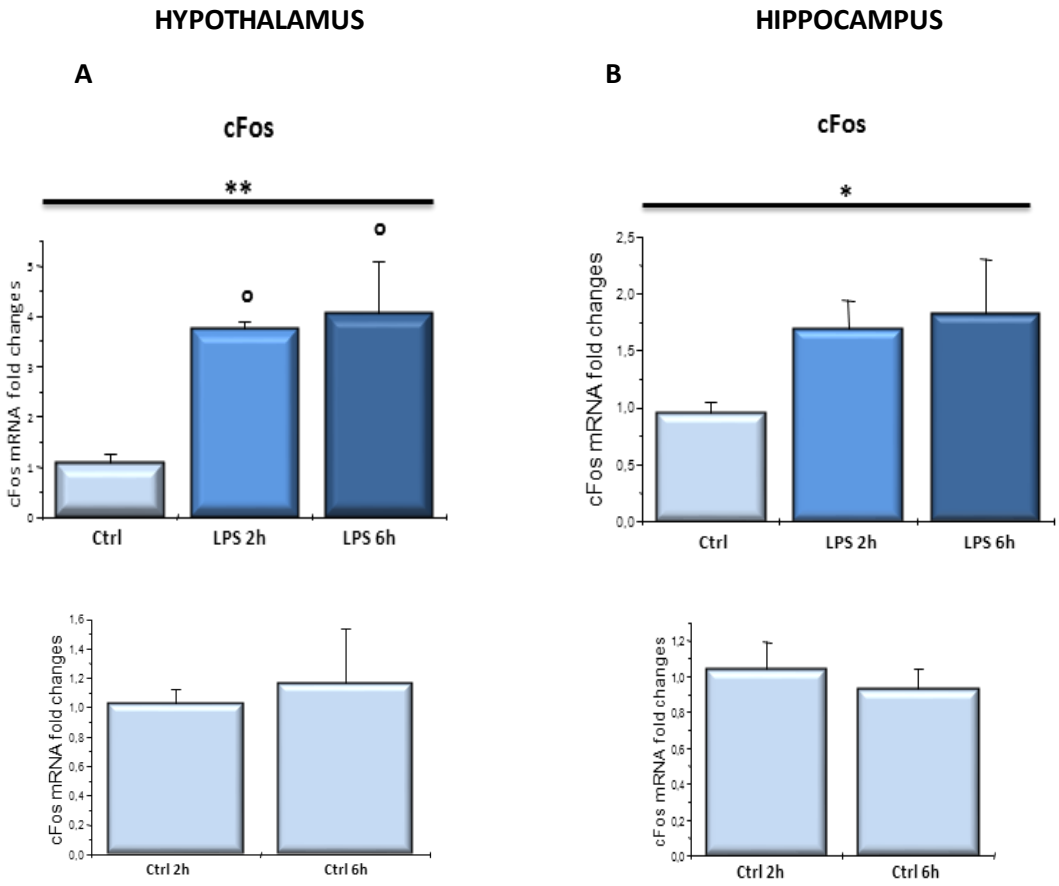

Figure 4. A peripheral LPS challenge (830 µg/Kg, i.p.) induced the expression of c-Fos 2 hours after exposure to LPS which remained high until 6 hours in the rat hypothalamus (A) and hippocampus (B). Adult rats were injected i.p. with LPS (830 µg/Kg, n=6) or with saline (n=6). The relative expression levels of c-Fos were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes of treated animals above the expression of control (saline injected) animals. Bars indicate the mean ± S.E.M.; *statistical significant relative to difference between LPS-treated and control animals **p<0.01, *p<0.05 (One-way ANOVA); ° p<0.01 (*post hoc* Dunnet). No effect comparing 2 hours control group and 6 hours control group (One-way ANOVA) was observed.

5. AIM 2

Epigenetic modifications of both DNA and histone proteins are now accepted as fundamental mechanisms by which neurons adapt their transcriptional response to developmental and environmental cues. Epigenetic changes allow to modify gene expression on the basis of cell requirements and environmental stimuli (Zhou et al., 2011). Environmental influences, such as, inflammation or stress, play a key role in determining the susceptibility to disease and in particular to brain disease (Benatti et al., 2012). Exposure to inflammatory agents provokes the release of stress-related molecules by the immune system involving the CNS and thus causing alterations in ordinary neuro-immune crosstalk.

While, epigenetic processes seem to fix information from the environment in neurons, accurate knowledge concerning these underlying processes is still rather limited.

One of the main epigenetic modifications, known to play a crucial role in the regulation of transcriptional activity, is on histone proteins which cause a remodeling of the chromatin conformation and, subsequently, an alteration in the transcriptional activity. Particularly, studies here focused on a specific modification; the simultaneous phosphorylation on a residue of Serine-10 and acetylation on a residue of Lysine-14 in the N-terminal of the histone H3.

Little is known about the exact role of this modification in regulating transcription of specific genes, involved in pathological processes, but recent data suggest its capacity to activate transcriptional response.

Histone H3 phospho-acetylation is dynamic and reversible and could serve to amplify the readout of upstream signaling pathways causing greater changes in the overall charge density of tails that lead to greater changes in the chromatin structure of target genes (Rea et al., 2000; Cheung et al., 2000b; Thomson et al., 2001; Nowak et al., 2000).

Given this, and once established the presence of an inflammatory status in our model, the second aim of my thesis was to investigate the effect of an inflammatory stimulus (LPS) on the phospho-acetylation of histone H3 in the two main brain areas examined.

So, histone H3 phospho(Ser-10)acetylation(Lys-14) protein expression was studied at 2 and 6 hours after LPS injection in the nuclear protein fraction of rat hypothalamus and hippocampus.

6. RESULT 2

6.1 A short term LPS challenge induced a strong phospho-acetylation of histone H3 protein levels in the rat hypothalamus and hippocampus

In order to test the effect on the modification of histone H3 of an LPS-induced inflammatory stimulus, a specific antibody was used to measure the expression level of phospho-acetylation in the nuclear fraction extracted from the rat hypothalamus and hippocampus at 2 and 6 hours after the systemic administration of LPS (830 µg/Kg i.p.).
Here, a comparison between all the control samples, at 2 and 6 hours, was performed in samples from the rat hypothalamus and hippocampus.

Hypothalamus

In the hypothalamus, results revealed a main effect of treatment, but not of time, on the phospho(S10)-acetyl(K14)H3/H3 ratio [one-way ANOVA $F(1;16) = 21{,}305$, $p < 0.01$) suggesting that the effect depended exclusively on LPS and not on the time of treatment.
Post hoc analyses showed that LPS enhanced phospho-acetylation of H3 protein levels with respect to total H3 levels at 2 hours following treatment ($p < 0.01$) with respect to control (Figure 5A). Whereas the total H3 protein levels were not influenced (Figure 5C).
Six hours after LPS injection, H3 phospho-acetylation protein levels decreased and the mean effect disappeared compared to 2 hours and as well as when comparing to controls.

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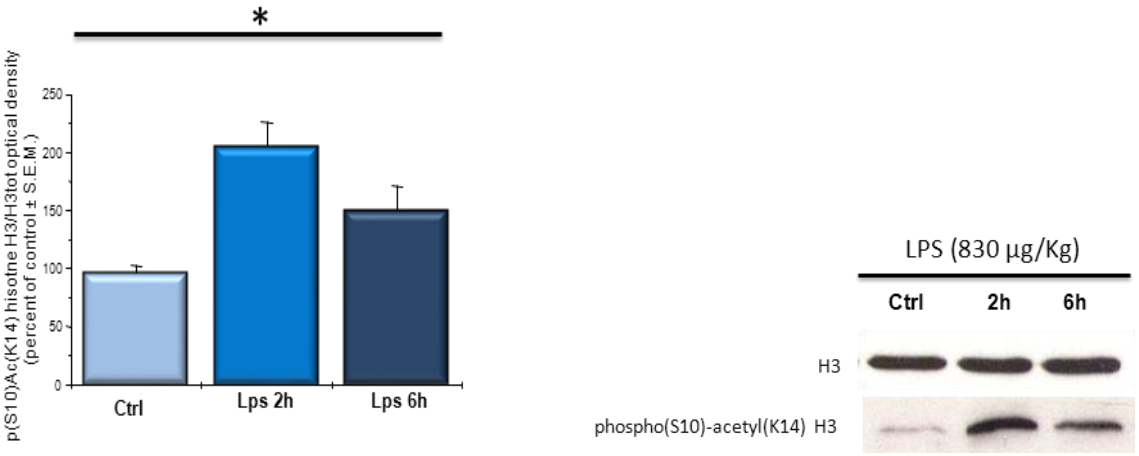
A **B**



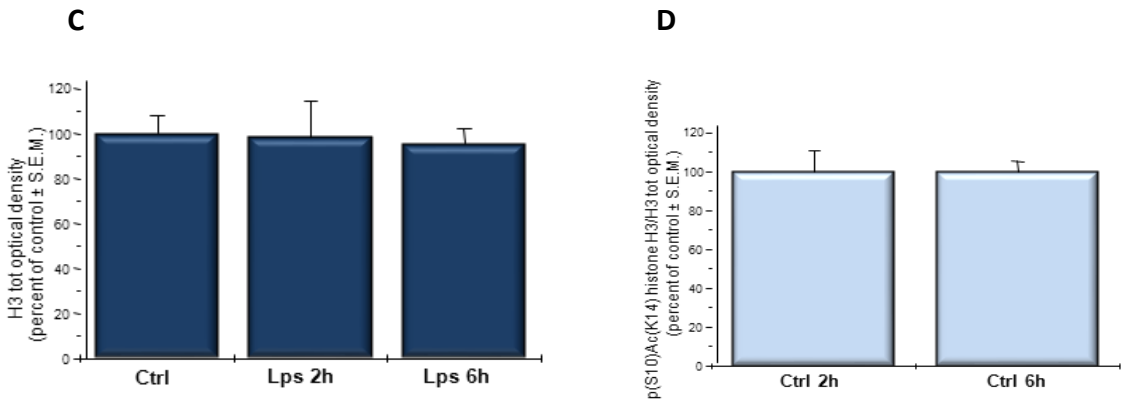


Figure 5. A systemic injection of LPS (830 µg/Kg i.p. n=6) enhanced phospho(S10)-acetyl(K14)H3 protein levels above those of total H3 2 hours after LPS exposure in the hypothalamus (A). No changes were observed in controls with respect to phospho(S10)-acetyl(K14)H3 neither at 2 hours nor at 6 hours post LPS (D). Nuclear proteins extracted from rat hypothalamus were measured by Western Blot. Phospho-acetyl-H3 protein levels were normalized on total histone H3 levels and both proteins were detected on the same blot. Total H3 protein levels were not affected by LPS treatment nor by time (C). A representative blot of phospho(S10)-acetyl(K14)H3 detected is shown (B). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.; * $p<0.01$ among the groups (one-way ANOVA).

Hippocampus

Instead, in the hippocampus, statistical analysis demonstrated a significant effect of LPS treatment [one-way ANOVA: $F(1;17) = 14{,}711$, $p < 0.01$] and of time [one-way ANOVA: $F(1;17) = 16{,}260$, $p < 0.01$] for the phospho-acetylation of H3/H3 ratio, while LPS treatment did not modify total H3 protein levels (Figure 6C). The two main effects were characterized, however, by a significant interaction between the two factors [one-way ANOVA: $F(1;17) = 16{,}260$, $p < 0.01$], indicating that the effect of LPS was not the same with respect to the time points considered. In fact, *post hoc* analyses confirmed a specific increase of H3 phospho-acetylation protein levels with regard to total H3, 2 hours following the LPS treatment ($p < 0.01$) (Figure 6A) and not at 6 hours, with respect the control group. Comparing the control groups at two time points, no differences were observed (Figure 6D).

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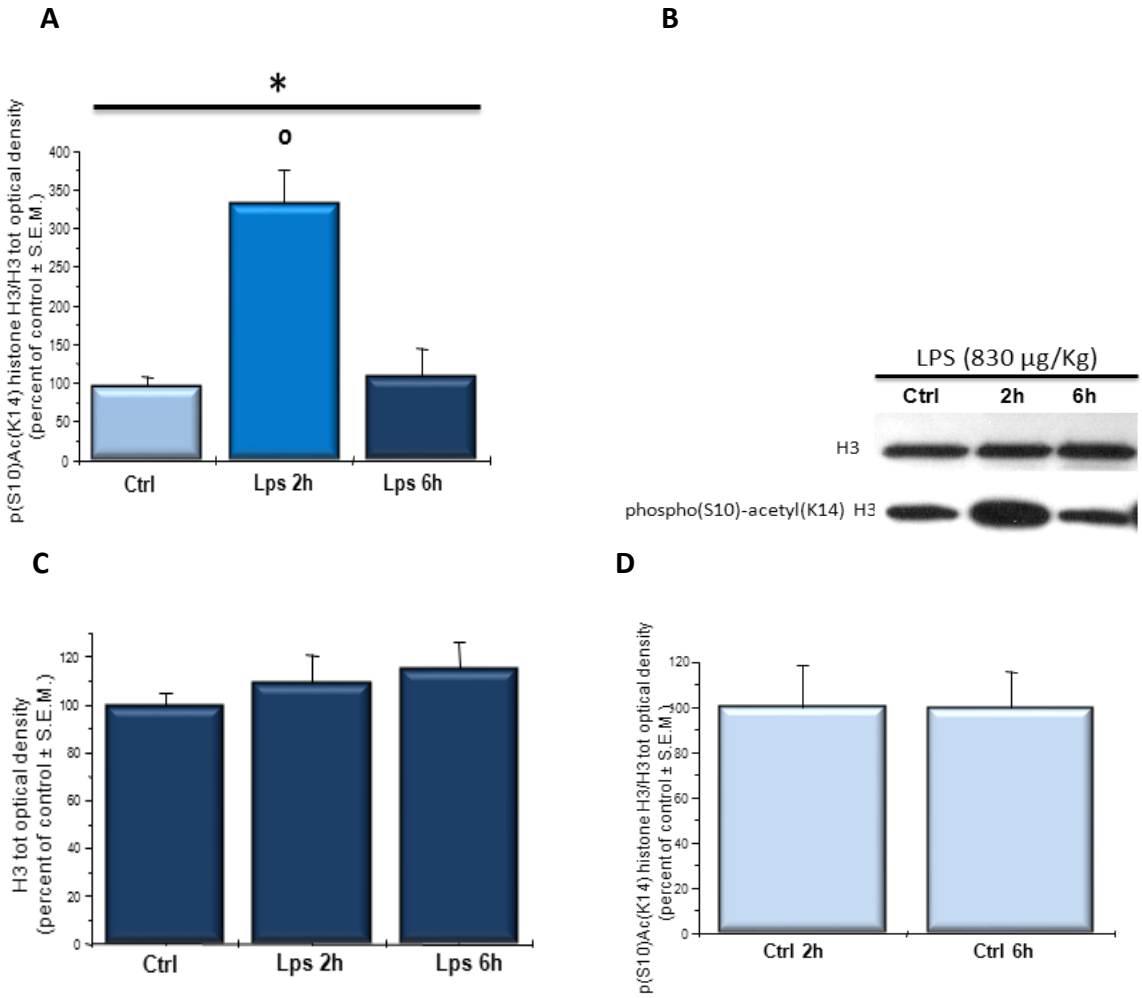

Figure 6. A systemic injection of LPS (830 µg/Kg i.p. n=6) enhanced phospho(S10)-acetyl(K14)H3 protein levels above those of total H3 2 hours after LPS exposure in the hippocampus (A). No changes were observed in controls with respect to phospho(S10)-acetyl(K14)H3 neither at 2 hours nor at 6 hours post LPS (D). Nuclear proteins extracted from rat hippocampus were measured by Western Blot. Phospho-acetyl-H3 protein levels were normalized on total histone H3 levels and both proteins were detected on the same blot. Total H3 protein levels were not affected by LPS treatment in every time points considered (C). A representative blot of phospho(S10)-acetyl(K14)H3 detected is shown (B). Optical density was expressed as % of control (saline-treated) group. Each column represents mean ± S.E.M.; *$p<0.01$ among the groups (one-way ANOVA).°$p<0.01$ with respect to the control group (*post hoc*).

7. AIM 3

The concept of chromatin remodeling addresses a key challenge in neurobiology of how stable changes in gene expression are induced in neurons and glia to produce long-lasting changes in behavior (Colvis et al., 2012). The nervous system responds to complex metabolic signals, diverse cellular processes and environmental changes by gene transcription responses and synthesis of new proteins, mediated by the dynamic remodeling of chromatin architecture transacted by an ever expanding array of enzymes and associated signal transduction pathways (Citri et al., 2007; Colvis et al., 2005). Moreover, literature indicates that environmental stimuli result in altered patterns of transcription in the brain.

The mammalian brain depends on numerous complex and highly regulated mechanisms to appropriately activate or silence gene programs in response to environmental input and developmental cues. At the molecular level, these events are controlled by activity-dependent signaling pathways that mediate gene expression by modifying the activity, localization, and/or expression of transcriptional-regulatory enzymes in combination with alterations in chromatin structure in the nucleus (McClung and Nestler, 2008).

Bacterial or viral infection represent one of the main environmental "experiences" organisms most often meet in their lifetime and, as such, may determine important alterations in the regulation of gene transcriptional responses through epigenetic processes.

Experiments of aim 1 and 2 showed that LPS induced a massive transcriptional activity of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, the enzyme iNOS and the early gene c-Fos, and, at the same time, enhanced the phospho-acetylation of histone H3.

Given the premise, the hypothesis tested was that phospho-acetylation of histone H3, induced by LPS, play a role in the regulation of the transcription of the main inflammatory mediators produced in the immune response. To test this hypothesis we used a chromatin immuno-precipitation (ChIP) assay to examine the effect of LPS on the phospho-acetylation status of histones H3 in the promoter region of IL-1β, IL-6, iNOS and c-Fos genes, induced by LPS-challenge.

8. RESULT 3

8.1 Histone H3 phospho(S10)-acetylation(K14) mediated the gene transcription response induced by LPS-challenge differently in hypothalamus and hippocampus

The phospho-acetylation of histone H3 in transcriptional activation by opening chromatin conformation has now been widely accepted (Cheung et al., 2000b).

Here we hypothesized that LPS affects the phospho-acetylation status of histones H3 in the promoter region of IL-1β, IL-6, iNOS and c-Fos genes. First the chromatin fragment was immunoprecipitated using a specific antibody for phospho(S10)-acetyl(K14) histone H3 (ChIP), then, the expression of the IL-1β, IL-6, iNOS and c-Fos genes on the promoter region was tested using a qualitative PCR and specific primers pairs (see Material and Methods for further details). Target gene transcription in promoter regions were differentially regulated with respect to brain area and time since LPS treatment.

In the hypothalamus, a representative qualitative PCR showed the effect of H3-phospho-acetylation in regulating the transcription of the iNOS promoter and, to a lesser extent, of c-Fos and IL-6 promoters (Figure 7a,b,d), while absent for the IL-1β expression (Figure 7c) 2 hours after administration of LPS administration. All samples were compared to total chromatin (input) and to the negative control without antibody.

Differently, in hippocampus the effect of H3-phospho-acetylation was observed only with respect to iNOS promoter expression (Figure 7f).

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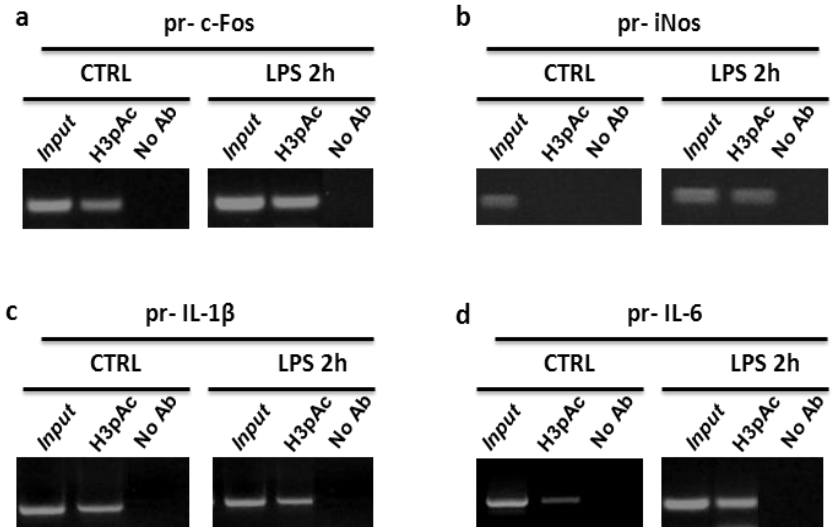

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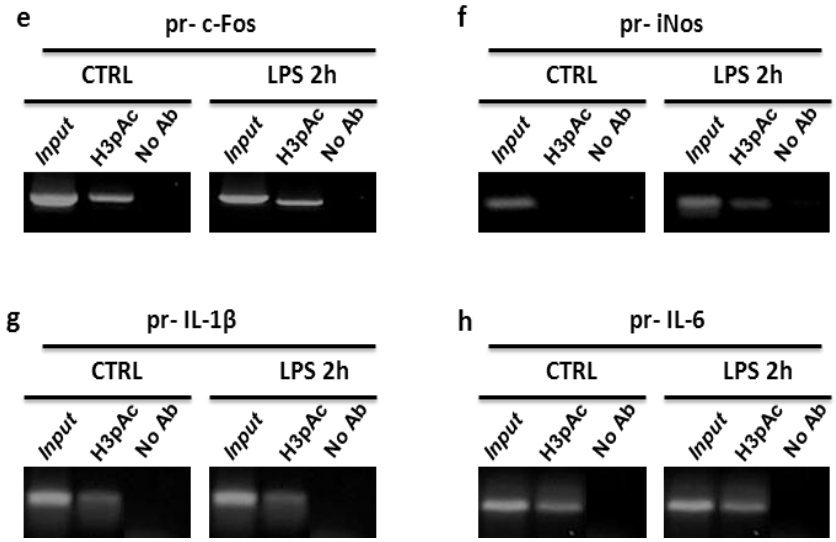


Figure 7. Phospho-acetylation of histone H3 regulated the transcription of IL-1β, IL-6, iNOS and c-Fos gene promoters differently in hypothalamus and in hippocampus of treated rats with respect to saline group 2 hours after exposure to LPS (830 μg/Kg i.p.). Qualitative PCR on the ChIP fragments showed an increase in the expression of IL-6, iNOS and c-Fos promoters in hypothalamus (a,b,d) and only an increase in the iNOS expression in hippocampus (f). Input samples represent the total of chromatin. No antibody sample was used as negative control of the immunoprecipitation.

Moreover, 6 hours after LPS treatment, data indicated that the H3-phospho-acetylation effect was time of treatment dependent, in the hypothalamus, only observable on iNOS promoter (Figure 8b). Instead, no changes were detected for IL-1β, IL-6 and c-Fos expression (Figure 8a,c,d). In the hippocampus, qualitative PCR did not reveal differences in the expression any of the target genes examined when comparing with LPS immunoprecipitated samples and with control samples (Figure 8e,f,g,h).

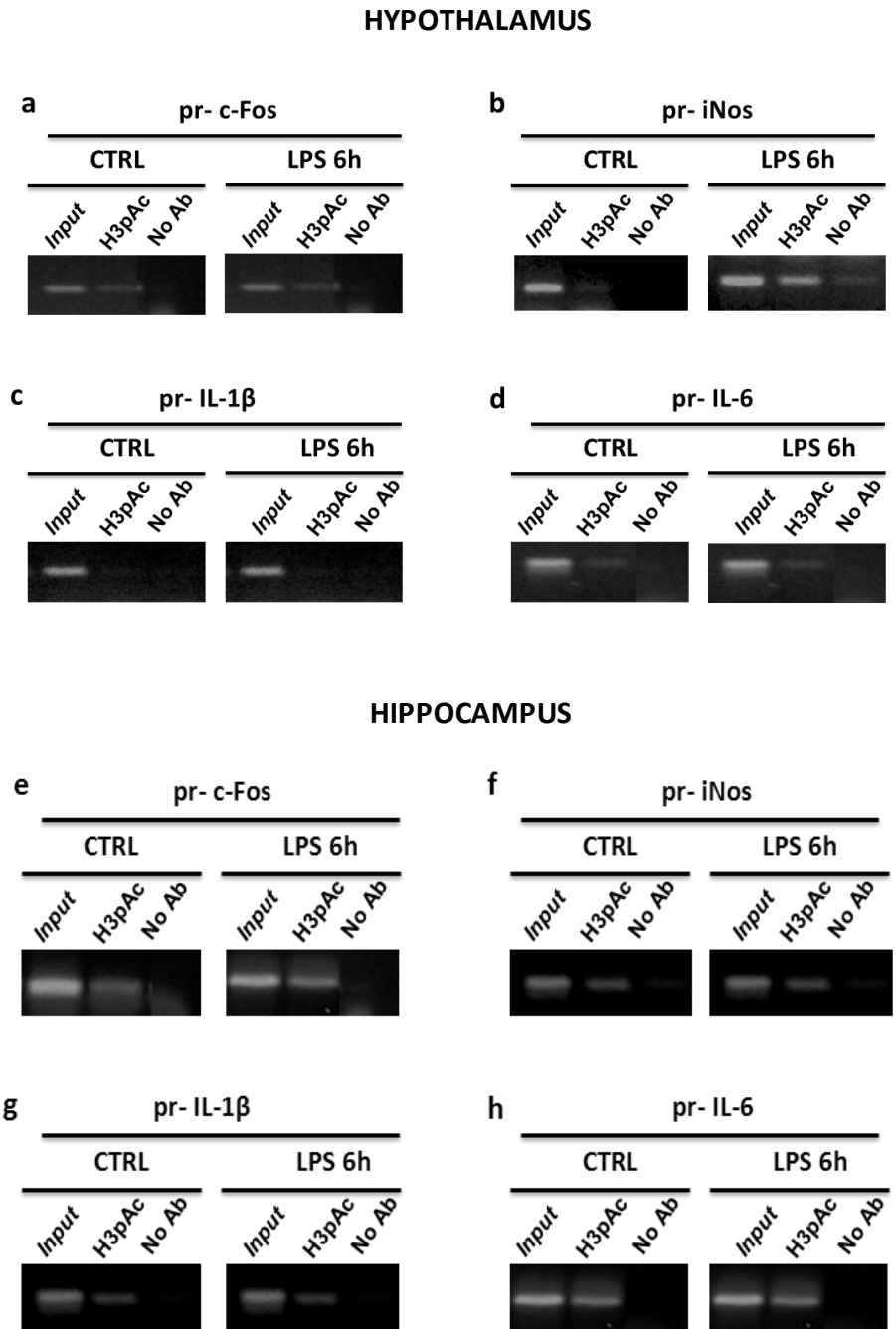


Figure 8. Six hours after a systemic LPS administration (830 µg/Kg i.p.), the effect of H3-phospho-acetylation was observed only on the transcription of iNOS gene promoter (b) in the hypothalamus, while no changes were revealed for IL-1β, IL-6 and c-Fos expression (a,c,d). In the hippocampus of treated rats, qualitative PCR on the ChIP fragments did not show alterations in the expression of IL-1β, IL-6, iNOS and c-Fos promoters (e,f,g,h). Input samples represent the total of chromatin. No antibody sample was used as negative control of the immunoprecipitation.

9. AIM 4

Recent data suggest that signaling pathways, particularly the mitogen-activated protein kinase (MAPK) and mitogen and stress-activated kinases (MSK) cascades, once activated, can phosphorylate residues on histone tails including S10 which may explain its critical role in inducing epigenetic modifications. While MSK has no known acetylase activity it is able to phosphorylate cAMP responsive element (CRE) binding protein (CREB) which after dimerization binds to CRE sites in the promoters of many genes thereby stimulating gene expression (Figure 7). Furthermore, CREB recruits a number of histone modifying enzymes to the chromatin including p300 and/or CREB binding protein (CBP), both of which have HAT activity (Mifsud et al., 2011).

CREB was first identified as a key transcription factor and, in the nucleus, CREB can be modified in multiple ways leading to changes in activity or stability of the protein. In particular, the phosphorylation of CREB at Ser-133 was required for transcription activation by CREB (Montminy et al, 1986) and its ability to influence the expression of genes.

Moreover, phosphorylation and activation of CREB recruits CBP, a transcriptional co-activator with intrinsic HAT activity, which regulates the local chromatin structure as part of CREB-dependent activation of nuclear gene transcription (Ogryzko et al., 1996; Sweatt et al., 2008; Bito et al., 2003; Johannessen et al., 2004; McClung et al., 2008). Furthermore, an increase in CREB activity was observed in the hippocampus following training in certain tasks, or memory provoking stimuli (Silva et al., 2003).

Another transcription factor known to be activated by inflammatory stimuli is NF-κB which orchestrates gene expression involved in biological processes such as inflammation, immune responses, development and differentiation, and apoptosis. NF-kB plays a crucial role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes.

Newly released NF-kB transcription factors form active complexes and translocate into the nucleus to induce expression of their target genes. Research has shown that LPS binds CD14 molecule (CD14) on the cell membrane which transfers LPS to lymphocyte antigen 96 (MD-2) and TLR4. TLR4, via binding MyD88, induces NF-kB-activating cascade.

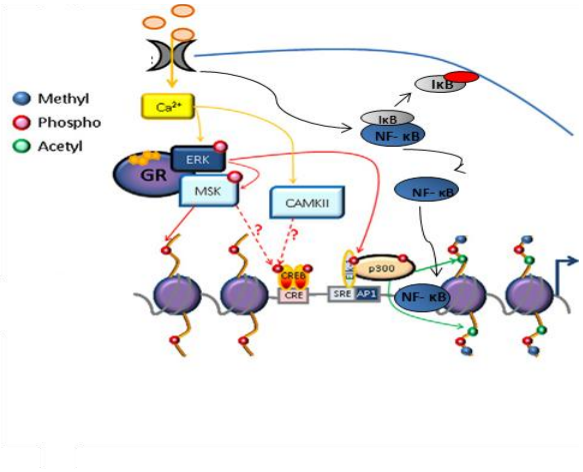


Figure 7. Representation of the phosphorylation pathway of CREB and NF-kB pathway in the transcription activation.

Given this, specific aim 4 experiments focused on better understanding the possible signaling pathways mobilized and responsible for LPS-induced histone H3 phospho(S10)-acetylation(K14). We investigated the effect of LPS at 2 and 6 hours on:

A. the activation of the transcription factor CREB, evaluating the phosphorylation of CREB at Ser-133 in the nuclear protein fraction of rat hypothalamus and hippocampus;
B. the translocation from cytoplasm to nucleus of NF-kB protein in rat hypothalamus and hippocampus.

10. RESULT 4

10.1 In the hippocampus a significant increase of phospho-CREB was present in the nucleus 2 hours after LPS-treatment

To further investigate which pathways mediated LPS signaling at the chromatin level, the phosphorylation of CREB at Ser-133 was analyzed in the cell nucleus of rat hypothalamus and hippocampus.

T-Test revealed a significant increase of phospho-CREB/CREB ratio (t =2,37833; p=0.041) at 2 hours post LPS treatment in hippocampus of rats with respect to saline-treated animals (Figure 9A), while no changes were observed for phospho-CREB in the hypothalamus (Figure 9B).

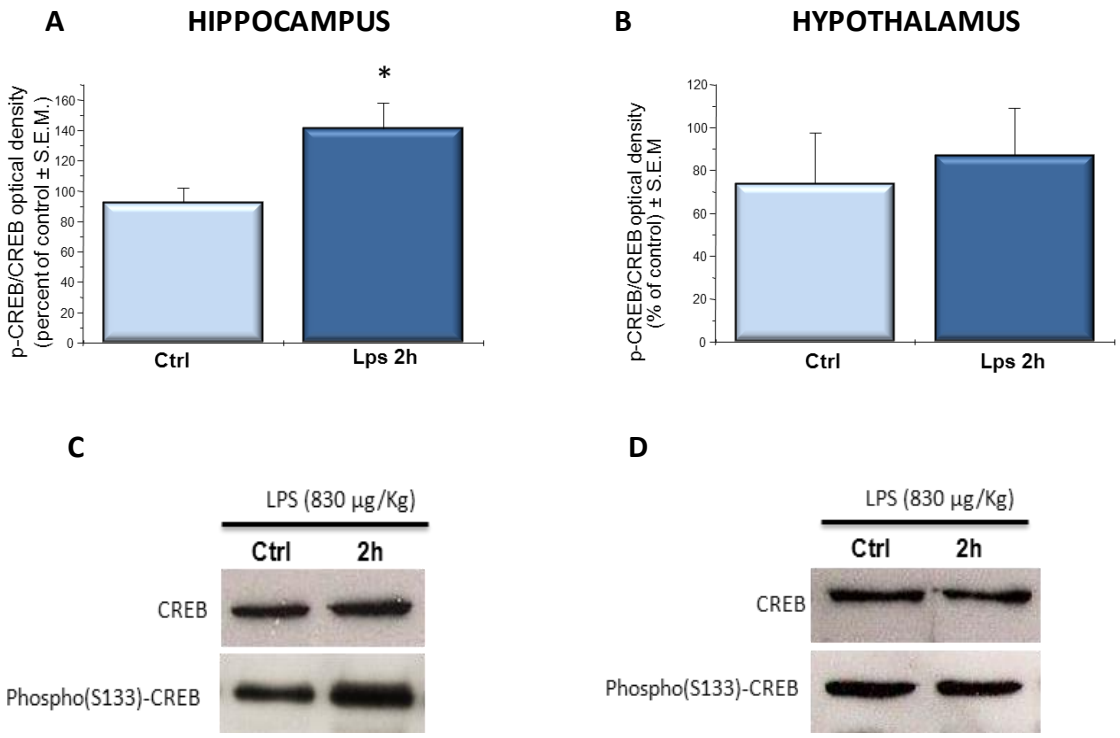


Figure 9. Peripheral injection of LPS (830 µg/Kg i.p. n=6) enhanced phospho-CREB protein levels above those of CREB 2 hours after LPS in the hippocampus (A) with respect to control group (n=6), but not in the hypothalamus (B). Phospho-CREB protein levels were normalized on CREB levels and both proteins were detected on the same blot. A representative blot of phospho-CREB/CREB detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.; * statistical significant difference between LPS-injected and control (saline-injected) animals. *p<0.05.

At 6 hours after treatment, LPS did not affect phospho-CREB protein levels with respect to CREB levels. No significative differences were observed neither in the hypothalamus nor in the hippocampus (Figure 10A,B).

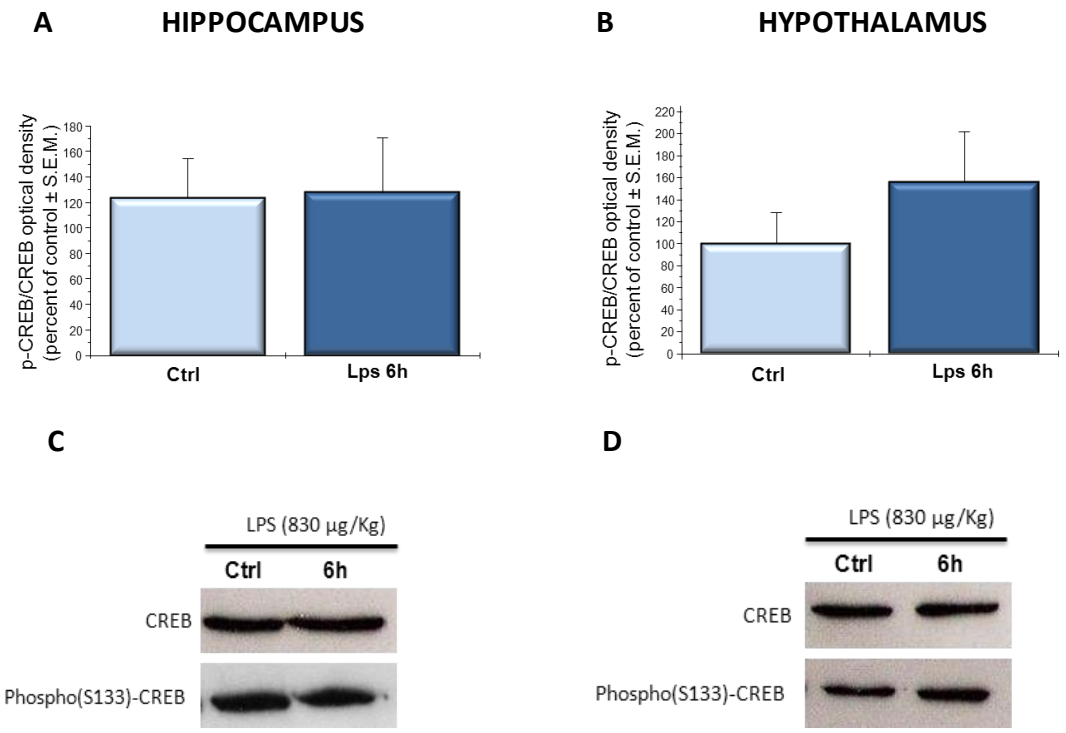


Figure 10. Peripheral injection of LPS (830 µg/Kg i.p. n=6) had no effect on phospho-CREB protein levels after 6 hours with respect to the control group (n=6), in the rat hippocampus (A) and hypothalamus (B). Phospho-CREB protein levels were normalized on CREB levels and both proteins were detected on the same blot. A representative blot of phospho-CREB/CREB detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.; * statistical significant difference between LPS-injected and control (saline-injected) animals. *p<0.05.

10.2 LPS treatment did not influence NF-kB protein levels in the rat hypothalamus and hippocampus after 2 and 6 hours following injection

NF-kB is a versatile molecule necessary for regulating transcriptional activity and plays an active role in inflammatory responses. The protein consists of different subunits and originates in the cytoplasm already assembled then translocates to the nucleus where they act as a transcription factor.

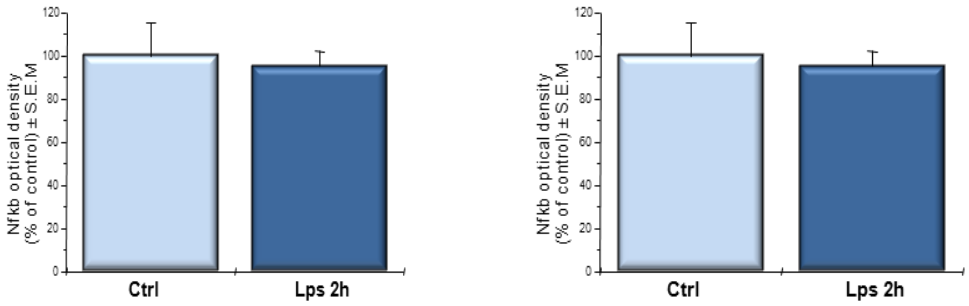
In particular, the p65 subunit of NF-kB is thought to have an additional synergy domain (in addition to the activation domain) and, together with the subunit p50, forms the DNA binding nuclear form of the transcription factor. For this reason, in this part of the study, NF-kB (p65) protein levels were measured in the nuclear and cytoplasmic fractions.

Results showed no significant effect of LPS to induce the translocation of NF-kB (p65) from the cytoplasm to the nucleus both 2 hours or 6 hours after receiving LPS in rat hypothalamus and hippocampus.

More specifically, *t-Test* analysis did not indicate changes in NF-kB (p65) protein levels at 2 hours after LPS treatment in the nucleus (Figure 11A) and in the cytoplasm (Figure 11B) of rat hypothalamus.

HYPOTHALAMUS

A **NUCLEUS** **B** **CYTOPLASM**



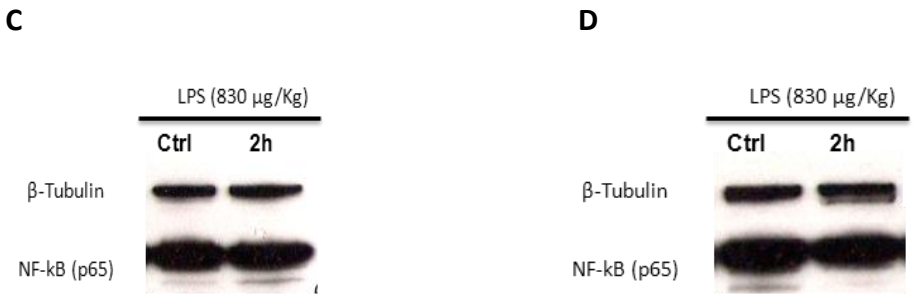
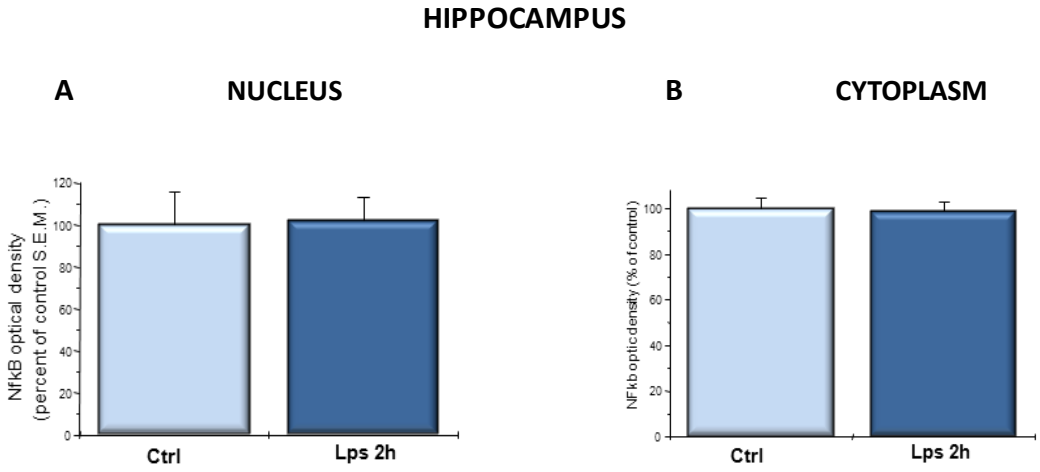


Figure 11. Systemic injection of LPS (830 µg/Kg i.p. n=6) did not affect NF-kB (p65) protein levels above those of standard β-Tubulin 2 hours post LPS in the nucleus (A) and in cytoplasm (B) with respect to the control group (n=6). Nuclear and cytoplasmic proteins extracted from rat hypothalamus were measured by Western Blot. NF-kB (p65) protein levels were normalized on β-Tubulin levels and both proteins were detected on the same blot. A representative blot of NF-kB (p65) detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.

The same pattern was displayed for the hippocampus: no changes in NF-kB (p65) protein expression were observed in the nucleus (Figure 12A) and in the cytoplasm (Figure 12B) 2 hours following LPS treatment compared to controls.



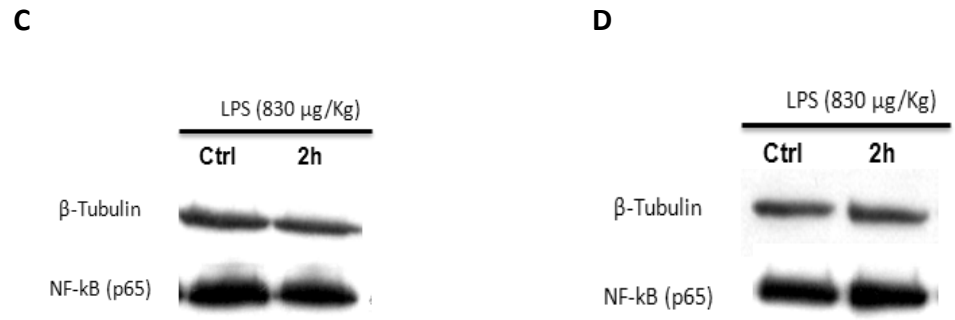
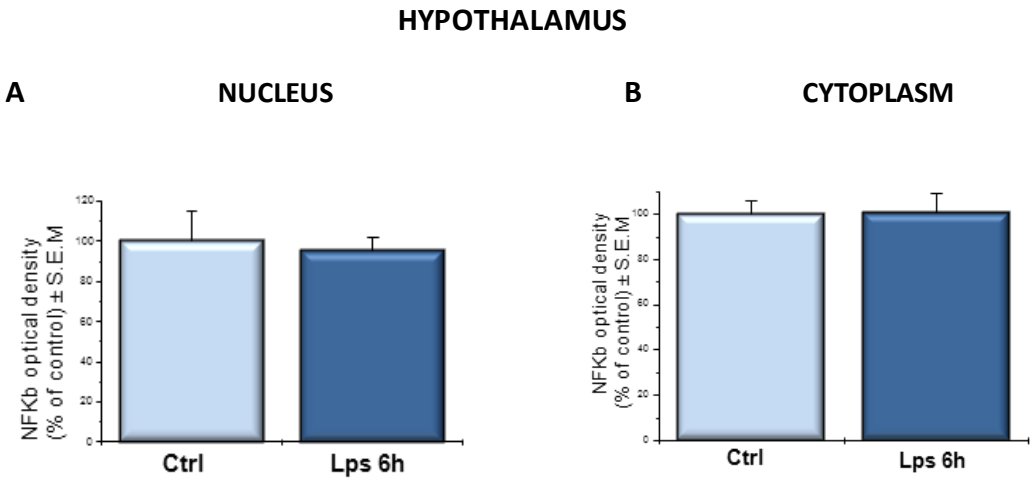


Figure 12. Systemic injection of LPS (830 µg/Kg i.p. n=6) did not affect NF-kB (p65) protein levels above those of standard β-Tubulin 2 hours post LPS in the nucleus (A) and in cytoplasm (B) with respect to the control group (n=6). Nuclear and cytoplasmic proteins extracted from rat hippocampus were measured by Western Blot. NF-kB (p65) protein levels were normalized on β-Tubulin levels and both proteins were detected on the same blot. A representative blot of NF-kB (p65) detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.

Finally, NF-kB (p65) protein expression was tested 6 hours after LPS administration. Here, also *t-Test* analyses did not result in significantly different protein levels in nuclear nor cytoplasmic fractions of the rat hypothalamus (Figure 13) and hippocampus (Figure 14).



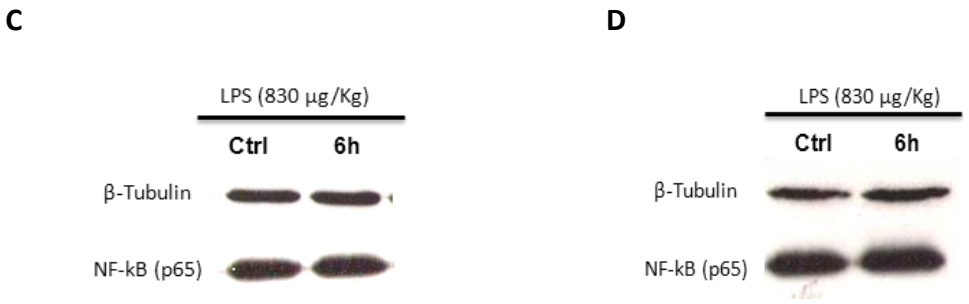


Figure 13. Systemic injection of LPS (830 µg/Kg i.p. n=6) did not affect NF-kB (p65) protein levels above those of standard β-Tubulin 2 hours post LPS in the nucleus (A) and in cytoplasm (B) with respect to the control group (n=6). Nuclear and cytoplasmic proteins extracted from rat hypothalamus were measured by Western Blot. NF-kB (p65) protein levels were normalized on β-Tubulin levels and both proteins were detected on the same blot. A representative blot of NF-kB (p65) detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M.

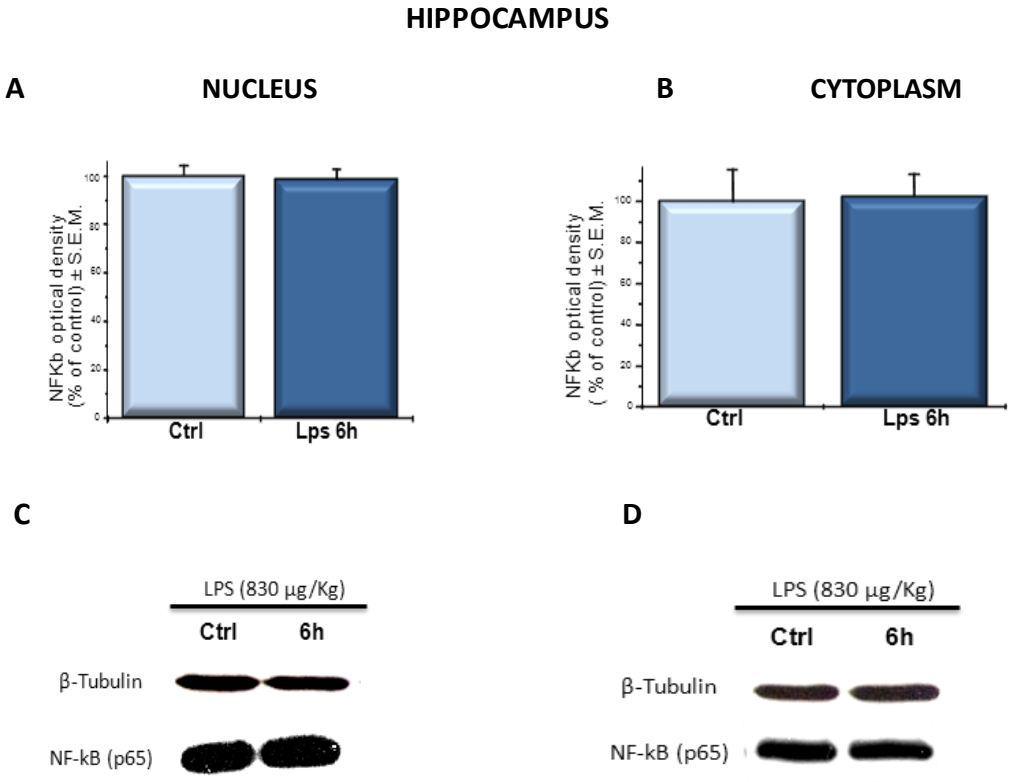


Figure 14. Systemic injection of LPS (830 µg/Kg i.p. n=6) did not affect NF-kB (p65) protein levels above those of standard β-Tubulin 6 hours post LPS in the nucleus (A) and in cytoplasm (B) with respect to the control group (n=6). Nuclear and cytoplasmic proteins extracted from rat hippocampus were measured by Western Blot. NF-kB (p65) protein levels were normalized on β-Tubulin levels and both proteins were detected on the same blot. A representative blot of NF-kB (p65) detected is shown (C,D). Optical density was expressed as % of the control (saline-treated) group. Each column represents mean ± S.E.M..

11. AIM 5

Epigenetic mechanisms are an integral part of a multitude of brain functions that range from basic cellular tasks to the development of the nervous system to higher order cognitive processes (Graff et al., 2011). Therefore, a thorough characterization of the epigenetic status of the brain is critical for understanding the molecular basis of its function in health and disease. Epigenetic processes are extremely important both for the establishment of cell-type–specific identities in the nervous system (Takizawa et el., 2001) and in mediating environmentally induced changes in the adult brain, being a critical component of various processes and conditions including memory formation, responses to stress, depression and drug addiction (Feng et al., 2010; LaPlant et al., 2010; Labonte B et al., 2012; Suderman et al., 2012). The brain, however, is characterized by a multifaceted complexity, including heterogeneity of cell types, such as neurons and glia, as well as subpopulations within these cell types. These cell types are differentially distributed among brain regions that themselves are heterogeneous in cytoarchitecture, connectivity and function. Hence, to achieve meaningful insight into the epigenetic landscape of the brain, the epigenetic marks should be studied within individual cell types in the specific brain regions. Indeed, recent reports have clearly demonstrated significant differences in epigenetic modification patterns between neuronal and non-neuronal cells and suggested that the previously reported epigenetic variation among brain regions could be largely due to differences in neuron to glia ratios (Guintivano et al., 2013). Recently, it has become clear that excessive neuronal excitation induces chromatin remodeling in neurons, thereby altering gene expression. Especially in the hippocampus, chromatin remodeling may be responsible for dynamic processes including synaptogenesis, long-term potentiation, dendritic remodeling and neurogenesis that are associated with learning and memory formation (Day et al., 2011). A substantial increase in H3 phosphorylation was detected in neurons in specific brain structures. Many studies have demonstrated that several stimuli, such as stress and seizures, increase H3 phosphorylation in hippocampal neurons, and this increase depended on neuronal excitation (Tetsuji Mori et al., 2013). Histone H3 phosphorylation at Ser-10 and acetylation at Lys-14 are frequently used markers for detecting histone modification (Healy et al., 2012; Sng et al., 2006; Chandramohan et al., 2007).

After observing the effect of LPS on histone H3 phospho-acetylation and its role in the regulation of the transcription of some specific genes, this particular aim was to extent the study on the characterization and the distribution of histone H3 phospho(S10)-acetylation(K14) in specific brain cell populations. Specific levels of neuron, microglia and astrocyte staining were analyzed, with specific antibodies, in the rat hypothalamus and hippocampus of LPS-treated rats (830 μg/Kg) and control treated rats.

12. RESULT 5

12.1 Marked activation of microglial cells to H3-phospho-acetylation after LPS-induced immune challenge

Here the hypothesis was tested that the modification of H3pS10acK14 upon systemic exposure to LPS was specific with respect to cerebral cell type. In particular, neurons, astrocyte and microglial cells of rat hypothalamus and hippocampus were analyzed by immunostaining respectively with specific antibodies including anti-neuronal nuclear protein (NeuN), anti-glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule 1 (Iba-1).

In this more exploratory part of my thesis research, several qualitative representative images are depicted and analyzed with respect to the two brain areas of interest. Among the various sections examined, the dentate gyrus (DG) of hippocampus (Figure 19, 20) and, respectively, the arcuate nucleus (Arc) (Figure 17, 18) and the parvocellular paraventricular nucleus (PVN) (Figure 15, 16) of the hypothalamus have been the focus of our attention.

No astroglial activation (GFAP staining) was detected 2 and 6 hours following LPS administration, nor was immunoreactvity observed for H3pS10acK14 among the different experimental groups examined in neither area of interest (data not shown).

Hypothalamus

In the hypothalamus, particularly in the parvocellular paraventricular nucleus, an increase in positive cell nuclei were observed for H3pS10acK14, 2 hours after LPS treatment (Figure 15H) with the same intensity maintained after 6 hours (Figure 15I) in LPS-treated rats with respect to saline treated animals (Figure 15G). Cell nuclei were evidenced by the nucleic acid stain, DAPI (Figure 15A,B,C).

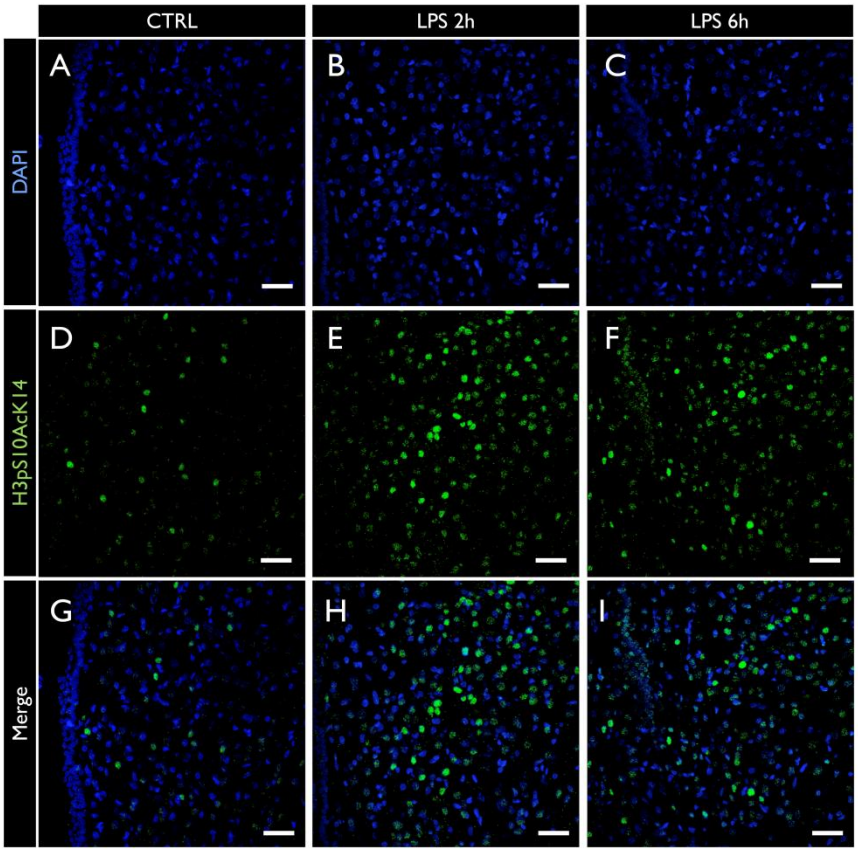


Figure 15. Increased immunoreactivity of H3pS10acK14 in cell nuclei of parvocellular paraventricular nucleus of hypothalamus. In panels A,B,C cell nuclei were stained by DAPI (blue). H3pS10acK14 (green) resulted to be enhanced in cells 2 and 6 hours after receiving LPS (830 µg/Kg i.p) compared to control (D,E,F). Merge images (G,H,I) show the co-localization of H3pS10acK14 with DAPI. Scale bar = 50 µm

In order to better understand which cell population was more affected by H3-phospho-acetylation, sections were labelled with NeuN and Iba-1. Co-localization of NeuN with H3pS10acK14 revealed neuronal activation for H3pS10acK14 already at 2 hours post LPS (Figure 16A) and lasted for up to 6 hours (Figure 16B). High magnification showed a morphological activation (Figure 16D) of microglial cells assuming the amoeboid/phagocytic form only 6 hours after LPS stimulus, while no changes were observed after 2 hours (Figure 16C). Simultaneously, the reactivity of microglial cells for H3pS10acK14 modification matched the microglia morphological activation 6 hours after LPS-administration but not after 2 hours, when the epigenetic process seemed to be exclusively neuronal (Figure 16C,D). This suggests that and immune challenge such as exposure to LPS, is capable to induce microglia activation for a

sustained period of time and, that as hypothesized, epigenetic mechanism could mediate this immune response.

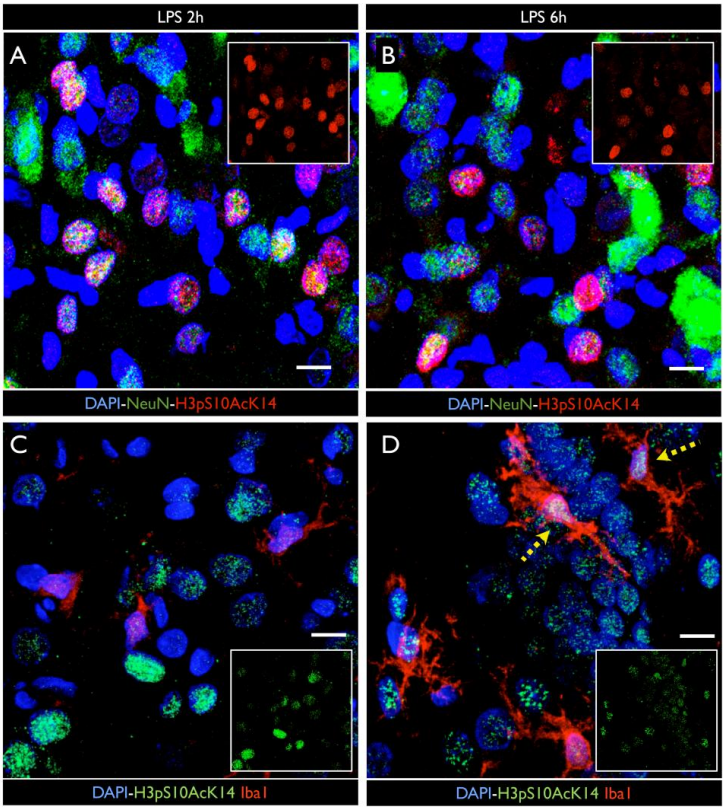


Figura 16. Co-localization of H3pS10acK14 with neurons and microglia markers in parvocellular paraventricular nucleus of the hypothalamus. Confocal microscopy images of brain cryosections labeled with DAPI (blue), H3pS10acK14 (red, panel A,B)(green, panel C,D), NeuN (green, panel A,B) and Iba1 (red, panel C,D) are represented. High magnification panels A and B show activated neurons to the H3pS10acK14 immunoreactivity 2 and 6 hours after LPS injection (830 µg/Kg i.p). LPS induced morphological activation of microglia cells and the simultaneous reactivity to H3pS10acK14, 6 hours following treatment, and it is shown by the co-localization between H3pS10acK14 and Iba1 (image D). Scale bar = 10 µm (A-D).

A similar pattern was observed in the arcuate nucleus of the hypothalamus: LPS induced a significant increase in H3pS10acK14 modifications in the cell nucleus 2 hours (Figure 17E) and 6 hours (Figure 17F) after exposure to LPS, with respect to controls (Figure 17D).

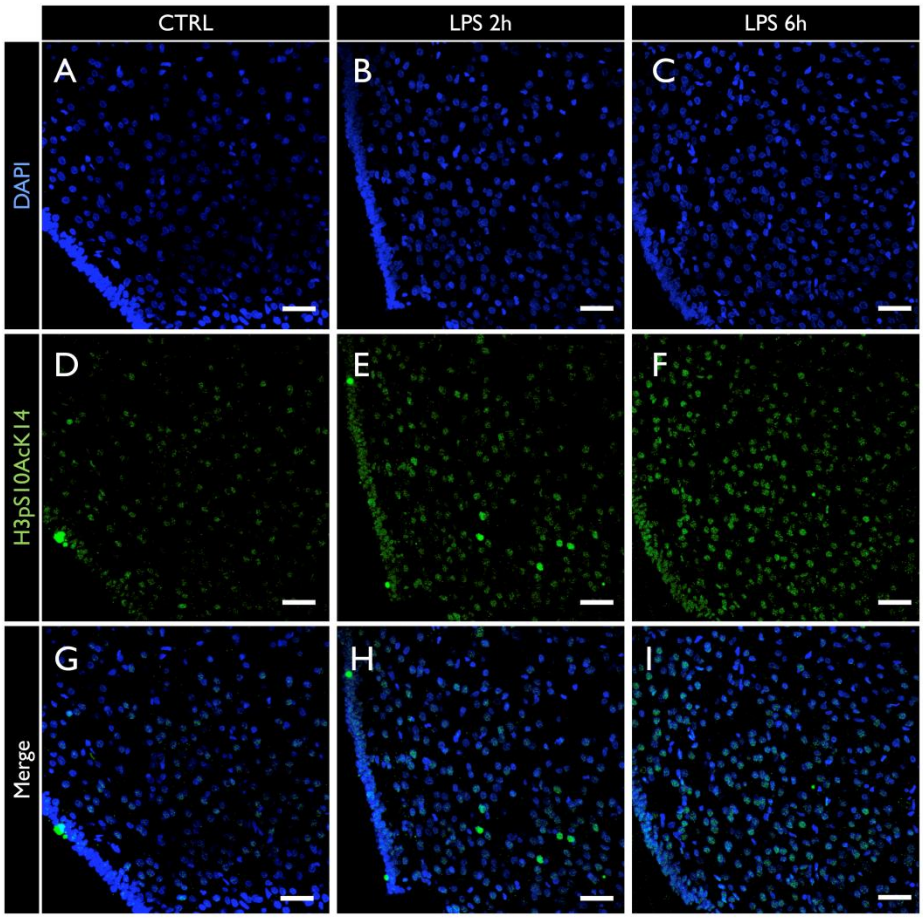


Figura 17. Increased immunoreactivity of H3pS10acK14 in cell nuclei of arcuate nucleus of the hypothalamus. In panels A,B,C cell nuclei were stained by DAPI (blue). H3pS10acK14 (green) resulted to be enhanced in cells 2 and 6 hours after receiving LPS (830 µg/Kg i.p) compared to control (D,E,F). Merge images (G,H,I) show the co-localization of H3pS10acK14 with DAPI. Scale bar = 50 µm

Labelling sections with H3pS10acK14 and Iba-1 resulted in the co-localization of these 6 hours following LPS injection, suggesting the response of microglia cells to LPS signal and towards the H3pS10acK14 modification (Figure 18B). Moreover, a neuronal reactivity for H3pS10acK14 was appreciable at the same time, deducible by the typical morphology of the neuronal nucleus where signal was more concentrated (Figure 18B). Less extent immunoreactivity for H3pS10acK14 was evident 2 hours after LPS treatment where signal resulted more scattered then 6 hours (Figure 18A).

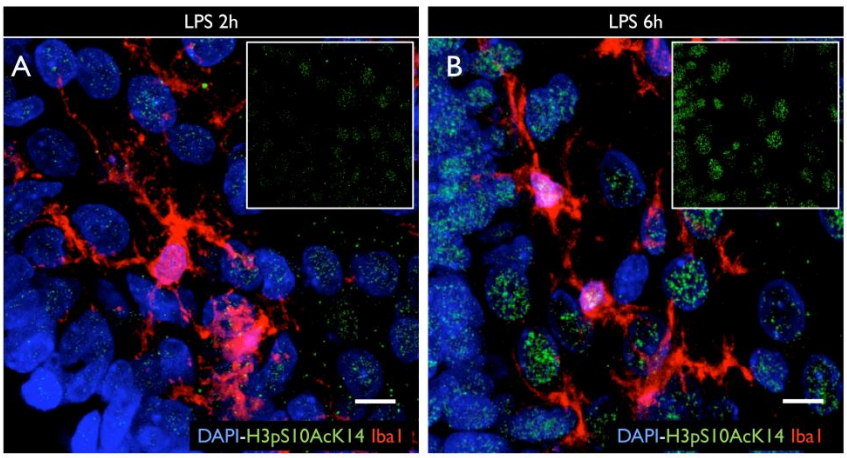


Figura 18. Immunoreactivity of H3pS10acK14 in neuron and microglia cells of arcuate nucleus of the hypothalamus. Confocal microscopy images of brain cryosections labeled with DAPI (blue), H3pS10acK14 (green) and Iba1 (red) are represented. High magnification panels A and B show activated neurons for H3pS10acK14 especially 6 hours after LPS injection (830 µg/Kg i.p.). Six hours following LPS treatment a morphological activation of microglial cells was observed together the simultaneous reactivity to H3pS10acK14 modification, showing by the co-localization between H3pS10acK14 and Iba1 (image B). Scale bar = 10 µm.

Hippocampus

In the hippocampus, general framework showing the co-localization of H3pS10acK14 with DAPI revealed a high immunoreactivity of H3pS10acK14 at 2 hours after LPS treatment, and disappeared after 6 hours returning to levels similar to the controls (Figure 19D,E,F). Moreover, given the mainly neuronal nature of the granular layer of the hippocampus, it was possible to assume that H3pS10acK14 immunoreactivity concern primarily neuronal cells.

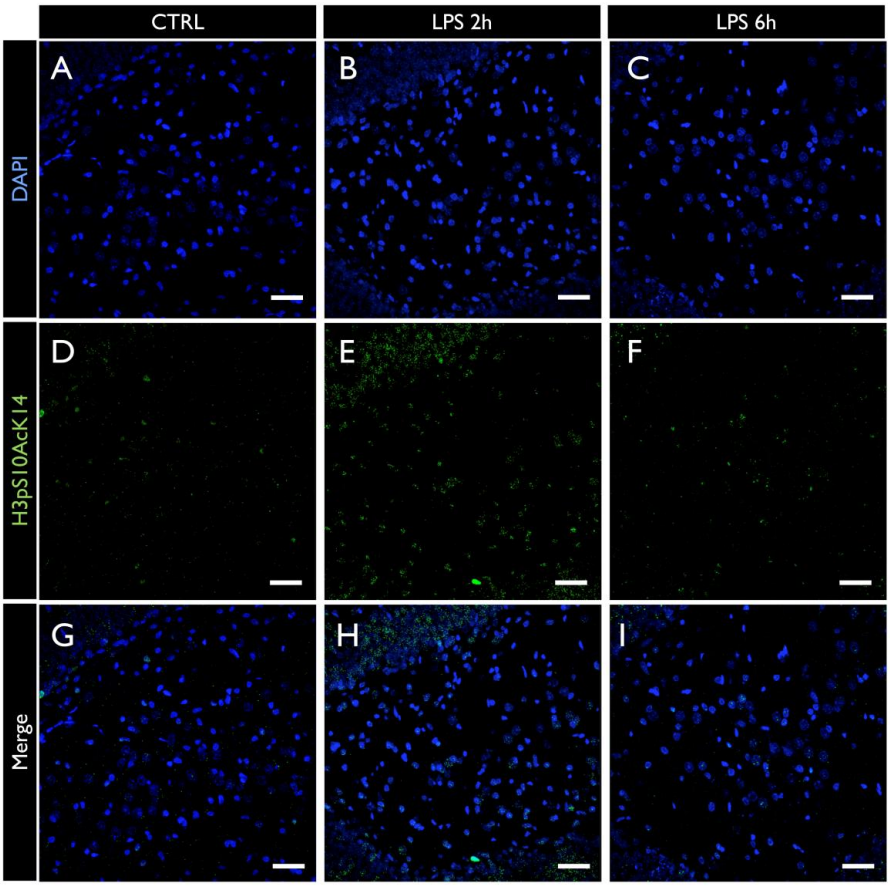


Figura 19. Increased immunoreactivity of H3pS10acK14 in granular neuronal cells of the DG. In panels A,B,C cell nuclei were stained by DAPI (blue). H3pS10acK14 (green) resulted to be enhanced in cells after 2 hours (E) and decreased levels were observed 6 hours (f) after receiving LPS (830 µg/Kg i.p.) compared to control (D,E,F). Merge images (G,H,I) show the co-localization of H3pS10acK14 with DAPI. Scale bar = 50 µm

Also in this case, a closer image about the immunoreaction between H3pS10acK14 and Iba1 was presented: in agreement with that seen in hypothalamus, it was detectable the activation of microglia which assumed a amoeboid/phagocytic form only 6 hours following LPS stimulus (Figure 20B) while no changes were detectable after 2 hours (Figure 20A). Besides, after 2 hours, H3pS10acK14 immunoreactivity resulted more concentrated in neuronal cells, deducible by the typical morphology of the neuron nucleus where signal was present (Figure 20A) unlike 6 hours where staining was more scattered.

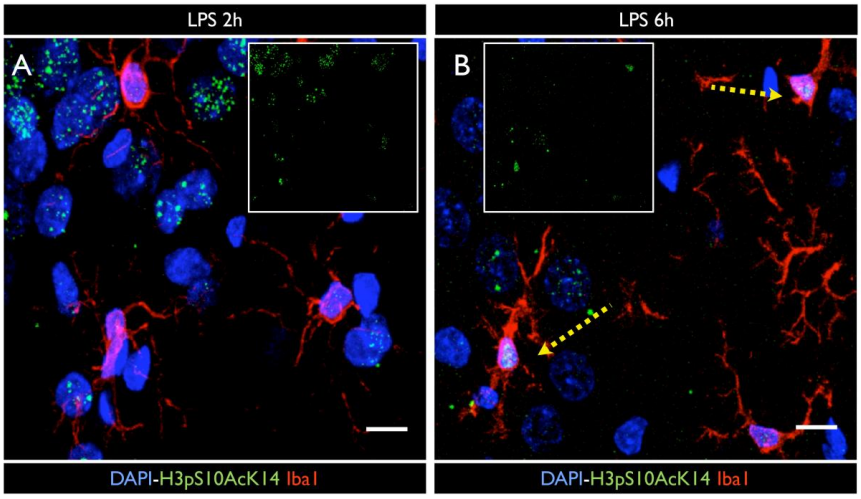


Figure 20. Immunoreactivity of H3pS10acK14 in neuron and microglia cells of hippocampus. Confocal microscopy images of brain cryosections labeled with DAPI (blue), H3pS10acK14 (green) and Iba1 (red) are represented. High magnification panels A and B show some activated neurons for H3pS10acK14, 2 hours after LPS injection (830 µg/Kg i.p.). Six hours following LPS treatment no neurons were activated, but a morphological activation of microglial cells was observed together the simultaneous reactivity to H3pS10acK14 modification, showing by the co-localization of H3pS10acK14 and Iba1 (image B). Scale bar = 10 µm.

This evidence supported the hypothesis that, in hippocampus, a first neuronal response, toward the histone H3 phospho(S10)-acetylation(K14) induced by LPS, was observable and only later it became mainly inflammatory, given the activation for both morphology and H3pS10acK14 reactivity of the microglia. In hypothalamus, a different neuronal response was highlighted at the two time points in the two type of nucleus examined (Arc-PVN), while the same correlation was found in microglial response, evident at 6 hours after LPS treatment, toward the histone H3 phospho(S10)-acetylation(K14) induced by LPS.

This morphological study allowed to notice that microglia were the prevalent cell population which respond to LPS and, simultaneously, to the histone H3 phospho(S10)-acetylation(K14) modification, induced in turn by LPS.

13. AIM 6

Inflammatory responses in turn may damage neurons; a bacterial endotoxin as lipopolysaccharide initiates a number of major cellular events that play critical roles in the pathogenesis of inflammatory responses.

Several cell types have been listed as active contributors of neuroinflammation, among them microglia are considered to be critical cellular components (Kettenmann et al., 2013). Microglia are the innate immune effector cells in the CNS and play an important role in immune response in the brain influencing both physiological and pathological processes. These inflammatory cells are constantly surveying their external environment and rapidly respond to a variety of molecules that signal changes in CNS homeostasis. In response to these signals, microglia influence neuronal connections, modulate the functions of other glia, and mediate inflammatory responses to disease or injury. In parallel with the regulation of inflammatory responses outside of the CNS, investigators have observed that microglia are capable of heterogeneous responses to exogenous and endogenous signals. While much of this molecular and morphological heterogeneity is regulated by gene transcription, there is ample evidence that microglial behavior is determined, in part, by epigenetic regulation (Garden, 2013).

At present, it is still not clear what role microglia play in epigenetic processes, but recent studies have demonstrated that modifications including DNA methylation, histone modification, and non-coding RNAs have fundamental role in modulating neuroinflammation.

Moreover, cultured microglia constitute a powerful tool to study how different agents modify microglial activation and help characterize molecules and pathway involved in microglial activation (Gresa-Arribas et al., 2012)

Given this, to further enhance our knowledge regarding the role of epigenetic regulation of neuro-inflammation, in the last part of my thesis work, a specific primary mouse microglia cell line, BV-2 was used (Blasi et al., 1990). In order to better understand the epigenetic mechanisms following a LPS treatment (100 ng/ml), in microglia, three hypothesis were tested:

a- LPS-treatment affects the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, iNOS, and the immediate early gene c-Fos at 4 and 8 hours after exposure of BV-2 to LPS;

b- histone H3 phospho(Ser-10)acetylation(Lys-14) protein expression, using a specific antibody, at 4 and 8 hours after LPS treatment, differs from control treatment;

c- Altered expression exists at the promoter level of the pro-inflammatory cytokines IL-1β and IL-6, iNOS and the immediate early gene c-Fos, on the immuno-precipitated fragment.

14. RESULT 6

14.1 LPS treatment induced an increase in the expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the enzyme iNOS and a decrease of the immediate early gene c-Fos in BV-2 cells

Microglia are considered to play a critical role in the immune response in the brain by influencing physiological and pathological processes. In response to exogenous signals, microglia can rapidly produce a variety of molecules that influence CNS homeostasis. A large number of studies using cultured microglia have shown temporal profiles of morphological change and the release of inflammatory mediators in LPS-induced microglia activation. In particular, within a short time span (1 and 2 hours) LPS induces the production of IL-1β, IL-6, TNF-α and NO, but the peak time of the maximum level of pro-inflammatory citokines was at 6 hours. After having reached their high point each target follows a different trend (Nakamura et al., 1999).

For this reason, an exploratory study using murine microglia cells, BV-2, was started to investigate the expression of the main pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, iNOS and the early gene c-Fos at 4 and 8 hours after LPS treatment (100 ng/ml).

Preliminary results showed a main effect of LPS on IL-1β, IL-6 and TNF-α mRNA expression in BV-2 microglia cells with respect the controls (Figure 21). Given the preliminary experiments, a small number of samples was used, but statistical analysis demonstrated that in all cases, low values of standard deviation were present which underlined the propriety of the data and the experimental conditions. However, this study represents an exploratory study that requires additional experiments to confirm these interesting data.

A non-parametric test revealed a significant increase in IL-1β, IL-6 and TNF-α mRNA levels 4 hours after LPS treatment. While peak levels of IL-6 and TNF-α (Figure 21B,C) were observed after 4 hours, IL-1β mRNA (Figure 21A) levels further increased until 8 hours after treatment. In general, BV-2 responded to LPS treatment with an high production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α.

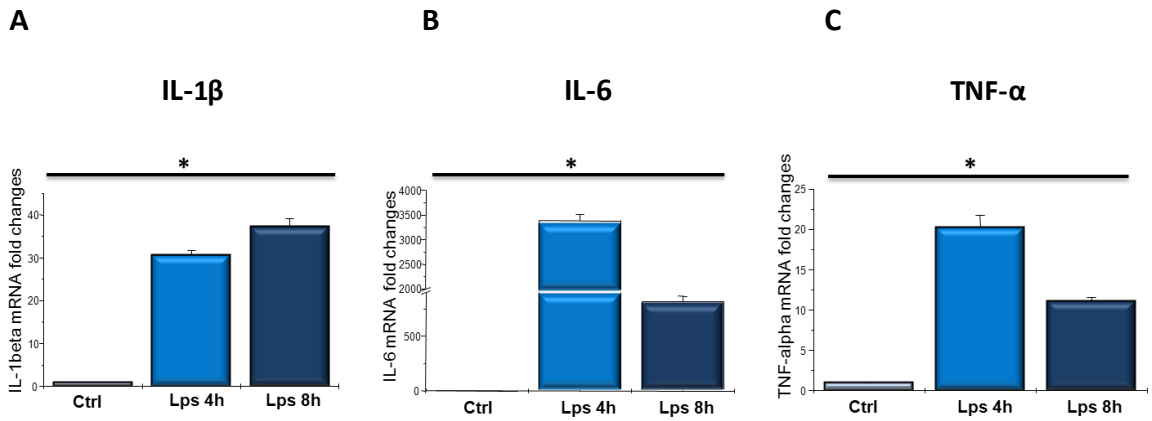


Figure 21. LPS treatment of BV-2 microglia (100 ng/ml) induced the expression of the pro-inflammatory cytokines IL-1β (A), IL-6 (B) and TNF-α (C) at 4 hours. Histograms show that, at 8 hours after LPS, IL-6 and TNF-α mRNA levels decreased. The relative expression level of IL-1β, IL-6 and TNF-α were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes of LPS treated samples above the expression of controls (PBS). Bars indicate the mean ± S.E.M.; *statistical significant relative to difference between LPS-treated and control samples. *p<0.05 (One-way ANOVA).

In the same context, the expression of iNOS and the early gene c-Fos were investigated and one-way ANOVA demonstrated a different trend.

INOS mRNA significantly increased at 4 and 8 hours following LPS treatment (Figure 22A) indicating a main effect of LPS treatment on microglia iNOS induction; in contrast, the expression of the immediate early gene c-Fos was down-regulated by LPS treatment ($p < 0.01$) (Figure 22B). *Post-hoc* tests showed a decrease of c-Fos mRNA at 4 hours ($p < 0.01$) with an even greater reduction 8 hours ($p < 0.01$) after LPS treatment as compared to PBS-treated cells.

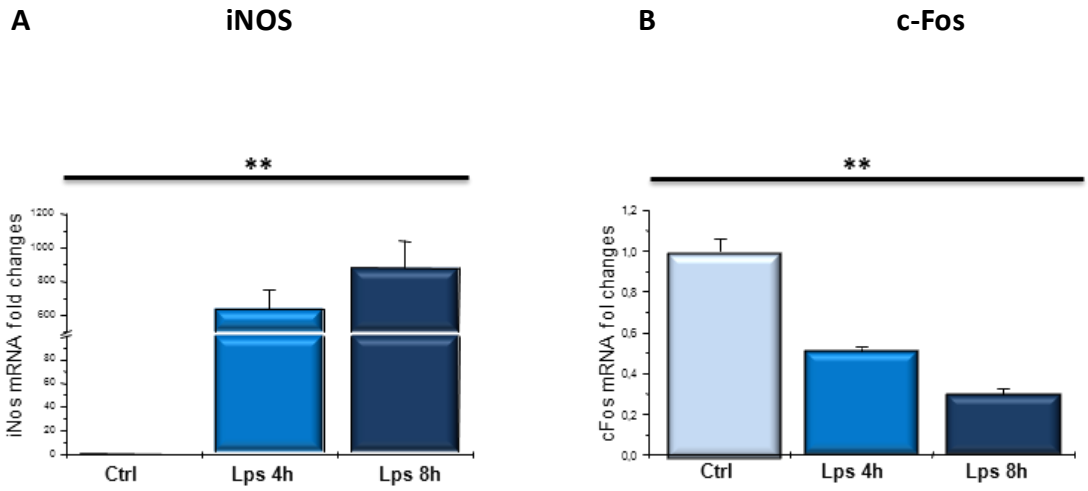
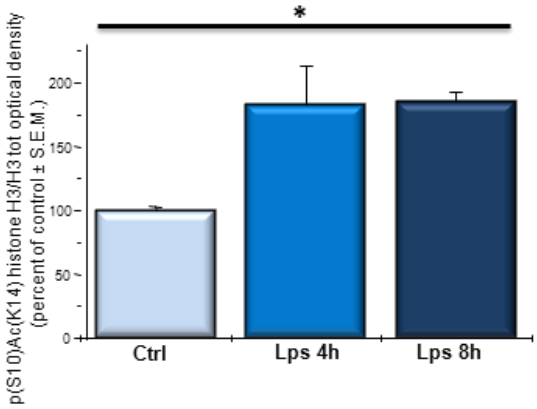


Figure 22. (A) LPS treatment of BV-2 microglia (100 ng/ml) induces the expression of iNOS mRNA after 4 hours and reached higher levels after 8 hours. (B) LPS treatment of BV-2 microglia (100 ng/ml) determined a decrease in c-Fos mRNA expression after 4 and 8 hours. The relative expression level of iNOS and c-Fos were evaluated by Real Time PCR using specific primers. Data were expressed as fold-changes above the expression of the respective control (PBS). Bars indicate the mean ± S.E.M.; *statistical significant difference between LPS-treated and control samples. **p<0.01 (One-way ANOVA).

14.2 LPS treatment induced the phospho(S10)-acetylation(K14) of H3 protein levels in BV-2 cells

In a subsequent experiment, we further tested the effect of LPS (100 ng/ml) on the phospho(S10)-acetylation(K14) of histone H3 in microglial cells. Here, the expression of H3-phospho-acetylation protein levels in the chromatin-enriched fraction extracted from BV-2 cells was studied.
Statistical analysis revealed an effect of LPS on phospho(S10)-acetylation(K14) of histone H3 protein levels which increased after 4 and 8 hours of treatment (*post-hoc* p = 0.043, p = 0.037) respectively with respect to controls. No changes were detected between the two time points examined, suggesting that H3-phospho-acetylation levels remained high (Figure 23).

A



B

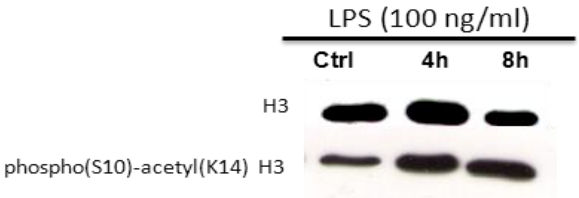


Figure 23. LPS treatment (100 ng/ml) of BV-2 microglia enhanced phospho(S10)-acetyl(K14)H3 protein levels with respect to total H3 4 hours after LPS and were maintained high to 8 hours post LPS (A). Chromatin-enriched proteins extracted from BV-2 were measured by Western Blot. Phospho-acetyl-H3 protein levels were normalized on total histone H3 levels and both proteins were detected on the same blot. A representative blot of phospho(S10)-acetyl(K14)H3 detected is shown (B). Optical density was expressed as % of control (PBS-treated) group. Each column represents mean ± S.E.M.; * $p<0.05$ among the groups (one-way ANOVA).

14.3 Histone H3 phospho(S10)-acetylation(K14) influenced iNOS gene transcription response induced by LPS in BV-2 cells

Next, the effect of LPS on the phospho-acetylation status of histones H3 in the promoter region of IL-1β, IL-6, iNOS and c-Fos genes was investigated.

Once chromatin was immunoprecipitated, using a specific antibody for phospho(S10)-acetyl(K14) histone H3, the expression of IL-1β, IL-6, iNOS and c-Fos genes on the promoter region was evaluated by a qualitative PCR (see Material and Methods for further details).

As shown in Figure 24, no significant differences in expression were observable for IL-1β, IL-6 and c-Fos promoter-genes neither at 4 nor at 8 hours after the LPS treatment of BV-2. While, interestingly, a different expression of iNOS promoter was displayed 4 and 8 hours after LPS with respect to controls.

These data need further testing to more substantially support and confirm our hypothesis.

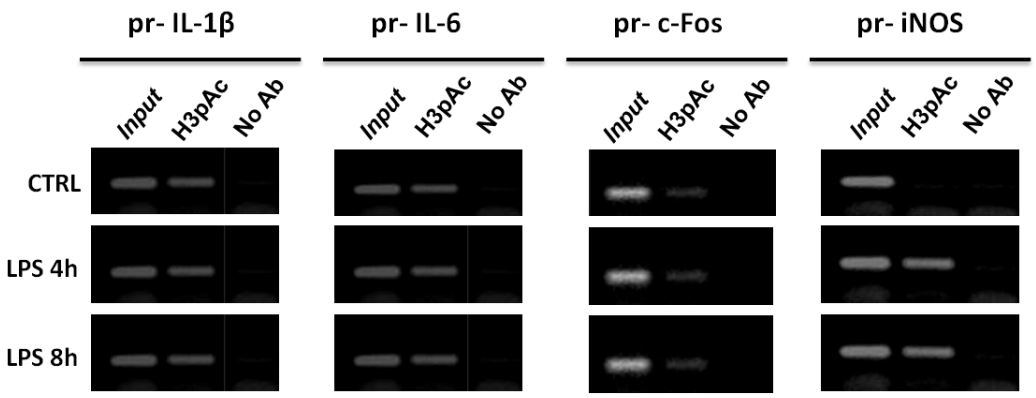


Figure 24. Phospho-acetylation of histone H3, induced at 4 and 8 hours after LPS treatment (100 ng/ml) did not affect the regulation of the transcription of IL-1β, IL-6 and c-Fos gene promoters in BV-2 cells. Differences in expression of iNOS were evident 4 and 8 hours after LPS with respect to control. Qualitative PCR on the ChIP fragments was used to evaluate the expression of IL-6, iNOS and c-Fos promoters. Input samples represent the total of chromatin. No antibody sample was used as negative control of the immunoprecipitation.

15. DISCUSSION

The general objective of this thesis was the role of epigenetic mechanisms in regulating gene expression in various areas of the brain and the diverse types of brain cells populating these areas in response to an inflammatory stimulus. With a set of experiments pertaining to six specific aims we have tried to respond to each specific hypothesis that generally tested the involvement of epigenetic mechanisms and the inflammatory activity of LPS in the brain by studying:

- the transcriptional activity in the brain,
- the modification of histone H3,
- the role of histone H3 modification in regulating the transcription activity,
- the activation of some specific signaling pathways
- and the involvement of specific cell types

Overall, the main results of these experiments suggest that the induction of epigenetic processes are brain area and cell-type specific, involving only some brain cell populations, and are conditioned by time since exposure to an inflammatory insult as well as the nature of the insult.

The nervous system coordinates complex physiological and behavioral changes to permit a rapid adaptation to environmental conditions and the maintenance of homeostasis. The CNS responds to metabolic signals, diverse cellular processes and environmental changes by gene transcription and synthesis of new proteins, mediated by the dynamic remodeling of chromatin architecture transacted by an ever expanding array of enzymes and associated signal transduction pathways (Citri et al. 2007; Colvis et al., 2005).

Epigenetic modifications are responsible for the translation of external input in functional responses; modifications of both DNA and histone proteins are now emerging as fundamental mechanisms by which neurons adapt their transcriptional response to environmental cues, such as, immune stimuli or stress (Toyokawa et al., 2012). Moreover, epigenetic mechanisms may play a role in the information storage in the CNS. At the molecular level, these events are controlled by activity-dependent signaling pathways that mediate gene expression by modifying the activity, localization, and/or expression of transcriptional-regulatory enzymes in combination with alterations in chromatin structure in the nucleus (McClung and Nestler, 2008).

The general purpose of this study was to investigate the role of a specific epigenetic modification, histone H3 phospho(S10)-acetylation(K14), in a brain inflammatory condition generated by a systemic administration of the bacterial endotoxin LPS. Interest in this modification arises from the growing knowledge concerning its involvement in regulating neuronal gene expression in response to a variety of stimuli. Moreover, the role of histone phospho-acetylation is known to

play a role in a large variety of diseases, such as stress, drug addiction, chronic electroconvulsive seizure and cancer, and it is progressively emerging in other psycho-social disorders like psychiatric and neurodegenerative diseases.
On the other hand, the most intense interest in neuroinflammation arises from its potential role in chronic degenerative diseases: the brain coordinates and regulates many aspects of the inflammatory acute phase, such as producing inflammatory mediators and other molecules which may begin to explain the behavioral response to disease as fatigue and depression, and how psychological state can influence susceptibility to disease and recovery. When acute phase responses persist in an inappropriate and excessive way, inflammation becomes a chronic state and the common consequence is irreversible neuronal loss and atrophy due to regenerative failure.

In this research, the study of the modification of histone H3 phospho-acetylation in a LPS-induced inflammatory condition was approached step by step, first in *vivo* (rat model of neuro-inflammation) and, finally, in *vitro* (BV-2 cell line) was started.
Intraperitoneal injection of LPS induces a systemic inflammatory response that generates the sickness syndrome, a condition characterized by physiological and behavioral symptoms commonly associated with infectious diseases (Hart, 1988). Symptoms include anorexia (Wisse et al., 2007), lethargy (Hopwood et al., 2009) memory impairments (Vereker et al., 2000, Shaw et al., 2001) and a depressive-like state (Dantzer et al., 2008). These symptoms are orchestrated by the LPS-dependent induction of pro-inflammatory cytokines, primarily IL-1β, IL-6 and TNF-α (Breder et al., 1994; Laye et al., 1994; Quan et al., 1999; Gatti and Bartfai, 1993) produced both in the periphery and in the brain. These mediators, acting at different levels of the CNS, have been described to determine profound alterations in neurological and endocrine functions particularly in those brain areas belonging to the limbic system. The study focused, in fact, on two area, the hypothalamus and the hippocampus, two areas implicated in the neurobiological mechanisms underlying the behavioral, cognitive, emotional and motivational alterations of cytokine-induced sickness behavior as well as the physiological response to infection (Dantzer et al, 2009; Andrè et al., 2008; Frenois et al., 2007).

Given this, in the first part of the thesis, to ensure that peripheral LPS injection was effective in inducing an inflammatory status, mRNA levels of the major pro-inflammatory cytokines were analyzed in the rat hypothalamus and hippocampus. The expression of IL-1β, IL-6, TNF-α in the hypothalamus was strongly increased 2 hours following exposure to LPS, and remained high for at

least up to 6 hours after lps treatment. In the hippocampus, LPS induced the expression of the IL-1β, IL-6 mRNA levels, but not of TNF-α 2 hours after receiving LPS; at 6 hours post LPS, all cytokines were strongly enhanced.

The effect of LPS on the expression of these cytokines appeared more intense in the hypothalamus with respect to the hippocampus (Andrè et al., 2008). The authors, who also showed a distinct time-course of these LPS effects between the two brain areas, attributed the difference of intensity to the diversity in the density of microglial and/or macrophage-like cells, although there are no clear data confirming this hypothesis.

After this, interest was direct towards another protagonist in the immune response to inflammation, iNOS, responsible of NO synthesis. NO plays a very important role in the control of neuronal activity in all brain areas: it is thought to mediate long-term potentiation in the hippocampus, while, in the hypothalamus, NO stimulates corticotropin-releasing hormone (CRH), prolactin releasing factor, growth hormone-releasing hormone (GHRH), and somatostatin, lutenizing hormone-releasing hormone (LHRH). Nitric oxide generated by iNOS has been implicated in many CNS pathologies including brain infections, injuries and neurological disorders (Bagasra et al., 1995; Adamson et al., 1996; Vodovotz et al., 1996).

Six hours after stimulation with LPS, strongly enhanced expression of iNOS was observed both in the hypothalamus and hippocampus. In the hippocampus, enhanced iNOS mRNA levels were present already at 2 hours. The induction of iNOS in the central nervous system leads to severe neuronal damage in the hippocampus casing memory impairment while in the hypothalamus iNOS leads to decreased fever and neuroendocrine response to infection, suggesting its potential role in the pathogenesis of neurodegenerative diseases (McCann SM, 1997). Once expressed, iNOS has a continuously high and long-lasting activity.

Finally, in the same context, c-Fos gene expression was induced 6 hours following a LPS challenge in both areas as already described (Frenois et al., 2007), but in the hypothalamus, the increased level of c-Fos was observed after 2 hours. C-Fos is an immediate early gene used as a marker for functionally activated neurons (Sagar et al., 1988) and its up-regulation may reflect neuronal activation in response to the central action of pro-inflammatory cytokines on brain areas coordinating the changes of systemic immune stimulation (Konsman et al., 1999).

Once established the presence of an inflammatory status, the hypothesis was tested that the inflammatory response to LPS- is mediated by epigenetic processes. For this reason, in the next study, histone H3 phospho(S10)-aceytlation(K14) modification was analyzed in an LPS induced inflammatory condition. Data indicated a main effect of LPS on histone H3 phospho(S10)-

aceytlation(K14) protein levels in the rat hypothalamus and hippocampus. H3 phospho(S10)-aceytlation(K14) was affected by treatment, resulting in a marked increase in the protein levels after exposure to LPS. A different effect of time was observed for the two areas because in the hippocampus a decrease of H3-phospho-acetylation to baseline levels was observed 6 hours after LPS administration while in the hypothalamus, at 6 hrs post LPS, modification remained highly expressed. Consequently, with regard to H3 modification, a different pattern characterized the two brain regions examined, which could be explained by different intensity and distribution of the various types of brain cells. Little is known about this modification and its timing of induction. What this study demonstrated, however, is that the activation of H3-phospho-acetylation was rapidly and strongly triggered by the LPS-induced immune challenge.

At this point, two main effects induced by LPS emerged in the rat hypothalamus and hippocampus: 1) LPS treatment (830 µg/Kg i.p.) induced a massive transcriptional response mediated by immune system cells. 2) LPS treatment (830 µg/Kg i.p.) induced the phospho(S10)-acetylation(K14) of histone H3.

Previously, the role of epigenetic modifications in regulating the cellular transcriptional activity was explained; specifically, epigenetic processes generate a remodeling of the chromatin determining an active or repressive conformation of gene transcription activity.

On the basis of this, the next study aimed at, first, to understand if H3-phospho-acetylation could have a role in modulating the transcription of the main genes examined above, and second, to investigate which target genes could be regulated in a specific manner by modification in H3-phospho-acetylation in the two brain areas considered. Analyzing the expression of IL-1β, IL-6, iNOS, and c-Fos promoters on the chromatin fragment receptive to H3-phospho-acetylation, a different effect of LPS was observed. Interestingly, target genes transcriptional regulation in the promoter regions were differentially modulated with respect to brain area and time.

In the hypothalamus, increased expression of iNOS, IL-6 and, to a lesser extent, c-Fos promoters was observed, 2 hours after treatment. In fact, at this time point, peak levels of the histone H3-phospho-acetylation were detected. A different pattern was observed in the hypothalamus 6 hours after LPS stimulus: at this later time point only the expression of the iNOS promoter gene was enhanced. As described before, the effect of LPS on H3-phospho-acetylation started to decrease after 6 hours while the expression of the pro-inflammatory cytokines, as well as iNOS and c-Fos was maintained high. This probably could be explained by the decreased effect of LPS on H3-phospho-acetylation in the hypothalamus while high levels of H3-phospho-acetylation are necessary for the regulation of the transcription. Our data are in agreement with other studies

that show the enrichment of histone H3 phospho(S10)-acetylation(K14) in the c-Fos promoter after exposure to stress (Gutierrez-Mecinas et al., 2009). It therefore seems likely that c-Fos expression is induced by phospho-acetylation of histone H3 and may be the link between signaling pathway activation and changes in target gene expression (Mifsud et al., 2011).
Regarding the hippocampus, ChIP assay showed increased expression of the iNOS promoter in LPS immunoprecipitated samples 2 hours after LPS exposure with respect to controls. Instead, no differences in expression were observed for all targets analyzed 6 hours following LPS treatment: this is in accordance with the non-presence of H3-phospho-acetylation that, as shown previously, were reduce to baseline levels.
This was only a qualitative and preliminary demonstration that deserves a more in depth analysis, however, learning more about the regulation of gene transcription may provide the basis for the study of new gene therapies.

In the next study, the possible signaling pathways leading to the activation of histone H3-phospho-acetylation was studied. Different studies demonstrate that MSK phosphorylates residues on histone tails including Serine10. However, MSK has no known acetylase activity, but can phosphorylate CREB which in turn recruits a number of histone modifying enzymes to the chromatin including p300 and/or CREB binding protein (CBP), both of which have histone acetyl-transferase (HAT) activity (Vecsey et al., 2007). The hypothesis tested in aim 4 was that CREB is implicated in H3-phospho-acetylation. Given that the phosphorylation of CREB at Ser-133 was necessary for its activation and transcription (Montminy et al, 1986), the phospho-CREB protein levels were measured in the nucleus. Results showed that, in the hypothalamus, there were no changes at 2 hours neither at 6 hours after LPS stimulus, indicating a likely non-influence of this brain region to the CREB activity. In contrast, in the hippocampus an increase of phospho-CREB was observed 2 hours following LPS treatment, but not at 6 hours. This confirms many data concerning the activation of CREB in the hippocampus after psychological stress, neuronal insult (Walton and Dragunow, 2000) and extracellular signals, as well as its involvement in synaptic plasticity and long-term memory, processes severely compromised in brain damage (Oike et al., 1999; Bourtchouladze et al., 2003; Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2005, 2006).
As explained before, different functional activity of these brain areas is characterized also by a diverse reactivity to psychological stressor. Furthermore, the effect of the time seems to play a significant role: an increase in phospho-CREB was observed only at 2 hours post LPS, suggesting that this pathway is activated in a short and transient way.

Moreover, given the HAT activity of CREB, these findings suggest that chromatin modification, via histone acetylation, could be the major molecular pathway involved in the regulation of transcription underlying memory storage, while the molecular mechanisms by which increased histone acetylation affects memory and synaptic plasticity remain unknown.

Finally, our findings validate the involvement of CREB in the production of cytokines (Mayer et al., 2013). In addition to phospho-CREB, another transcription factor, known to be activated by inflammatory stimuli, was investigated: NF-kB. NF-kB plays a crucial role in immune and inflammatory responses. After a stimulus, different components and subunits are involves in the activation of NF-kB determining its translocation to the nucleus where binds to a DNA consensus sequence on target genes and promotes transcription of a variety of genes like cytokines receptors, cytokines, prostaglandins, chemokines, and a few neuromediators (Miyamoto and Verma, 1995). The DNA binding nuclear form of the transcription factor NF-kB is usually a heterodimer which typically includes one of 50 kDa (p50) and one of 65 kDa (p65).

Evaluating NF-kB(p65) protein levels in the cytoplasmatic and nuclear fractions, differences were observed in the hypothalamus and hippocampus of LPS-treated rats with respect to controls. No difference was observed in NF-kB(p65) protein levels at 2 and 6 hours post LPS exposure.

A possible explanation is that the activation of NF-kB was rapidly triggered by LPS, whereby at 2 and 6 hours its activity at the chromatin level ended. Moreover, cytokine expression levels resulted very high at these two time points indicating that the transcriptional activation by NF-kB had already occurred. As demonstrated, a self-limited inflammatory response involves the successful elimination of the inflammatory stimulus within the first 2 hours post-endotoxin administration followed by a subsequent resolution within the following 24 hours (Foteinou et al., 2009). An alternative explanation may be that sustained and persistent pro-inflammatory signaling down-regulates the activity of NF-kB resulting in fading of NF-kB control. Here the hypothesis was tested that the activation of NFkB signaling serves as the representative signaling controller of the pro-inflammatory genetic switch underpinning the manifestation of transcriptional responses. An inadequate control of its transcriptional activity can be associated with the peak of a hyperinflammatory response (Foteinou et al., 2009).

Subsequently, in the next part of my thesis, another model was employed to study the question put forward in aim 5. A thorough characterization of the epigenetic status of the brain is critical for understanding the molecular basis of its function in health and disease. Epigenetic processes are extremely important both for the establishment of cell-type–specific identities in the nervous

system (Takizawa et al., 2001) and in mediating environmentally induced changes in the adult brain. To this end, the cellular characterization and cell distribution of histone H3 phospho(S10)-acetylation(K14) modification was analyzed in the rat hypothalamus and hippocampus. The study revealed that specific areas of the examined areas are most affected by the modification. In the hypothalamus, the arcuate nucleus was chosen, known to regulate feeding and energy balance, and the parvo-cellular para-ventricular nucleus which governs the neuroendocrine stress cascade. In the hippocampus, the granule cell layer of dentate gyrus zone was analyzed because of its critical role in the main processes conducted by this area, including learning, memory and spatial coding, and for its supposed function as a preprocessor of incoming information.

Interestingly, results from these experiments indicate that a correlation exists between evidence obtained in study 2 and the data obtained from the immunohistochemical analysis. In the hypothalamus, LPS induced an increase in histone H3 phospho(S10)-acetylation(K14) 2 hours after exposure to LPS which was maintained for up to 6 hours post LPSW exposure. In the hippocampus, LPS induced an increase in histone H3 phospho(S10)-acetylation(K14) 2 hours after LPS exposure which returned to baseline levels after 6 hours. So, detection of an increase in histone H3 phospho(S10)-acetylation(K14) protein levels by Western Blot was confirmed by the morphological study with a similar but not same trend in both areas considered. Furthermore, precise analyses showed the co-localization of phospho(S10)-acetyl(K14)H3 with neuronal and microglial markers suggesting a key role of these cell types in epigenetic processes. Recent reports have clearly demonstrated significant differences in epigenetic modification patterns between neuronal and non-neuronal cells and indicated that the epigenetic variations among brain regions could be largely owing to differences in neuron to glia ratios (Guintivano et al., 2013).

In the hippocampus, a first neuronal response was present for the histone modification induced by LPS. While, in the hypothalamus, the neuronal response was differently expressed at the two time points depending on the hypothalamic nucleus examined. It is becoming increasingly clear that excessive neuronal excitation is capable of inducing chromatin remodeling in neurons, thereby altering gene expression. Especially in the hippocampus, the brain region characterized by extensive neuroplasticity, it has been suggested that chromatin remodeling is responsible for a series of dynamic processes including synaptogenesis, long-term potentiation, dendritic remodeling and neurogenesis, that are associated with learning and memory formation (Day et al., 2011). With respect to astroglia, any type of phospho(S10)-acetyl(K14)H3 activation was induced by LPS in both areas studied (data not shown).

As hypothesized, the cell population that responded to a higher degree to LPS was microglia. Interestingly, the activation of microglia induced by LPS matched perfectly with the reactivity for

histone H3 phospho(S10)-acetylation(K14) only 6 hours following the LPS stimulus. Activated microglia become deramified and develop an enlarged cell body with several short, thickened processes. This morphological transformation occurs within hours of initial activation and coincides with microglial homing and adhesion to damaged neurons (Kloss et al., 1999; Raivich et al., 1999). It is well known that the active contribution of microglia to neuroinflammation serves as a main inducer of the immune response in the brain: the morphological change induced by LPS after 6 hours indicates the activation of these cells to produce pro-inflammatory cytokines IL-1β, IL-6, TNF-α, iNOS. However, in first part of this study, the induction of pro-inflammatory mediators was demonstrated to be triggered starting at 2 hours post inflammatory challenge. This suggests that pro-inflammatory cytokine production by microglial cells starts rapidly, once activated by an inflammatory insult, but before the morphological transformation, a process that needs more time. It is also important to note that microglia are not the sole producers of cytokines following an CNS insult. Astrocytes have also been implicated in the generation of pro-inflammatory mediators involved in neurodegenerative disorders (Lin et al., 2010; Lu et al., 2011; van Neerven et al., 2010). Recent findings are leading us to appreciate the idea that microglia regulate neuronal function and homeostasis under physiological conditions, in the absence of immune challenge or inflammation, which raises the possibility that disruption of such fundamental processes may contribute to pathological conditions characterized by neuronal or synaptic dysfunction (Frick et al., 2013).

In the last part, a new study approach was explored. The general purpose was to better understand epigenetic mechanisms in CNS cells, especially microglia cells, how these can regulate transcriptional responses as well as the signaling pathway involved. Given the interesting results of study 5, an exploratory study was started using the mouse microglia BV-2 cell line (widely used as a model to study microglia). First, the transcriptional response of BV-2 to LPS was investigated after 4 and 8 hours following LPS exposure: the expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and iNOS was strongly induced with peak levels 4 hours after LPS treatment. IL-1β and iNOS mRNA maintained high levels for up to six hours post LPS. These data confirm the activation of microglia after an immune challenge and are in agreement with a large number of studies on cultured microglia that demonstrate a peak level in the release of pro-inflammatory mediators around 6 hours post LPS exposure. Moreover, , others as well as our study, indicate 6 hours post inflammatory challenge to display the most morphological change in microglia (Nakamura et al., 1999).

Curiously, the expression of c-Fos decreased after the LPS treatment in BV-2 cells; this is probably because c-Fos is prevailing as a neuronal marker and reflects neuronal activity, so this result is compliant with its absence in microglia. Furthermore, c-Fos is an immediate early gene whose expression is very rapid and transient and therefore difficult to be observed at a prolonged time post immune challenge.

Then, BV-2 responds to an LPS-induced inflammatory stimulus in a longer time frame such as 4 and 8 hours following the exposure to bacterial endotoxin, like observed *in vivo* experiments.

Subsequently, given the activation of histone modifications observed in the immunohistochemical analysis, the next aim was to better understand the epigenetic response of BV-2 cells to LPS, exploring histone H3 phospho(S10)-acetylation(K14) expression. As in rats, the protein levels of histone H3 phospho(S10)-acetylation(K14) increased after 4 hours and was kept up after 8 hours post LPS treatment indicating that microglia can respond epigenetically to the LPS influence, therefore, suggesting a correlation with the data obtained *in vivo*.

Given the H3pS10acK14 modification and the expression of pro-inflammatory mediators in BV-2 cells, we next tested the effect of LPS on the phospho-acetylation status of histones H3 in the promoter region of IL-1β, IL-6, iNOS and c-Fos genes in BV-2 cells. Preliminary data obtained by ChIP displayed an increased expression of the iNOS promoter 4 and 8 hours after treatment with LPS, indicating a regulation of the transcription due to the H3-phospho-acetylation status induced by LPS. This result is in accordance with the data obtained *in vivo,* thus, providing the foundations to further extend the study of this particular transcriptional regulation. Using a model like this is important for understanding how epigenetic mechanism can modulate the transcriptional response of specific brain cells to inflammatory challenges, such as LPS. These data provide a step further in the understanding of the epigenetic mechanism that regulates the transcriptional response of activated microglia. However, this is just a tiny step, many more studies are needed to unravel microglial signaling pathways in the CNS in response to inflammatory insult.

Finally, this thesis had as general aim to study the mechanisms and the signaling pathways involved in the effects of an inflammatory insult, such as exposure to LPS, in the hippocampus and the hypothalamus of rats. As an extensive literature accumulates studying LPS and its role in inducing a neuroinflammatory-like condition in the CNS, the underlying processes by which LPS induces neuroinflammation are not completely clear yet. Results from this work strongly suggest that epigenetic modifications can be one of these underlying mechanisms that may play a fundamental role in mediating the effects of LPS in various parts of the brain, involving different cell types mainly due to the capacity to regulate gene transcription. Precisely, the hightened

activation of microglial cells both *in vivo* and *in vitro* opens the possibility of a direct action of LPS on the CNS. Furthermore, the simultaneous presence of the histone H3-phospho(S10)-acetylation(K14)modification in microglia, induced by LPS, justifies and stimulates future study of this cell population.

Finally, results obtained on BV-2 cells make this model extremely suitable to study and enhance our understanding of the molecular mechanism involved in epigenetic processes and may help to accelerate research programs while at the same time reducing the necessity of animal experimentation.

In conclusion, results exposed in this thesis indicate that the CNS can adapt its functional activity to environmental changes such as an inflammatory insult generated by a systemic exposure to bacterial lipopolysaccharide. Data indicate that LPS affects the CNS and induces the activation of a broad response which determines molecular, physiological and behavioral changes.

It is not yet clear whether the effect of LPS is direct or mediated by other factors, but the presence of a substantial activation, both *in vivo* and *in vitro*, of specific brain cells and of particular pathways suggests a direct role of the endotoxin on the CNS. The simultaneous presence of a marked response by microglial cells and, particularly, the epigenetic modifications involved allow us to conclude that the reaction and adaptation of the brain to a systemic infection is area and cell type specific and differ according to the time since exposure to the inflammatory insult.

16. MATERIALS AND METHODS 1

16.1 Animals and treatments

Adult Sprague-Dawley male rats were used in this study. Animals were housed in polycarbonate cages with ad *libitum* access to food and water throughout the study, and maintained under a 12:12 light –dark cycle in an ambient temperature of 21±3°C with relative humidity controlled. Animals were checked for signs of discomforts indicated by animal care and use guidelines (National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)). All procedures were carried out in accordance with the EC guidelines (EEC Council Directive 86/609 1987), Italian legislation on animal experimentation (Law Decree 116/92).

LPS (830 µg/Kg/ml *Escherichia Coli* serotype 0127:B8, Sigma Aldrich L3129) was injected intraperitoneally (i.p.) (n=6) and an equal volume of vehicle (saline) was used as a control (n=6). Tissues were harvested 2 hrs and 6 hrs after injection and stored at -80°C until RNA extraction.

16.2 RNA extraction and RT-PCR

Total RNA extraction was performed using TRIzol® reagent (Sigma®, St.Louis, MO,USA) followed by clean-up step on GenElute™ Mammalian Total RNA Miniprep Kit and DNAse treatment to remove genomic contamination following the manufacturer's instructions (Sigma®, St.Louis, MO,USA). 2 µg of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA) in 40 ul of reaction mix.

16.3 Real Time PCR

Real time PCR was performed in ABI PRISM 7900 HT (Life Technologies Corporation, Carlsbad, CA, USA) using Power SYBR Green mix (Life Technologies Corporation, Carlsbad, CA, USA). We used specific primers to detect the expression levels of the following genes at the final concentration of 150 nM. The mRNAs levels were normalized for each well to endogenous control glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (Table 1).

Target	NCBI GenBank	Forward	Reverse
IL-1β	NM_031512.2	ACTCGTGGGATGATGACGA	TCACATGGGTCAGACAGCAC
IL-6	NM_012589.1	CTTCACAAGTCGGAGGCTTA	AGTGCATCATCGCTGTTCAT
TNF-α	NM_012675.3	CCACCACGCTCTTCTGTCTA	TGATCTGAGTGTGAGGGTCTG
iNos	NM_012611	ACTTTTAGAGACGCTTCTGAG	ACATGTCTGTGACTTTGTTGC
c-Fos	NM_022197	CTCCAAGCGGAGACAGAT	GGCTGCCAAAATAAACTCCA
GAPDH	NM_017008.3	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

Table 1. Designed sequences of primer on target mRNA

The cycle parameters were: 95°C 10 min and 95°C 15 s, 60°C 1 min for 40 cycles. Single PCR products were subjected to a heat dissociation protocol (gradual increase of temperature from 60°C to 95°C) and agarose gel separation to verify the absence of artifacts, such as primer-dimers or non-specific products. Direct detection of PCR products was monitored by measuring an increase in fluorescence intensity caused by binding of SYBR GREEN I dye to neo-formed strand DNA during the amplification phase. Ct (cycle threshold) value was determined by the SDS software 2.2.2 (Life Technologies Corporation, Carlsbad, CA, USA) and was utilized to calculate mRNA fold changes using the data delta ct (ΔΔCt) method. The equation used was $2^{\Delta\Delta ct}=2^{(CtX-CtR)Reference-\ (CtX-CtR)target}$, where CtX was the threshold cycle of the gene of interest and CtR was the threshold cycle of the house-keeping gene (that was: GAPDH). For an appropriate application of comparative ΔΔCt method, it was demonstrated that amplification efficiency of the target gene and endogenous control gene was approximately equal. Each cDNA sample was run in triplicate and the mean values were used to calculate the gene expression levels.

16.4 Statistical analysis

The relative quantity of the diverse genes expression was analyzed by the ΔΔCt method using as calibrator average of the control group (Saline-injected) animals. The mRNA levels of the target genes were normalized on the intensity of the house-keeping gene, GAPDH. Ct values were obtained by an interpolate study. The effect of LPS on the expression pattern of different genes were analyzed with a one factor analysis of variance (ONE-WAY ANOVA) between the LPS- and Saline-treated animals in hypothalamus and hippocampus (*p* values below 0.05 were considered significant).

17. MATERIALS AND METHODS 2

17.1 Animals and treatments

Adult Sprague-Dawley male rats were used in this study. Animals were housed in polycarbonate cages with ad *libitum* access to food and water throughout the study, and maintained under a 12:12 light –dark cycle in an ambient temperature of 21±3°C with relative humidity controlled. Animals were checked for signs of discomforts indicated by animal care and use guidelines (National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)). All procedures were carried out in accordance with the EC guidelines (EEC Council Directive 86/609 1987), Italian legislation on animal experimentation (Law Decree 116/92).

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17.2 Protein extraction

For protein extraction, tissues were homogenized by a potter (30 strokes at 60 rpm) in a volume 20 times the weight of tissue of Lysis Buffer (Hepes 10 mM, EGTA 0.1 mM, Sucrose 0.28 M, in the presence of a protease inhibitor Complete 1X (Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitor, 10 µl Na_3VO_4, 50 µl NaPP, 20 µl NaF adding before using for each ml of buffer). A fraction of the lysate was collected (total extract) and the remaining fraction was centrifuged at 1,500g for 5 min at 4°C. The surnatant was collected (cytoplasm fraction) and the pellets were resuspended in a Low Salt Buffer (Hepes 20 mM, $MgCl_2$ 2 mM, KCl 0,1 M, Glycerol 25%, Dithiothreitol 5 mM adding before using). The fraction was centrifuged at 1,500 g for 5 min at 4°C. The surnatant was discarded and the pellets were resuspended in a High Salt Buffer (Hepes 20 mM, $MgCl_2$ 2mM, KCl 1M, EDTA 1mM, Glycerol 25%, Dithiothreitol 5 mM), then vortex vigorously and kept in ice for 30 min. Finally the lysate was centrifuged at 13,200 rpm for 30 min at 4°C. The surnatant was collected and the pellets were resuspended in a Resuspension Buffer (NaCl 120mM, Hepes 20 mM, EGTA 0,1 mM, Dithiothreitol 0,1 mM in the presence of a protease and phospahatase inhibitor (nuclear enriched extract). Protein concentration was determined using standard protocol Coomassie® reagent (Pierce). Total, cytosolic and nuclear fractions were stored at -20°C.

17.3 Western blotting

Western blotting were carried out on 4 µg of nuclear enriched extract for phospho(S10)-acetyl(K14) histone H3, histone H3, phospho-CREB, CREB, NF-κB detection. NF-κB protein levels were measured also on 4 µg of cytosolic extracts. Nuclear and cytosolic extracts were mixed with sample buffer for each sample just before the SDS-PAGE (14% polyacrilamide for phospho-acetyl histone H3 and histone H3, 10% for other antibodies). Electrophoresis was performed using Bio-Rad Protean III mini-gel apparatus with 14% and 10% acrylamide gel run at 200 V for 45. Proteins were transferred to PVDF membranes (Millipore®) using Bio-Rad trans-blot apparatus at 100 V for 1 hr. The membranes were blocked for 2 hrs with 5% milk non-fat dry milk in TBS-Tween 20 followed by incubation, overnight at 4°C, with primary antibodies: Anti-phospho(Ser10)-acetyl (Lys14)-Histone H3 1:1000 in Blocking-buffer (Histone H3 phospho(Ser-10)acetyl(Lys-14) rabbit polyclonal antibody, Millipore®); anti-histone H3 1:1000 in Blocking-buffer (Histone H3 rabbit polyclonal antibody Millipore®); anti-phospho-CREB 1:1000 in PAD (phospho-CREB (Ser133) rabbit polyclonal antibody, Cell Signaling®); anti-CREB 1:1000 in PAD (CREB rabbit polyclonal antibody, Cell Signaling®); anti-NF-κB 1:1000 in Blocking-buffer (NF-κB(p65) rabbit polyclonal antibody, Abcam®); anti-β-Tubulin 1:1000 in Blocking-buffer (β-Tubulin rabbit polyclonal antibody, Santa Cruz D-10 TEBU-Bio). After washings membranes were incubated 1 h at RT with secondary antibody in Blocking-Buffer: anti- rabbit IgG-HRP-linked (Cell Signaling®) 1:5000 for all targets. After 3 washing steps, bands were detected using Immobilon Western Chemiluminescent HRP (Millipore®). The protein levels were calculated by measuring the peak densitometric area of the autoradiografy analyzed with the image analyzer (GS-690 BIORAD). Phosphorylated and un-phosphorylated proteins run and were detected on the same blot.

The optical densities (OD) of phospho-acetyl histone H3 signal was normalized according to the OD of total histone H3. The optical densities (OD) of phospho-CREB, CREB, NF-κB signal was normalized according to the OD of β-tubulin and the OD of phospho-protein was normalized also with the respective OD of un-phosphorylated protein. Ratios were expressed as percent of control ± S.E.M.

17.4 Statistical analysis

Western blotting data were analyzed with analysis of variance (ANOVA). Multiple comparisons between different time points of LPS treatment were conducted for each target with one-way ANOVA. All mean differences were considered statistically significant $p < 0.05$.

18. MATERIALS AND METHODS 3

18.1 Animals and treatments

Adult Sprague-Dawley male rats were used in this study. Animals were housed in polycarbonate cages with ad *libitum* access to food and water throughout the study, and maintained under a 12:12 light –dark cycle in an ambient temperature of 21±3°C with relative humidity controlled. Animals were checked for signs of discomforts indicated by animal care and use guidelines (National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)). All procedures were carried out in accordance with the EC guidelines (EEC Council Directive 86/609 1987), Italian legislation on animal experimentation (Law Decree 116/92).

LPS (830 µg/Kg/ml *Escherichia Coli* serotype 0127:B8, Sigma Aldrich L3129) was injected intraperitoneally (i.p.) and an equal volume of vehicle (saline) was used as a control. Tissues were harvested 2 hrs and 6 hrs after injection and stored at -80°C until chromatin extraction.

18.2 Chromatin extraction and sonication

Chopped hippocampus and hypothalamus were cross-linked with formaldehyde (1% final concentration) for 15 min at room temperature and the reaction was stopped by adding glycine to final concentration 0,125 M for 5 minutes at room temperature. Fixed tissues were washed twice with ice-cold PBS added of a protease inhibitor Complete 1X (Protease Inhibitor Cocktail Tablets, Roche) and homogenized by potter (15 strokes at 60 rpm) in 1 ml of PBS in the presence of protease inhibitor Complete 1X. After a centrifugation at 5,000 rpm for 5 min at 4°C, pellet was resuspended in Cellular Lysis Buffer (Tris-HCl ph 8.0 10mM, NaCl 10 mM, Igepal 0.2%, in the presence of a protease inhibitor Complete 1X and phosphatase inhibitors, 10 µl Na_3VO_4 , 50 µl NaPP, 20 µl NaF adding before using for each ml of buffer) and kept in ice for 15 min.
The lysate was centrifuged at 5,000 rpm for 5 min at 4°C then surnatant was collected and the pellet resuspended in Nuclear Lysis Buffer (Tris-HCl ph 8.0 50 mM, EDTA 10 mM, SDS 0,5 %, in the presence of a protease inhibitors Complete 1X and phosphatase inhibitors) and kept on ice for 30 min. The chromatin was sonicated 4 times (30 sec on / 30 sec off) at 270 setting in Labsonic sonicator (B.Braun International®) to produce 500-1000bp fragments then was centrifuged at 14000 rpm for 10 min. The cleared supernatant was used immediately in ChIP experiments or stored at -80°C.

18.3 Chromatin Immunoprecipitation (ChIP)

Once the chromatin has been appropriately sonicated, measure the protein concentration of the lysate using standard protocol Coomassie® reagent (Pierce). The volume of lysate for 1 ug/ul was diluted to a final volume of 2 ml in a mixture of 9 parts Dilution buffer (Triton X-100 1.1%, 1.2 mM EDTA pH 8, 16.7 mM Tris-HCl pH 8 and 167 mM NaCl) and 1 part of Lysis buffer (SDS 1%, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8) and pre-cleared for 2 hours, rotating at 4°C, with 10 ul of protein G Agarose beads 50% slurry (KPL, Gaithersburg MD, USA). Samples were centrifuged at 2,000 rpm for 5 min and the surnatant was incubated with 3 µl of the antibody phospho(Ser10)-acetyl(Lys14) histone H3 (Millipore®) overnight rotating at 4°C. Simultaneously, 10 ul of the protein G beads were incubated with a pre-blocked mix consisting of 9:1 (Dilution: Lysis), 100 ug/ml BSA and 500 ug/ml sheared salmon sperm DNA in a final volume of 100 ul for each chromatin and rotate overnight at 4°C. After immuno-precipitation, 10 µl of protein G pre-blocked beads, previously washed, were added to each sample and incubated for 2 hours while rotating at 4°C. The unbound material was recovered as input and beads were washed 3 times with Wash Buffer (1% Triton X-100, 0.1% SDS, 150mM NaCl, 2mM EDTA pH 8.0, 20mM Tris-HCl pH 8.0) end once with a Final Wash Buffer (1% Triton X-100, 0.1% SDS, 500mM NaCl, 2mM EDTA pH 8.0, 20mM Tris-HCl pH 8.0). ChIPed material was eluted by two 15 minute incubations at room temperature with 250 ul Elution Buffer (SDS 1%, 0.1 M NaHCO3). Chromatins, including input samples, were cross-linked reverse and DNA was submitted to RNAse and proteinase K digestion by incubating before at 37°C for 1 hour then at 65°C for 4 hours minimum and finally extracted by phenol-chloroform. Immuno-precipitation reactions were performed in duplicate using a no-Antibody sample as a non-specific control.

18.4 Qualitative PCR analysis

Immunoprecipitated DNA of hypothalamus and hippocampus was analyzed by qualitative PCR analysis using GoTaq® Flexi DNA Polymerase (Promega® Italia, Milan, Italy). The promoter region of our gene of interest was amplified with the following primers:

Target	NCBI GenBank	Forward	Reverse
IL-1β	NC_005102.3	TCAGGACTTAAATGTTCAGC	AAGGGGTGGCAGACTATGAC
IL-6	NC_005103.3	CCCACCCTCCAACAAAGAT	ACAGACATCCCCAGTCTCATA
iNos	NC_005109.3	CCACTATGCTGCCCAAACTA	CCAGGTTCCAAATGACCAGT
c-Fos	NC_005105.3	AACCATCCCCGAAATCCTA	GAGCGGAACAGAGAAACTGG

Aliquots of chromatin obtained before immunoprecipitation were also analyzed (Input DNA).

19. MATERIALS AND METHODS 4

19.1 Animals and treatments

Adult Sprague-Dawley male rats were used in this study. Animals were housed in polycarbonate cages with ad *libitum* access to food and water throughout the study, and maintained under a 12:12 light –dark cycle in an ambient temperature of 21±3°C with relative humidity controlled. Animals were checked for signs of discomforts indicated by animal care and use guidelines (National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)). All procedures were carried out in accordance with the EC guidelines (EEC Council Directive 86/609 1987), Italian legislation on animal experimentation (Law Decree 116/92).

LPS (830 µg/Kg/ml *Escherichia Coli* serotype 0127:B8, Sigma Aldrich L3129) was injected intraperitoneally (i.p.) (n=6) and an equal volume of vehicle (saline) was used as a control (n=6). Animals were sacrified after 2 hrs and 6 hrs for immunofluorescence histochemistry assay.

19.2 Animal procedures and sample preparation

For histological processing, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). Intracardial perfusion was performed with 4% paraformaldehyde and 0.2% picric acid in Phosphate Buffered Saline (PBS) (100 mL/15min) preceded by an infusion of 50 ml of 0.9% NaCl saline containing heparin sodium (5000U/L); then, brains were dissected out. The brains were post-fixed in the same solution for 12 h, rinsed in 15% sucrose in PBS for approximately 12 h and then in a 30% sucrose in PBS for 1 day. The brains were frozen using dry ice, and coronal 50 µm thick sections series were cut at a cryotome, washed three times in cold PBS 1X and stored at -20°C in a glycerol-PBS solution until use. As control, also saline solution was i.p. injected with the same procedure.

19.3 Immunohistochemistry for brain sections

Brain sections were processed for multiple immunofluorescence histochemistry according to the following protocol. After five washes with PBS 1X pH 7.4 for 10 min, blocking was performed for 1 h at room temperature in a PBS 1X solution containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA). Incubation with anti-phospho(Ser-10)acetyl(Lys-14) H3 histone antibody

(Millipore®), anti-NeuN (Millipore®), anti Iba1 (Millipore®) diluted in 0.3% Triton X100, 1% normal serum (NS) and PBS 1X was performed overnight at 4°C. Before incubation with anti-NeuN brain sections were treated with citrate ph 6 for 20 min at 98°C, then 20 min at RT. After three washes in PBS/0.1% Triton X100, incubation with monoclonal mouse secondary antibody in 0.2% Triton X100, 1% NS and PBS was carried out for 90 min at RT. After washing three times with PBS for 10 min, brain sections were placed on gelatinised glass slides, dried and, after incubation with DAPI, mounted for confocal microscopy analysis.

20. MATERIALS AND METHODS 5

20.1 Cells and treatments

The immortalised murine microglia cell line BV-2 was a gift of Dr. Samuele Peppoloni (Department of Diagnostic and Clinic Medicine and Public Health, University of Modena, Modena). Cells were maintained at 37°C and 5% CO_2 in RPMI medium supplemented with 10% of heat-inactivated (56°C, 30 min) fetal bovine serum (Hyclone ™ Fetal Bovine Serum(U.S), Thermo Scientific™), 200 mM L-glutamine, 50 mg/ml streptomicyn. Cells were seeded and grown in T25- and T75- flask for 48-72h. Treatment was performed using 100 ng/ml of LPS (*Escherichia Coli* serotype 0127:B8, Sigma Aldrich L3129) after 4 and 8 hours, while the control (0 min) received phosphate-buffered saline (PBS). For each time-point we used 3 T25-flasks with ~ 2 x 10^6 cells in each one.

20.2 RNA extraction and RT-PCR

Total RNA extraction was performed using TRIzol® reagent (Sigma®, St.Louis, MO,USA) followed by clean-up step on GenElute™ Mammalian Total RNA Miniprep Kit and DNAse treatment to remove genomic contamination following the manufacturer's instructions (Sigma®, St.Louis, MO,USA). 2 µg of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA) in 40 ul of reaction mix.

20.3 Real Time PCR

Real time PCR was performed in ABI PRISM 7900 HT (Life Technologies Corporation, Carlsbad, CA, USA) using Power SYBR Green mix (Life Technologies Corporation, Carlsbad, CA, USA). We used specific primers to detect the expression levels of the following genes at the final concentration of 150 nM. The mRNAs levels were normalized for each well to endogenous control glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (Table 2).

Target	NCBI GenBank	Forward	Reverse
IL-1β	NM_008361	TGAAAGCTCTCCACCTCAATG	CCAAGGCCACAGGTATTTTG
IL-6	NM_031168	CTTCACAAGTCGGAGGCTTA	CAAGTGCATCATCGTTGTTC
TNF-α	NM_013693.2	GGCCTCCCTCTCATCAGTTC	CACTTGGTGGTTTGCTACGA
iNos	NM_010927.3	ACGAGACGGATAGGCAGAGA	GAGTAGTAGCGGGGCTTCAA
c-Fos	NM_010234.2	CACTCCAAGCGGAGACAGAT	GGCTGCCAAAATAAACTCCA
GAPDH	NM_017008.3	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

Table 2: Designed sequences of primer on target mRNA

The cycle parameters were: 95°C 10 min and 95°C 15 s, 60°C 1 min for 40 cycles. Single PCR products were subjected to a heat dissociation protocol (gradual increase of temperature from 60°C to 95°C) and agarose gel separation to verify the absence of artifacts, such as primer-dimers or non-specific products. Direct detection of PCR products was monitored by measuring an increase in fluorescence intensity caused by binding of SYBR GREEN I dye to neo-formed strand DNA during the amplification phase. Ct (cycle threshold) value was determined by the SDS software 2.2.2 (Life Technologies Corporation, Carlsbad, CA, USA) and was utilized to calculate mRNA fold changes using the data delta ct (ΔΔCt) method. The equation used was $2^{\Delta\Delta ct}=2^{(CtX-CtR)Reference-\ (CtX-CtR)target}$, where CtX was the threshold cycle of the gene of interest and CtR was the threshold cycle of the house-keeping gene (that was: GAPDH). For an appropriate application of comparative ΔΔCt method, it was demonstrated that amplification efficiency of the target gene and endogenous control gene was approximately equal. Each cDNA sample was run in triplicate and the mean values were used to calculate the gene expression levels.

20.4 Statistical analysis

The relative quantity of the diverse genes expression was analyzed by the ΔΔCt method using as calibrator average of the control group (PBS). The mRNA levels of the target genes were normalized on the intensity of the house-keeping gene, GAPDH. Ct values were obtained by an interpolate study. The effect of LPS on the expression pattern of different genes was analyzed with a one factor analysis of variance (ONE-WAY ANOVA) between the LPS- and PBS- treated cells (*p* values below 0.05 were considered significant).

20.5 Protein extraction

Cultured cells (~ 6 x 10^6) were harvested by centrifugation, washed in PBS, and resuspended in solution A and solution A plus Triton X-100 (50:50) (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol) added of protease Complete 1X (Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitor, 10 µl Na_3VO_4 , 50 µl NaPP, 20 µl NaF adding before using for each ml of buffer) incubated on ice for 10 min and centrifuged at 1,300 x g for 4 min. Supernatant was harvested as cytoplasmatic fraction. Washed 3 times the isolated nuclei (pellet) with Solution A/0,1%TRITON-X100, then pellet was lysed in Solution B (3 mM EDTA, 0.2 mM EGTA, and just before use adding 1 mM Dithiothreitol, protease Complete 1X and phosphatase inhibitor)

and incubated on ice for 30 min. the lysate was centrifuged at 1,700 x g for 4 min. Supernatant was collected as soluble nuclear fraction. Washed 3 times the isolated chromatin (pellet) with solution B, it was centrifuged at 10,000 x g for 1 min. Final pellet obtained was resuspended directly in Laemmli and PBS (50:50). Protein concentration was determined using standard protocol Coomassie® reagent (Pierce). Cytosolic, nuclear and chromatin enriched fractions were stored at -20°C.

20.6 Wetern Blot analysis

Western blotting were carried out on 4 µg of chromatin enriched extract for phospho(S10)-acetyl(K14)-H3 detection. Nuclear and cytosolic extracts were mixed with sample buffer for each sample just before the SDS-PAGE (10% polyacrilamide). Electrophoresis was performed using Bio-Rad Protean III mini-gel apparatus with 14% and 10% acrylamide gel run at 200 V for 45. Proteins were transferred to PVDF membranes (Millipore®) using Bio-Rad trans-blot apparatus at 100 V for 1 hr. The membranes were blocked for 2 hrs with 5% milk non-fat dry milk in TBS-Tween 20 followed by incubation, overnight at 4°C, with primary antibodies: Anti-phospho(Ser10)-acetyl (Lys14)-Histone H3 1:1000 in Blocking-buffer (Histone H3 phospho(Ser-10)acetyl(Lys-14) rabbit polyclonal antibody, Millipore®); anti-histone H3 1:1000 in Blocking-buffer (Histone H3 rabbit polyclonal antibody Millipore®). After washings membranes were incubated 1 h at RT with secondary antibody in Blocking-Buffer: anti- rabbit IgG-HRP-linked (Cell Signaling®, #7071) 1:5000. After 3 washing steps, bands were detected using Immobilon Western Chemiluminescent HRP (Millipore®). The protein levels were calculated by measuring the peak densitometric area of the autoradiografy analyzed with the image analyzer (GS-690 BIORAD). H3 phospho-acetylated and total H3 proteins run and were detected on the same blot. The optical densities (OD) of phospho(Ser10)-acetyl(Lys14)-H3 signal was normalized according to the OD of total histone H3. Ratios were expressed as percent of control ± S.E.M.

20.7 Statistical analysis

Western blotting data were analyzed with analysis of variance (ANOVA). Multiple comparisons between different time points of LPS treatment were conducted for the target with one-way ANOVA. All mean differences were considered statistically significant $p < 0.05$.

20.8 Chromatin extraction and sonication

Cells were cross-linked with formaldehyde (1% final concentration) for 15 min at room temperature and the reaction was stopped by adding glycine to final concentration 0,125 M for 5 minutes at room temperature. Fixed tissues were resuspended in Cellular Lysis Buffer (Tris-HCl ph 8.0 10mM, NaCl 10 mM, Igepal 0.2%, in the presence of a protease inhibitor Complete 1X and phosphatase inhibitors, 10 µl Na_3VO_4, 50 µl NaPP, 20 µl NaF adding before using for each ml of buffer) and kept in ice for 15 min. The lysate was centrifuged at 5,000 rpm for 5 min at 4°C then surnatant was collected and the pellet resuspended in Nuclear Lysis Buffer (Tris-HCl ph 8.0 50 mM, EDTA 10 mM, SDS 0,5 %, in the presence of a protease inhibitors Complete 1X and phosphatase inhibitors) and kept on ice for 30 min. The chromatin was sonicated 4 times (30 sec on / 30 sec off) at 270 setting in sonicator Labsonic(B.Braun Internationl®) to produce 500-1000bp fragments then was centrifuged at 14000 rpm for 10 min. The cleared supernatant was used immediately in ChIP experiments or stored at -80°C.

20.9 Chromatin Immunoprecipitation (ChIP)

Once the chromatin has been appropriately sonicated, measure the protein concentration of the lysate using standard protocol Coomassie® reagent (Pierce).

The volume of lysate for 1 ug/ul was diluted to a final volume of 2 ml in a mixture of 9 parts Dilution buffer (SDS 0.01%, Triton X-100 1.1%, 1.2 mM EDTA pH 8, 16.7 mM Tris-HCl pH 8 and 167 mM NaCl) and 1 part of Lysis buffer (SDS 1%, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8) and pre-cleared for 2 hours, rotating at 4°C, with 10 ul of protein G Agarose beads 50% slurry (KPL, Gaithersburg MD, USA).

Samples were centrifuged at 2,000 rpm for 5 min and the surnatant was incubated with 3 µl of the antibody phospho(ser-10)-acetyl(Lys-14) histone H3 (Millipore®) overnight rotating at 4°C. Simultaneously, 10 ul of the protein G beads were incubated with a pre-blocked mix consisting of 9:1 (Dilution: Lysis), 100 ug/ml BSA and 500 ug/ml sheared salmon sperm DNA in a final volume of 100 ul for each chromatin and rotate overnight at 4°C. After immuno-precipitation, 10 µl of protein G pre-blocked beads, previously washed, were added to each sample and incubated for 2 hours while rotating at 4°C. The unbound material was recovered as input and beads were washed 3 times with Wash Buffer (1% Triton X-100, 0.1% SDS, 150mM NaCl, 2mM EDTA pH 8.0, 20mM Tris-HCl pH 8.0) end once with a Final Wash Buffer (1% Triton X-100, 0.1% SDS, 500mM NaCl, 2mM EDTA pH 8.0, 20mM Tris-HCl pH 8.0). ChIPed material was eluted by two 15 minute

incubations at room temperature with 250 ul Elution Buffer (SDS 1%, 0.1 M NaHCO3). Chromatins, including input samples, were reverse-crosslinked and DNA was submitted to RNase and proteinase K digestion by incubating before at 37°C for 1 hour then at 65°C for 4 hours minimum and finally extracted by phenol-chloroform. Immuno-precipitation reactions were performed in duplicate using a no-Antibody sample as a non-specific control.

20.10 Qualitative PCR analysis

Qualitative PCR analysis was carried out using GoTaq® Flexi DNA Polymerase (Promega® Italia, Milan, Italy). DNA was extracted from immunoprecipitated chromatin of hippocampus and hypothalamus.

The promoter region of our gene of interest was amplified with the following primers:

Target	NCBI GenBank	Forward	Reverse
IL-1β	NC_000068.7	TGTGCATCTACGTGCCTACC	GTGTCATCGTGGTGGAAATG
IL-6	NC_000071.6	TAAGCACACTTTCCCCTTCC	TGAGCTACAGACATCCCCAGT
iNos	NC_000077.6	ATGTGTCCTGGGCGTGTT	GCTGCTGAGGGATTTTGTTC
c-Fos	NC_000078.6	TCCCTCCCTCCTTTACACAG	CCCGTCTTGGCATACATCTT

Aliquots of chromatin obtained before immunoprecipitation were also analyzed (Input DNA).

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