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# Fingerprints of Neural Activity after Peripheral Immune Challenges: An Experimental Study on the Communication between the Immune and Central Nervous Systems

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A Chouchou,
A Pascal,

« Ce qui s'apprend sans peine ne vaut rien et ne demeure pas »
René Barjavel

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**Chapter 5. General discussion**

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Raphaël Doenlen

# List of abbreviations

| | |
|---|---|
| ACTH | adrenocorticotropin hormone |
| Am | amygdala |
| AP | area postrema |
| BBB | blood brain barrier |
| BLA | basolateral nucleus of amygdala |
| CaN | calcineurin |
| CCK | cholecystokinin |
| CeA | central nucleus of amygdala |
| CNS | central nervous system |
| CORT | corticosterone |
| CRH | corticotropin releasing hormone |
| CS | conditioned stimulus |
| CsA | cyclosporin A |
| CTA | conditioning taste avoidance |
| CyPA | cyclophilin A |
| DA | dopamine |
| DVC | dorso-ventral complex |
| EEG | electroencephalogram |
| GABA | γ-aminobutyric acid |
| HPA | hypothalamo-pituitary axis |
| IC | insular cortex |
| IFN | interferon |
| IL | interleukin |
| i.p. | intra-peritoneal |
| IR | inmuno-reactive |
| IS | immune system |
| LC | locus ceoruleus |
| LHA | lateral part of hypothalamus |
| LPS | lipopolisaccharide |
| LV | lateral ventricule |

| | |
|---|---|
| NTS | nucleus tractus solitarii |
| Opt | optical tractus |
| PBN | parabrachial nucleus |
| PBS | phosphate buffer solution |
| POA/AH | preoptic area of anterior hypothalamus |
| PVH | paraventricular nucleus of hypothalamus |
| Rapa | rapamycin |
| s.c. | sub-cutaneus |
| SEB | staphylococcus enteretoxin B |
| SNS | sympathetic nervous system |
| SRBC | sheep red blood cell |
| TNF | tumor necrosis factor |
| US | unconditioned stimulus |
| VMH | ventro-medial part of hypothalamus |
| VPpc | ventroposterior parvicellular nucleus of the thalamus |
| 4V | 4e ventricule |
| 6-OHDA | 6-hydroxidopamine |

# Summary

Over the last three decades, several lines of evidence demonstrated the presence of a bi-directional communication that takes place between the central nervous system (CNS) and the immune system. In the present dissertation, it has been considered the immune system as a sensory organ that provides specific information of his own status to the CNS through several afferent pathways. In turn, the CNS is able to detect changes in the immune status induced by peripheral injection of immunomodulating agents. However, the specificity and the mechanisms by which the CNS detects or "senses" peripheral immune changes are poorly understood. The aim of the project was to elucidate whether the administration of immunostimulating or immunosuppressive agents in the periphery differently affects the patterns of electroencephalogram EEG in specific brain regions which were previously shown to play an important role in neuro-immune communication.

In the present study, the peripheral immune system was challenged either with (a) immunostimulating agents like lipopolysaccharides (LPS) of E. Coli and Staphylococcal enterotoxin B (SEB) or with (b) immunosuppressive agents like cyclosporin A (CsA) or rapamycin (Rapa). LPS is a major component of the outer membrane of Gram-negative bacteria that stimulates the secretion of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). In addition to a single LPS challenge, LPS tolerance was induced in a group of animal. In contrast to LPS naïve animals, LPS tolerant individuals do not show increases in plasma pro-inflammatory cytokines. SEB is a bacterial superantigen from Gram-positive bacteria that activates T cells and mainly stimulates the secretion of Th1-cytokines (e.g., IL-2 and IFN-γ). CsA elicits its immunosuppressive effects based on calcineurin (CaN) inhibition and Rapa induces an immunosuppressive effects based on a CaN-independent mechanism, mTOR inhibition.

Based on previous work done for the behavioral conditioning of immune function, the insular cortex (IC) and the amygdala (Am) were the selected brain regions studied. Local field potentials of the Am and IC were recorded during the course of the peripheral immune response in conscious freely moving animals (Chapter 2). Immunohistochemistry (IHC) of c-Fos was performed in the central nucleus of the Am (CeA), basolateral nucleus of the Am (BLA) and the IC after i.p. injection of different immunomodulating agents at different time points (Chapter 3). The time points for the c-Fos IHC were selected based on the different

peaks of EEG signal observed in the Am and the IC. At the same time, peripheral plasma cytokine concentrations were measured. In order to assess the role of the vagus nerve as a potential afferent pathway that may be involved in signalling peripheral changes to the CNS after peripheral injection of CsA, selective vagal deafferentation was performed and c-Fos IHC was analysed in the nucleus tractus solitarii (NTS), CeA, BLA and IC (Chapter 4).

The results demonstrated that the CNS specifically "sense" changes of the immune status induced by immunostimulating agents (LPS and SEB), by LPS tolerance or by immunosuppressive agents (CsA and Rapa). In addition, the spectral analysis of the EEG signal demonstrated that each immunomodulating agent has its own combination of changes in EEG frequency bands. The results also demonstrated that selective vagal deafferentation did not affect c-Fos level in the Am and IC after CsA injection. Therefore, the afferent fibers of the vagus nerve seem to be not essential in signalling CsA to the brain.

In conclusion, the data suggest that the CNS is able to specifically "sense" changes of the immune status by providing "fingerprints"of neural activity that may reflect peripheral modifications of the immune status after administration of different immunomodulating agents. However, additional afferent pathways have to be investigated in order to understand how a peripheral injection of the immunosuppressive drug CsA signals the CNS.

# Zusammenfassung

Während der letzten drei Jahrzehnte haben zahlreiche Versuchsansätze die bidirektionelle Kommunikation zwischen dem zentralen Nervensystem (ZNS) und dem Immunsystem dokumentiert. Dabei läuft der Informationsaustausch vom Gehirn zum peripheren Immunsystem über efferente Kommunikationswege, während Veränderungen in peripheren Immunfunktionen vom ZNS über afferente Wege wahrgenommen werden. Allerdings sind die Spezifität als auch die Mechanismen über die das ZNS periphere Immunveränderungen detektiert und weiterverarbeitet bisher kaum bekannt. Das Ziel dieser experimentellen Arbeit war daher die Analyse von ZNS-Aktivierungsmustern nach Gabe unterschiedlicher immunstimulierender bzw. immunsupprimierender Substanzen sowie die Analyse möglicher afferenter Mechanismen, über die diese Informationen aus dem peripheren Immunsystem an das ZNS weitergeleitet werden.

Um zu analysieren, ob verschiedene Immunstimuli das Muster der elektrischen Aktivität im Gehirn (EEG) unterschiedlich beeinflussen, wurden zum einen immunstimulierende Substanzen wie bakterielles Endotoxin (Lipopolysaccharid, LPS) oder das Superantigen Staphylcoccus Enterotoxin B (SEB) interperitoneal injiziert. Zum anderen wurden immunsupprimierende Substanzen wie Cyclosporin A (CsA) oder Rapamycin (Rapa) appliziert. LPS ist ein Hauptbestandteil der äusseren Zellmembran von Gram-negativen Bakterien, das die Sekretion proinflammatorischer Zytokine (z.B. IL-1$\beta$, IL-6, TNF-$\alpha$) induziert. Darüber hinaus kann durch konsektutive LPS-Injektionen eine Endotoxin-Toleranz erzeugt werden, bei der die nach einmaliger LPS-Gabe beobachteten ausgeprägten Zytokinanstiege ausbleiben. SEB ist ein Toxin Gram-positiver Bakterien das spezifisch T-Zellen stimuliert und die Sekretion von $Th_1$-Zytokinen (IL-2, IFN-$\gamma$) auslöst. CsA erzielt seine immunsuppressive Wirkung durch die Hemmung von Calcineurin (CaN) während Rapamycin aufgrund der Hemmung von mTOR (mamalian target of rapamycin) über einen CaN-unabhängigen Mechanisms die immunsuppressive Wirkung induziert.

Da in vorangegangenen Experimenten der Inselkortex (IC) und die Amygdala (Am) als neuronale Strukturen identifiziert wurden, die für die Konditionierung von peripheren Immunfunktionen essentiell sind, wurden diese Gehirnareale als Zielregionen für die Analysen ausgewählt. Das EEG-Signal in Am und IC wurde während des Verlaufs der durch

die unterschiedlichen Substanzen beeinflussten Immunantwort in frei beweglichen Ratten aufgezeichnet (Kapitel 2). In weiteren Experimenten wurde mittels Immunohistochemie die Expression des neuronalen Aktivierungsmarkers c-Fos im zentralen (CeA) und basolateralen Nukleus der Am (BLA) sowie im IC zu unterschiedlichen Zeitpunkten nach der Injektion der unterschiedlichen immunomodulierenden Substanzen bestimmt (Kapitel 3). Die Zeitpunkte für die Analyse der c-Fos Expression wurden basierend auf den unterschiedlichen Aktivierungsmustern des EEG-Signals in Am und IC ausgewählt. Parallel wurden Zytokinkonzentrationen im Plasma bestimmt. Um die Rolle des Vagusnervs als möglichen afferenten Kommunikationsweg von der Peripherie zum Gehirn nach der Applikation von CsA zu untersuchen, wurde in einem weiteren Experiment eine selektive Vagus-Deaffenzierung durchgeführt und die c-Fos Expression im Nucleus tractus solitarii (NTS), in der CeA, im BLA sowie im IC analysiert (Kapitel 4).

Insgesamt zeigen die Befunde, dass das ZNS Veränderungen im Immunstatus, die durch immunstimulierende Agenzien (LPS, SEB) aber auch durch immunsuppressive Substanzen (CsA, Rapa) hervorgerufen werden, spezifisch wahrnehmen und verarbeiten kann. Zusätzlich zeigte die Analyse des EEG-Signals, dass unterschiedliche immunmodulierende Substanzen verschiedene EEG-Muster im ZNS induzieren. Des weiteren konnte dokumentiert werden, dass die selektive Vagus-Deaffenzierung keine Effekte auf die c-Fos Expression in Am und IC nach CsA Applikation hat, was darauf hindeutet, dass die afferenten Projektionen des Vagusnervs nicht an der Weiterleitung der CsA induzierten, peripheren Immunveränderungen an das Gehirn beteiligt ist.

Zusammengefasst zeigen die Daten, dass das ZNS in der Lage ist Veränderungen im peripheren Immunsystem spezifisch zu detektieren. Diese experimentellen Daten bestätigen zum einen Befunde über die ZNS-Aktivierung nach peripherer Immunstimulation, zum anderen dokumentieren diese Ergebnisse erstmalig, dass eine spezifische ZNS-Aktivierung auch nach einer substanzinduzierten Suppression peripherer Immunfunktionen zu beobachten ist. Die deutet auf eine Immunstatus-spezifsche Signalverarbeitung im ZNS hin. In weiterführenden Experimenten müssen die afferenten Übertragungswege identifiziert werden, um zu klären über welche Mechanismen insbesondere eine periphere Immunsuppression vom ZNS detektiert und verarbeitet wird.

# Chapter 1.

# General introduction: behavioral conditioning as a model to study the communication between the immune and central nervous systems

Studies on the communication and the interaction between the central nervous system (CNS) and the immune system (IS) have developed over the last three decades into an extensive interdisciplinary field of research termed psychoneuroimmunology (PNI) (Blalock and Smith, 2007).

An elegant model to investigate the interactions between these systems is the behavioral conditioning paradigm (Reviewed in Riether et al., 2008). In these experiments the administration of an immunomodulating drug or substance, the unconditioned stimulus (US), is paired with a neutral stimulus, typically a taste or odour, the conditioned stimulus (CS). After one or several pairings of the CS with the US (the acquisition phase), re-exposure to the CS (the evocation phase) induced changes in the peripheral immune response, formerly elicited by the drug or substance, i.e. US (Ader, 2003). This taste-immune associative learning phenomenon is based on the intensive, and clinically relevant, interaction between the brain and the immune system, which has been documented particularly during the past two decades (Watkins and Maier, 2000; Tracey, 2002; Besedovsky and Rey, 2007; Nance and Sanders, 2007; Quan and Banks, 2007; Ziemssen and Kern, 2007). Experimental evidence demonstrates that the brain signals to the immune system via two distinct efferent pathways: via neural innervation of primary and secondary lymphoid organs, such as the thymus and the spleen (Felten et al., 1985; Elenkov et al., 2000; Nance and Sanders, 2007; Quan and Banks, 2007), or via humoral pathways comprising activation of the hypothalamus-pituitary-adrenal (HPA) axis (Besedovsky and del Rey, 1996). Corticotropin releasing factor (CRH) induces the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH reaches via the bloodstream the adrenal cortex and induces the release of corticosterone (CORT) (rat) or cortisol (humans). The release of CORT induces a negative feedback on the hypothalamus and the anterior pituitary (Fig. 1). Leukocytes bear intracellular and extracellular receptors for hormones, neurotransmitters and neuropeptides (Rook, 1999; Sanders and Straub, 2002). Therefore, alterations in plasma levels of these neuroendocrine mediators can induce tissue-specific changes in receptor expression of immune cells resulting in impaired cytokine production and gene expression. In parallel, the CNS impacts immune function via peripheral neural pathways like the sympathetic nervous system (SNS). The SNS innervates lymphoid organs such as the spleen and lymph nodes predominately via noradrenergic nerve fibers (Felten et al., 1985; Nance and Sanders, 2007; Quan and Banks, 2007), affecting

circulation and activity of adrenoceptor-expressing lymphocytes (Elenkov et al., 2000; Nance and Sanders, 2007).

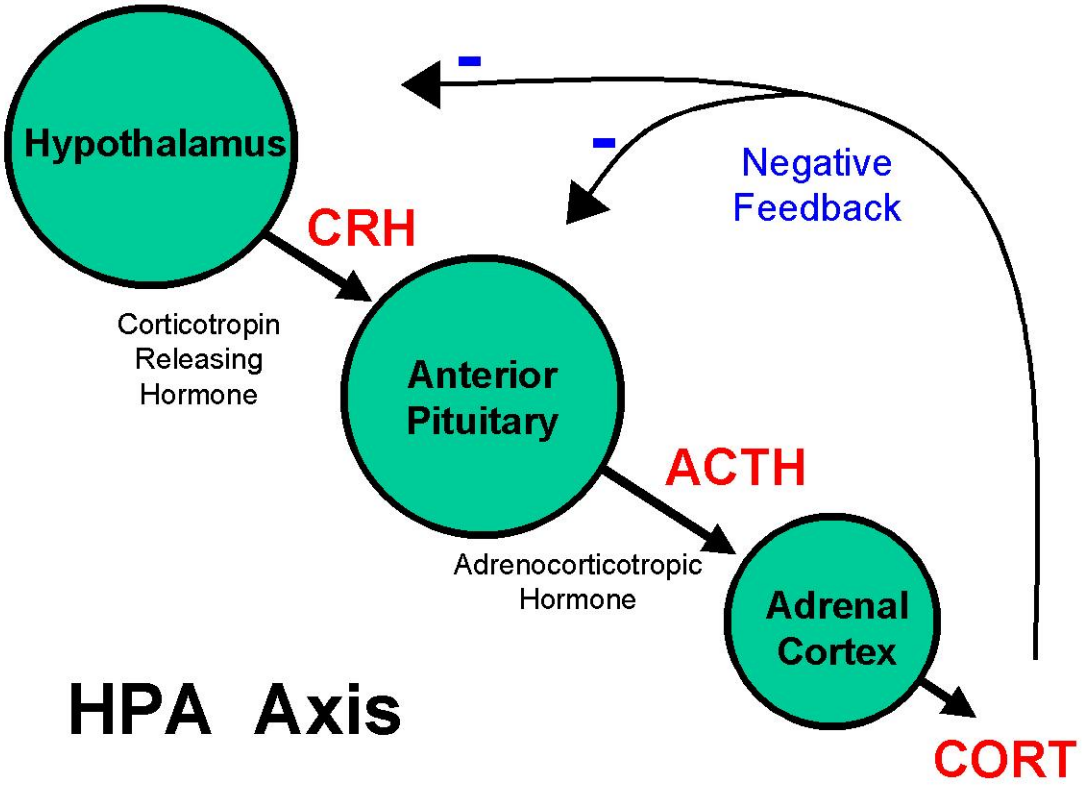


**Figure 1.** Schematic representation of the hypothalamus-pituitary-adrenal (HPA) axis.

In turn, the peripheral immune system gives the brain feedback about the interoceptive immune status through the afferent arm via neural and/or humoral afferent pathways. The neural pathway comprises signalling such as stimulation of the vagus nerve by cytokines, while the humoral pathway implements signalling such as peripheral cytokines crossing the blood brain barrier (BBB) via active or passive transport mechanisms (Gaillard, 1998; Banks et al., 2001; Quan and Banks, 2007). Neurons express receptors for pro-inflammatory cytokines (Diana et al., 1999; Morikawa et al., 2000), T cell cytokines (Neumann et al., 1997; Wang et al., 2001) and chemokines (Horuk et al., 1997). The pro-inflammatory cytokine IL-1β, for instance, activates the vagus nerve via receptors on sensory neurons (Goehler et al., 1998). These alterations in vagal activity are immediately transmitted to brain areas via the nucleus tractus solitarii (NTS), resulting in up-regulated IL-1β gene expression in microglia cells (Dantzer, 2004). This complex bi-directional network illustrated in figure 2 shows that the brain is capable of detecting signals released by an activated immune system. A major

issue for future research activities will be to elucidate the hierarchical, temporal and spatial communication patterns linking the brain and the peripheral immune system under normal conditions, and to understand in more detail how, when and where this interaction is disturbed under the different pathological conditions.

**Historical development: Conditioning of immune function**

S. Metalnikov and V. Chorine were always thought to be the pioneer workers in behavioral conditioning of immune functions. However, studies by I.I Makukhin in 1911 and reports by A. Voronov and I. Riskin in 1925 have now been cited as the first attempts to demonstrate "conditioned leukocyte functions" (Luk'Ianenko, 1961).

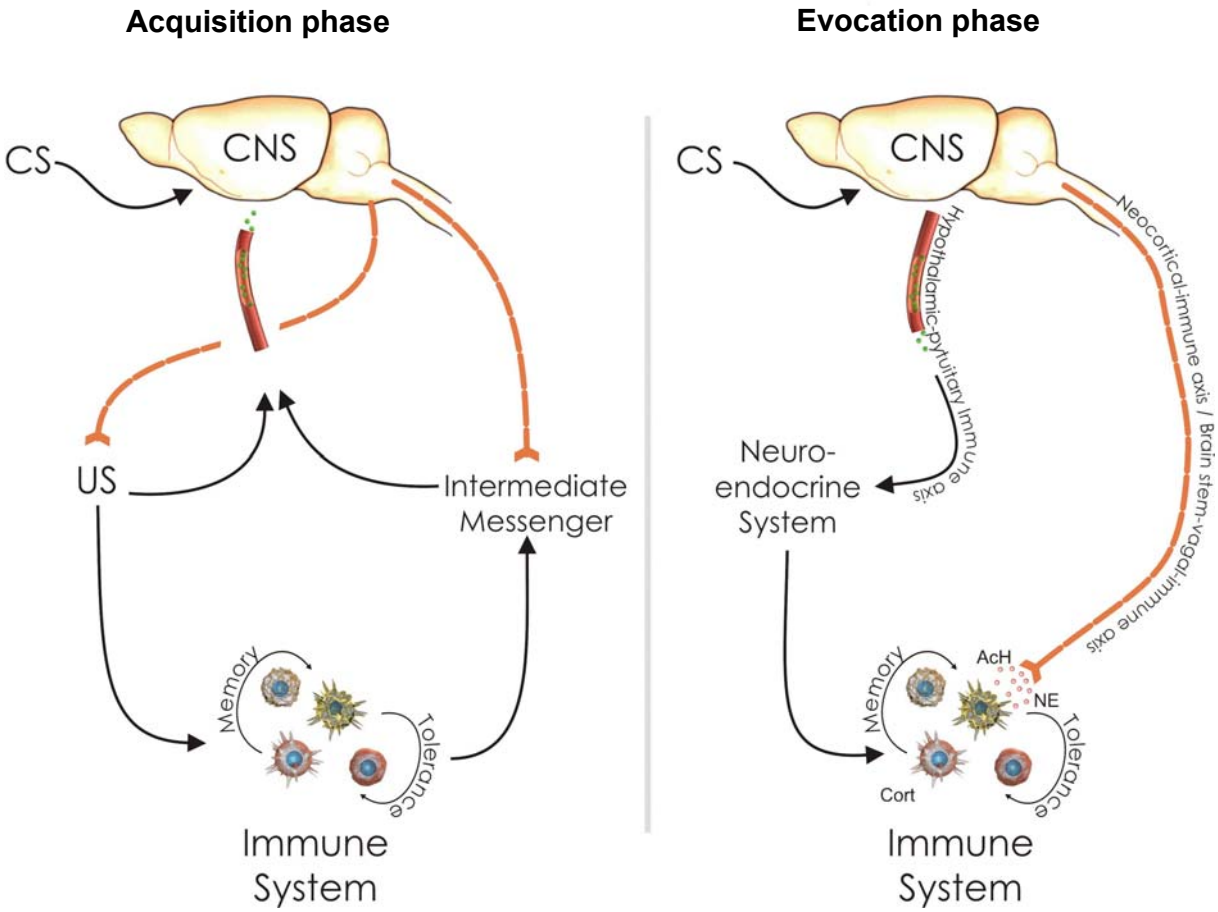


**Figure 2.** Theoretical framework for behavioral conditioning of immune function. At acquisition phase, there are two possible unconditioned stimuli (US) associated with a conditioned stimulus (CS). The US that is directly detected by the central nervous system (CNS) is defined as a '*genuine US*', whereas the one that needs one or more intermediary molecules to be released by another system before it can be detected by the CNS is called a '*sham US*'. For any US, *directly* or *indirectly* perceived, there are two possible afferent pathways to the CNS: the neural afferent pathway and the humoral afferent pathway. At evocation phase, there are two possible pathways by which the CNS can modulate immune functions: the humoral efferent pathway and the neural efferent pathway. The humoral efferent pathway may imply changes in neuro-hormones that directly or indirectly modify the immune response. The neural efferent pathway is supported by the direct innervations of primary and secondary lymphoid organs (Riether et al. 2008).

Nevertheless, in 1926 at the Pasteur Institute in Paris, Metalnikov S. and Chorine V. were the first researchers who employed the paradigm of Pavlovian conditioning in order to systematically affect immune responses. They injected guinea-pigs intraperitoneally with different antigens (Tapioca, B. Anthrax, Staphylococcus filtrate) (US) and associated each injection (10-20 times) with an external stimulus like skin scratching or a hot metallic plate placed (CS) on the skin. Fifteen days after the last acquisition trial, some animals were stimulated by the CS only, i.e. scratching or hot metallic plate. The results showed that the animals who were only re-exposed to the CS displayed the same magnitude of leukocyte reaction as the animals who indeed received the bacterial injection. These initial results were rapidly replicated during the following years (Nicolau and Antinescu-Dimitriu, 1929b, a; Ostravskaya, 1930). In the early 1950s, Noelpp et al. showed that asthmatic attacks in guinea-pigs can also be behaviorally conditioned. The anaphylactic reaction was monitored by assessing the movement of the thorax and the abdomen by "thoraco-abdomino-motography". Sensitized animals were conditioned by five contingent pairings of an auditory stimulus (CS) and exposure to an allergenic aerosol (US). At evocation phase, when the animals were exposed to the CS and the aerosols of an $H_2O$-spray, some animals displayed a conditioned allergic response to the CS (Noelpp and Noelpp-Eschenhagen, 1952a, b, c). Considerable attention was given to the question of conditioned immune effects by Soviet investigators (Luk'ianenko, 1961; Ader, 1981) who conducted studies in a basically similar manner to these performed by Metalnikov and Chorine. In 1975, Ader and Cohen provided the first evidence for conditioned immunosuppression by pairing the gustatory CS, saccharin, with the immunosuppressive drug cyclophosphamide (CY), the US, thereby attenuating the antibody response after CS re-exposure. This report of behaviorally conditioned immunosuppression initiated the interdisciplinary research field of PNI more than 30 years ago (Ader and Cohen, 1975).

Classical or Pavlovian conditioning is often described as the transfer of the response-eliciting property of a biologically significant stimulus (US) to another stimulus (CS) without that property (Pavlov, 1927; Carew and Sahley, 1986; Domjan, 2005). This transfer is thought to occur only if the CS serves as a predictor of the US (Rescorla and Wagner, 1972; Pearce, 1987; Rescorla, 1988). Thus, classical conditioning can be understood as learning about the temporal or causal relationships between external and internal stimuli to allow for the appropriate preparatory set of responses before biologically significant events occur.

During the last 30 years, enormous progress was made in elucidating the neuronal and molecular events that take place during association and consolidation of the memory trace in classical conditioning paradigms (Glanzman, 1995; Menzel and Muller, 1996; Berman and Dudai, 2001; Bermudez-Rattoni, 2004).

**Neural mechanisms underlying behavioral conditioning of immune function**

Most of the recent studies on behavioral conditioning of immune functions were confined to immunosuppressive alterations (Ader and Cohen, 1975). However, after the replication and acceptance of the conditionability of immuno-pharmacological responses, studies on the interaction between the immune system and the CNS were broadened resulting in the exploration of both directions of the same paradigm, i.e. conditioned immunosuppression as well as conditioned immuno-enhancement (Reviewed in Pacheco-Lopez et al., 2007). The following section will provide an overview of the neural mechanisms involved in behavioral conditioning of immune functions (Fig. 3).

The phenomenon of associating a flavour (food/drink) with possible immune consequences was experimentally appraised in rodents and humans by exploring the conditioned taste aversion/avoidance (CTA) paradigm (Garcia et al., 1955; Riether et al., 2008). Subjects learned to associate a flavour with a postprandial malaise (Bermúdez-Rattoni, 2004). A discrete neural network involved in taste-visceral associative learning was already reported (Sewards and Sewards, 2002; Sewards, 2004). Such a neural circuit consistently comprises sensory and hedonic pathways, including the NTS, parabrachial nucleus (PBN), medial thalamus, amygdala (Am) and insular cortex (IC) (Yamamoto et al., 1994; Yamamoto, 2007). Regarding the central processing of the gustatory CS and visceral US, cholinergic neurotransmission in the IC and Am seems to be essential during the initial stages of taste memory formation (Bermudez-Rattoni et al., 2004). Acetylcholine appears to codify the novelty of both the conditioned and the unconditioned stimulus (Acquas et al., 1996; Miranda et al., 2002). In particular, the IC is essential for the acquisition and retention of this kind of associative learning process (Bermudez-Rattoni and McGaugh, 1991; Cubero et al., 1999). It was postulated that the IC may integrate gustatory and visceral stimuli (Sewards and Sewards, 2001). In addition, the preponderant role of the IC in conditioned antibody production (Chen et al., 2004) was confirmed when assessing the neuronal activity marker c-Fos. The Am seems to play an important role during the formation of aversive ingestive

associations (Reilly and Bornovalova, 2005), and also seems to be relevant to limbic-autonomic interaction (Swanson and Petrovich, 1998). Based on the central findings of CTA, a series of studies investigated the involvement of the IC and the Am, which are reciprocally interconnected, in conditioned immunosuppression of antibody production (Ramirez-Amaya et al., 1998; Ramirez-Amaya and Bermudez-Rattoni, 1999). The IC and Am were identified as key structures in mediating conditioned immunosuppression after evoking taste-cyclophosphamide association.

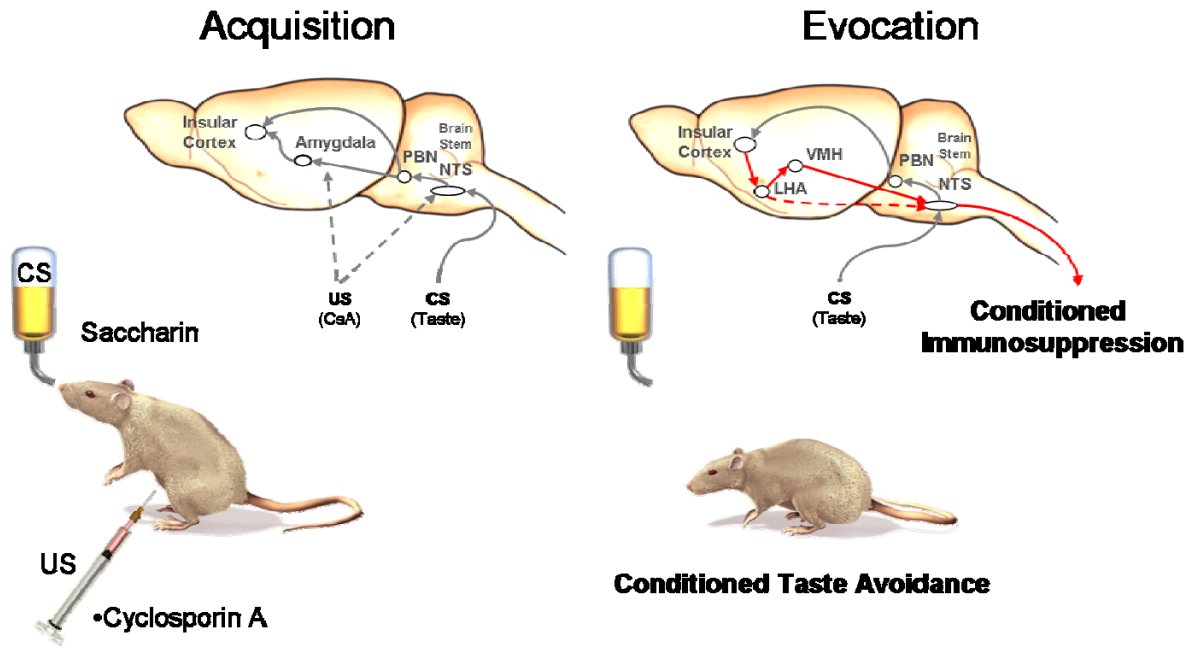


**Figure 3.** Schematic representation of brain-immune interaction in the model of saccharin-cyclosporin A agents conditioning. Two basic steps compose the conditioning protocol: an **acquisition phase** in which one or more CS-US pairings occur inducing an associative learning process, and an **evocation phase** in which the memory of such an association is retrieved after exposure to the CS. The information on the gustatory CS is centrally processed through brain stem relays (nucleus tractus solitarii (NTS) and parabrachial nucleus (PBN)), reaching the insular cortex (IC). The IC, together with the amygdala (Am), is indispensable in CS-US association processes, and is also necessary for evoking conditioned taste avoidance. Both the lateral hypothalamic area (LHA) and the ventromedial nucleus of the hypothalamus (VMH) are also essential for evoking conditioned immunosuppression in the periphery.

N-methyl-D-aspartic acid (NMDA)-induced lesions either in the IC or the Am before acquisition and before evocation demonstrated that IC lesions disrupt both acquisition and evocation of conditioned immunosuppression, while Am lesions merely effected acquisition. In addition, cortical and amygdaloidal glutamate releases were related to central visceral processing (Miranda et al., 2002). Moreover, the IC and the Am was shown to be involved in behavioral interactions that mediate conditioned enhancement of antibody production (Ramirez-Amaya and Bermudez-Rattoni, 1999). The ventromedial

hypothalamic nucleus (VMH), widely recognized as a satiety centre (Vettor et al., 2002), is intimately associated with sympathetic facilitation in peripheral tissues (Saito et al., 1989), including modulation of peripheral immune reactivity (Okamoto et al., 1996). More recently, the neural substrates involved in behaviorally conditioned immunosuppression by cyclosporine A (CsA) in rats were identified (Pacheco-Lopez et al., 2005), showing that excitotoxic brain lesions of the IC, the Am and the VMH modulate the conditioned immunosuppressive effects on the immune system, measured as splenocyte proliferation and cytokine production (IL-2 and IFN-$\gamma$). More specifically, these results indicate that the IC is essential for acquiring and evoking conditioned immunosuppression. In contrast, the Am seems to mediate the input of visceral information necessary at acquisition time, while the VMH appears to participate in the efferent output pathway to the immune system to evoke the behaviorally conditioned immune response.

Investigating the conditioned enhancement of natural killer (NK) cell activity in rodents, it was demonstrated that central catecholamines and glutamate are essential factors at the evocation stage (Hsueh et al., 1999; Kuo et al., 2001). Central and peripheral catecholamine contents were specifically depleted before the evocation phase by reserpine and 6-hydroxydopamine (6-OHDA) treatment, respectively. Since reserpine treatment impaired conditioned NK cell activity and 6-OHDA did not, central catecholamines seemed to be essential for memory retrieval during evocation (Hiramoto et al., 1990). These findings were confirmed by Hsueh et al 1999; pre-treatment with $\alpha$- and $\beta$-adrenoceptor antagonists or dopamine (DA)-1- and DA-2-receptor antagonists before evocation also blocked the effects inducing conditioned enhancement of NK cell activity (Hsueh et al., 1999). In addition, glutamate but not $\gamma$-aminobutyric acid (GABA) was required at evocation time (Kuo et al., 2001). It was shown that both the cholinergic as well as the serotonergic system are critical for triggering the conditioned NK cell response during the association and evocation phases (Hsueh et al., 2002). At association, acetylcholine seems to act through the nicotinic, $M_2$ and $M_3$-muscarinic receptors, whereas at evocation neither the latter receptors nor the $M_1$ receptors appear to affect the conditioned response (CR). In both the association and evocation phases, serotonin acts through the 5-$HT_1$ and 5-$HT_2$ receptors to modulate the CR (Hsueh et al., 2002). Furthermore, it was shown that naltrexone only blocked conditioned enhancement of NK cell activity when applied before re-exposure to the CS, suggesting that opiate receptors in the CNS mediate the conditioned response. In contrast, CS-US

association does not seem to involve endogenous opioids, since naltrexone administered prior to acquisition did not interfere with the conditioning process (Solvason et al., 1989).

**The afferent communication between the immune system and the central nervous system**

At present it is completely unknown when and how the CNS detects a US with immune consequences such as immunosuppressive drugs. Within the peripheral nervous system, the vagus nerve, with relays in brain stem nuclei, was proposed as the main neural afferent pathway during the immunosensory process, prior to the immune information reaching the forebrain structures (Goehler et al., 2000). Therefore, it is particularly relevant to determine the main neural afferent pathway, and to establish when and how the US employed in the conditioning model is detected by the CNS. Originally ascribed to the immune system, cytokines play an active and important role in the afferent communication between the immune system and the CNS (Besedovsky and del Rey, 1996a; Turnbull and Rivier, 1999; Dantzer, 2004a). It was reported that the CNS is able to detect or "to sense" changes in cytokine concentrations in the periphery, in particular changes in pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 or tumour necrosis factor alpha (TNF-$\alpha$), that also modulate spatial learning tasks, as well as long term potentiation (Gibertini, 1996; Schneider et al., 1998; Fiore et al., 2000; Banks et al., 2001a; Matsumoto et al., 2001; Rachal Pugh et al., 2001; Lynch, 2002; Matsumoto et al., 2002). In this context it was hypothesized that, within the CNS, cytokines play an important role during the acquisition time of behavioral conditioning.

Recent behavioral data from our laboratory indicate that endotoxin-tolerant animals categorize a relevant gustatory stimulus followed by a high dose of lipopolysaccharide (LPS) differently to naïve control animals (Pacheco-Lopez et al., 2008). Peripheral pro-inflammatory cytokines seem to control the strength of the taste-LPS engram, since immunocompetent cells may not work just as transducers but in some instances may be able to filter or amplify environmental information, depending on previous immunological experience (immune history). These data are supported by neuroanatomical data showing a lack of LPS-induced brain activation during endotoxin tolerance (Valles et al., 2005). In particular, important brain nuclei involved in taste-visceral associative learning such as the central nucleus of the amygdala (CeA) and locus coeruleus (LC) displayed reduced activity in

comparison to LPS-naïve controls. In addition, food allergic animals displayed increased brain activity following intra-oral antigenic challenge. This increase in activity can be abolished by previous repeated intake of lower doses of the same antigen resulting in an oral tolerance phenomenon (Basso et al., 2004; Costa-Pinto et al., 2006).

**Objectives and working hypothesis**

The communication between the immune and the central nervous system (CNS) is a well established concept (Besedovsky and Rey, 2007; Quan and Banks, 2007; Dantzer et al., 2008). Peripheral immune stimulation has repeatedly shown to alter brain activity (Saphier et al., 1987b, a; Saphier, 1989; Saphier et al., 1990; Valles et al., 2002; Beishuizen and Thijs, 2003; Chen et al., 2005; Dallaporta et al., 2007; Teeling et al., 2007). The brain is able to monitor peripheral immune stimulations by several pathways (Quan and Banks, 2007; Dantzer et al., 2008):

- The neural afferent pathway mainly through the vagus nerve (Bluthe et al., 1994; Watkins et al., 1994).
- The humoral pathway through the circumventricular organs and the choroid plexus (Quan et al., 1998; Vitkovic et al., 2000).
- The cytokine transporters pathway at the blood-brain barrier (BBB) (Banks, 2006).
- Cytokine receptors which are located on perivascular macrophages and endothelial cells of brain venules (Schiltz and Sawchenko, 2002; Konsman et al., 2004).

However, the capacity of the CNS to specifically "sense" different immunomodulating agents remains unclear. The aim of the study was to elucidate whether the administration of different immunomodulating agents in the periphery differently affects brain neural activity in a specific CNS region.

In the present study, the peripheral immune system was challenged either with (a) immunostimulating agents like lipopolysaccharides (LPS) of E. Coli and Staphylococcal enterotoxin B (SEB) or with (b) immunosuppressive agents like cyclosporin A (CsA) or rapamycin (Rapa). LPS is a major component of the outer membrane of Gram-negative bacteria that stimulates the secretion of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). In addition to a single LPS challenge, LPS tolerance was induced in a group of animal. In contrast to LPS naïve animals, LPS tolerant individuals do not show increases in plasma pro-inflammatory cytokines. SEB is a bacterial superantigen from Gram-positive bacteria that

activates T cells and mainly stimulates the secretion of Th1-cytokines (e.g., IL-2 and IFN-γ). CsA elicits its immunosuppressive effects based on calcineurin (CaN) inhibition and Rapa induces an immunosuppressive effects based on a CaN-independent mechanism, mammalian target of rapamycin (mTOR) inhibition.

Based on previous work done for the behavioral conditioning of immune function, the insular cortex (IC) and the Amygdala (Am) were the selected brain regions studied. These regions are integrative and associative brain areas that receive ascending visceral informations from the ventroposterior parvicellular nucleus of the thalamus, the parabrachial nucleus, the nucleus tractus solitarii and the lateral hypothalamic area (Cechetto and Saper, 1987; Krushel and van der Kooy, 1988; Allen et al., 1991; Shi and Cassell, 1998; Barnabi and Cechetto, 2001). Local field potentials of the Am and IC were recorded during the course of the peripheral immune response in conscious freely moving animals (Chapter 2). Immunohistochemistry (IHC) of c-Fos was performed in the central nucleus of the Am (CeA), basolateral nucleus of the Am (BLA) and the IC after i.p. injection of different immune stimuli at different time points (Chapter 3). The time points of the c-Fos IHC were selected based on the different peaks of EEG signal observed in the Am and the IC. At the same time, peripheral plasma cytokine concentration was measured. In order to assess the role of the vagus nerve as a potential afferent pathway that may be involved in signalling peripheral changes to the CNS after peripheral injection of CsA, selective vagal deafferentation was performed and c-Fos IHC was analysed in the nucleus tractus solitarii (NTS), CeA and IC (Chapter 4).

The results demonstrated that the CNS specifically "sense" changes of the immune status induced by immunostimulating agents (LPS and SEB), LPS tolerance or immunosuppressive agents (CsA and Rapa). In addition, the spectral analysis of the EEG signal demonstrated that each immunomodulating agent has its own combination of changes in EEG frequency bands. The results also demonstrated that selective vagal deafferentation did not affect c-Fos level in the Am and IC after CsA injection. Therefore, the afferent fibers of the vagus nerve seem to be not essential in signalling CsA to the CNS.

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# Chapter 2.

## Specific changes in the EEG of the amygdala and the insular cortex after peripheral injection of different immunomodulating agents in conscious adult rats

# Introduction

The pioneering work of Hugo Besedovsky and his colleagues in 1977 demonstrated that neuronal firing rates in the ventromedial nucleus of the hypothalamus increase at the peak of the primary antibody response (Besedovsky et al., 1977). These types of related work allowed Besedovsky and Edwin Blalock to propose that the immune system functions as a sense organ which informs the central nervous system about infection and injury. In 1984, Blalock published a paper titled "The Immune System as a Sensory Organ" (Blalock, 1984). However, the use of the expression "sensory organ" for the immune system has to be properly defined. A sensory organ is normally able to provide specific information to the CNS like the *modality*, the *intensity*, the *location* and the *duration* of the stimuli. These four aspects of the stimulus will constitute the "code" or the "pattern" of the stimulus that allows the CNS to specifically "sense" it. With this specific information, the brain will be able to discriminate several stimuli but also to provide the proper feedback response to each stimulus. For example after i.p. injection of LPS or SEB, the cytokine profile differs depending on which immune cells are stimulated, how long the cells will be stimulated and where. So the *modality* of a peripheral immune challenge will be which cytokines increase or decrease, for example LPS will stimulate the secretion of pro-inflammatory cytokines (IL-1$\beta$, IL-6 and TNF-$\alpha$) and SEB will mainly stimulate the secretion of Th1 cytokines (IL-2 and IFN-$\gamma$). Concerning the *intensity*, it will be determined by the quantity of each cytokine secreted. The *duration* will be for how long the increase or decrease of cytokines last until it is back to baseline level. The aspect of *location* will be in which organ the secretion of cytokine take place and/or which afferent pathway is used to signal to the brain (humoral vs. neural). In that sense, the immune system can indeed be classified as a sensory organ.

Based on hypothetical considerations that every chemical interaction within the brain is accompanied by electrical changes, a method called stereo-EEG was described in 1984 which started with intracerebral recording of brain field potentials after injection of different psychoactive drugs (Dimpfel and Decker, 1984). In 1985, the classification of three neuroleptic drugs, namely sulpiride, clozapine and haloperidol was established based on this stereo-EEG method. The authors showed that the recorded activity in the cortex, the striatum and the reticular core resulted in a "fingerprint" for each drug (Dimpfel and Decker, 1985). In 1986, the method was upgraded to a telemetry method called tele-stereo-EEG

which recorded the electrical activity from stereotactically defined brain location after injection of different psychoactive drugs (Dimpfel et al., 1986a; Dimpfel et al., 1986b). This method can not only be used to describe "fingerprint" of brain activity between different psychoactive drugs but also to detect the onset and time dependence of drug that act in the brain (Krugel et al., 2003).

Based on the paradigm of a bi-directional communication between the brain and the immune system, the tele-stereo-EEG can be used to record EEG signal of selected brain areas after injection of different immunostimulating agents (e.g., LPS or SEB) and immunosuppressive agents (e.g., CsA or Rapa). Recordings of multi-unit electrical activity in the CNS were made in the preoptic area/anterior hypothalamus (POA/AH) and in the paraventricular nucleus (PVN) of rats following sheep red blood cell (SRBC) immunization in sensitized animals (Saphier et al., 1987a, b; Saphier, 1989; Saphier et al., 1990). Significant increase in hypothalamic electrical activity was observed when serum antibodies were first detected. Induction of a second immune response to SRBC showed an increase in the electrical activity of the hypothalamus with an extended duration and with reduced amplitude compared to the first SRBC induction. In addition, administration of a cytotoxic drug, cyclophosphamide, prevented the generation of antibody and the increase of electrical activity in the hypothalamus.

In the first part of the present experiment, different immune responses were experimentally induced using either a single injection of LPS, SEB, cyclosporin A or rapamycin or repeated administration of LPS which leads to the development of LPS tolerance. Several studies demonstrated that peripheral injection of LPS induces an increase of pro-inflammatory cytokines (IL-1$\beta$, IL-6 and TNF-$\alpha$), adrenocorticotropic hormone (ACTH) and corticosterone (Cort) within 2 hours in plasma (Givalois et al., 1994; West and Heagy, 2002; Zhou et al., 2003). In addition, LPS challenge stimulates the release of bioactive lipids (e.g., prostaglandins), reactive oxygen species and activated coagulation components (Schletter et al., 1995). Consecutive injections of LPS induce immune tolerance to this antigen (Oberbeck et al., 2003). It has been described that c-Fos immunoreactivity and the febrile response are reduced in the preoptic area of the hypothalamus after induced LPS tolerance (Navarro et al., 2007). However, mRNA cytokine expression still occurs in the brain during endotoxin tolerance, whereas in the periphery this expression is blunted (Chen et al., 2005).

Peripheral injection of SEB stimulates the secretion of Th1 cytokines (IL-2 and IFN-γ) in plasma within one hour (Miethke et al., 1992; Huang and Koller, 1998; Serrats and Sawchenko, 2006). SEB induces increase of c-Fos in the brain that peaks at 2-3 hours after SEB injection (Serrats and Sawchenko, 2006). An accumulation of IL-2 mRNA is already detectable in lymph nodes and spleen within 1-2 hours after SEB injection and purified subsets of T cells from SEB-injected animals indicates that cluster of differentiation 4 $(CD4)^+$ cells have much more IL-2 mRNA than $CD8^+$ cells (Baschieri et al., 1993). In contrast to IL-2, mRNA of IFN-γ is mainly found in CD8 subset (Herrmann et al., 1992).

Cyclosporin A (CsA) and rapamycin (Rapa) are both immunosuppressive drugs but have two distinct signalling pathways to specifically inhibit T cells proliferation (for a review see Halloran, 1996). CsA binds to a cytosolic receptor called cyclophilin A (CyPA). The CsA-CyPA complex interacts and inhibits the $Ca^+$-dependent serine-threonine phosphatase, calcineurin (CaN). This protein is a critical component of the T cell receptor (TcR)-linked signal transduction pathway leading to cytokine gene transcription. CaN is activated by the increase in cytoplasmic free $Ca^+$ that results from TcR activation. The activated phosphatase targets the T cell specific transcription factor, nuclear factor of activated T cells (NFAT). The dephosphorylated cytoplasmic NFAT subunit is then able to translocate to the nucleus and associate with a nuclear subunit to form the active NFAT complex. The NFAT complex is required for expression of IL-2 and other cytokine genes. Therefore, by interfering with the TcR-mediated activation of CaN, CsA will block the transcription of IL-2 in T cells (Halloran, 1996).

Rapamycin, a lipophilic macrolide, binds to a cytosolic protein called FK506 binding protein 1A (FKBP12), the major FK506 binding protein. The Rapa-FKBP12 complex interferes specifically with the progression of G1-phase cells into synthesis phase. This complex targets and inhibits the function of mTOR protein (Abraham and Wiederrecht, 1996; Halloran, 1996). The mTOR protein is involved in cell-cycle regulation and governed the G1-phase checkpoint. Rapamycin generate then its anti-proliferative effects by inhibiting the function of mTOR.

The second part of the study was to record the EEG of two specific brain areas after injection of the four different immunomodulating agents (LPS, SEB, CsA and Rapa) and after induction of LPS tolerance with the tele-stereo-EEG method. The IC and the Am were the two brain areas selected based on previous work that showed the implication of these

two areas in behavioral conditioning of immune functions and there sensitization after injection of immunostimulating agents (Pacheco-López et al., 2005; Valles et al., 2005). Taking in consideration that the immune system signals to the CNS through different afferent pathway by eliciting different bioactive molecules depending of the nature of the immunomodulating agent, it was hypothesized that the CNS should be able to detect or "sense" the changes of immune status after injection of different immune stimuli. In addition, the pattern of EEG in IC and Am should be specific to the nature of the immunomodulating agent resulting in a "fingerprint" of neural activity for each immune stimulus.

# Materials & Methods

### *Animals*

Male Dark Agouti rats, weighing between 250-300 g, were obtained from Harlan Netherland (Horst, Netherlands). Animals were individually housed under a normal 12:12h light/dark schedule (lights on at 7 am) with food and water available *ad libitum*. The experiments were carried out following the current Swiss and German regulations for animal experimentation (Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance, German guidelines [BGBl. I, p. 1105]) and were approved by the relevant local animal ethics committees.

### *Surgical procedure*

Monopolar stainless steel EEG electrodes were respectively implanted under anaesthesia with xylazine hydrochloride (5 mg/kg, Rompun®, Bayer Health Care, Leverkusen, Germany) plus ketamine hydrochloride (90 mg/kg, Ketanest®, Parke-Davis GmbH, Karlsruhe, Germany) in the central amygdala (CeA) and in the insular cortex (IC), respectively, at coordinates relative to the Bregma (CeA: anterior -2.5 mm; lateral 4.3 mm; ventral 8.0 mm, IC: anterior +1.6 mm; lateral 4.8 mm; ventral 5.2 mm) (Fig. 4). A stainless steel screw used as indifferent electrode was positioned at the surface of the Cerebellum. The electrodes were soldered to a socket (TSE, Bad Homburg, Germany) and fixed with dental cement (Technovit 3040, Heraeus Kulzer, Wehrheim, Germany) on the skull. After surgery, the animals were treated i.m. with antibiotic (Retacillin compositum, i.m., 200,000 IE, Jenapharm, Jena, Germany), with analgesic Rimadyl, s.c., 5 mg/kg, Pfizer, USA) and with 5

ml of sterile Glucose solution (s.c., B. Braun Medical AG, Switzerland). Following surgery, the animals were housed separately and were allowed to recover for a period of at least 14 days.

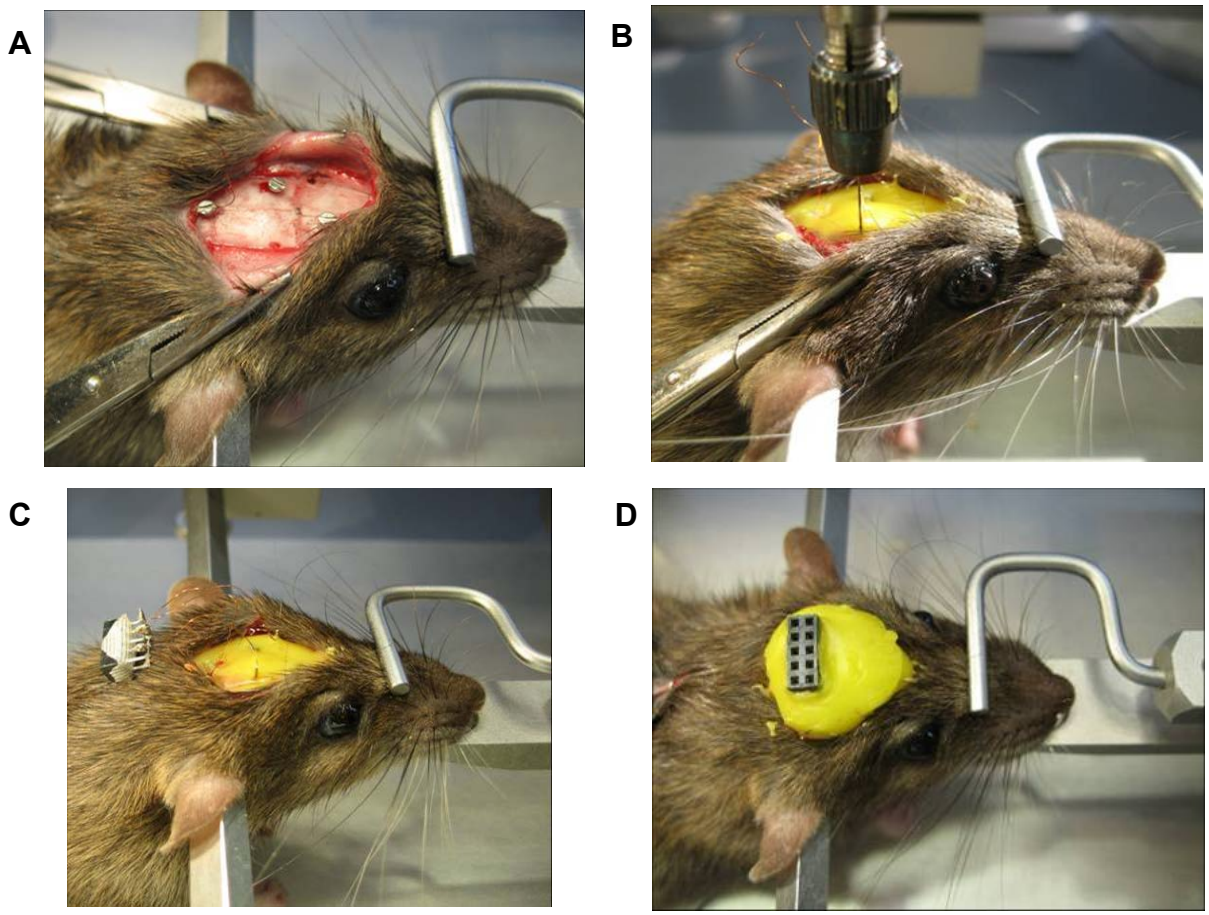


**Figure 4.** Surgical procedure. (A) Fixation of the reference electrode and two anchor screws. (B) Implentation and fixation with dental cement of one monopolar electrode. (C) Connection of the electrodes to the socket. (D) Fixation of the socket with dental cement.

### *Experimental protocol*

The animals were divided in six different treatment groups: (1) the LPS group was injected with lipopolysaccharides (LPS) from E. Coli (serotype: B5, 0.1 mg/kg, Sigma Aldrich, Germany), (2) the LPS tolerant (LPS Tol.) group received i.p. injections of LPS with increasing the doses (0.1, 0.2, 0.5, 0.5 and 0.5 mg/kg) over a period of 5 consecutive days (Oberbeck et al., 2003), (3) the SEB group was injected with Staphylococcus enterotoxin B (SEB, 1.0 mg/kg, Sigma Aldrich, Germany), (4) the CsA group received an injection of Cyclosporine A (CsA, 20 mg/kg, Sandimmun, Novartis, Switzerland), (5) the Rapa group was

injected with Rapamycin (Rapa, 3 mg/kg, LC Laboratories, Woburn, MA, USA) and (6) a naïve group of animal was treated with an equivolume of vehicle (1 ml sterile saline).

***Electroencephalography***

For the EEG recording, the animals were transferred to a separate room and allowed to adapt for 40 min (Fig. 5). Thereafter, 3 periods of 5 min were recorded each 15 min as reference period which constituted the EEG baseline of each animal. The particular immunomodulating agents were given in a volume of 1 ml by intraperitoneal (i.p.) injection and the first EEG recording started 20 min post-injection. To monitor the EEG of freely moving rats, a radio-transmitter (TSE, Bad Homburg, Germany) was fixed on the socket by plug connection. The recording procedure and the analysis of the EEG signal were based on previous work of Dimpfel et al., 1986. The EEG signals were telemetrically received via pulse position modulation and transmitted to the decoder. The EEG signal of the targeted brain areas (Am, IC) was recording during 5 min each 15 min. The recording procedure lasted 3 hours. For each record of 5 min, the EEG signal was computed for periods of 4 s by fast Fourier transformation and averaged on each channel (channel Am and channel IC) over time blocks of 5 min after artefact rejection. The obtained EEG signal corresponded to field potentials generated in the Am and the IC recorded by deep monopolar electrode. The data were expressed as percentage of the difference between the EEG signal recorded before the injection (baseline) and the EEG signal recorded after injection of the different immunomodulating agents. The data were displayed as absolute EEG power (results, Fig. 6) as well as continuous spectra of power intensity (results, Fig.7). The continuous spectra of power intensity were divided into six frequency bands (Hz): 0.6-4.0 ($\delta$-band), 4.0-8.0 ($\theta$-band), 8.0-9.5 ($\alpha$1-band), 9.5-13 ($\alpha$2-band), 13-19 ($\beta$1-band) and 19-30 ($\beta$2-band) (Krugel et al., 2003).

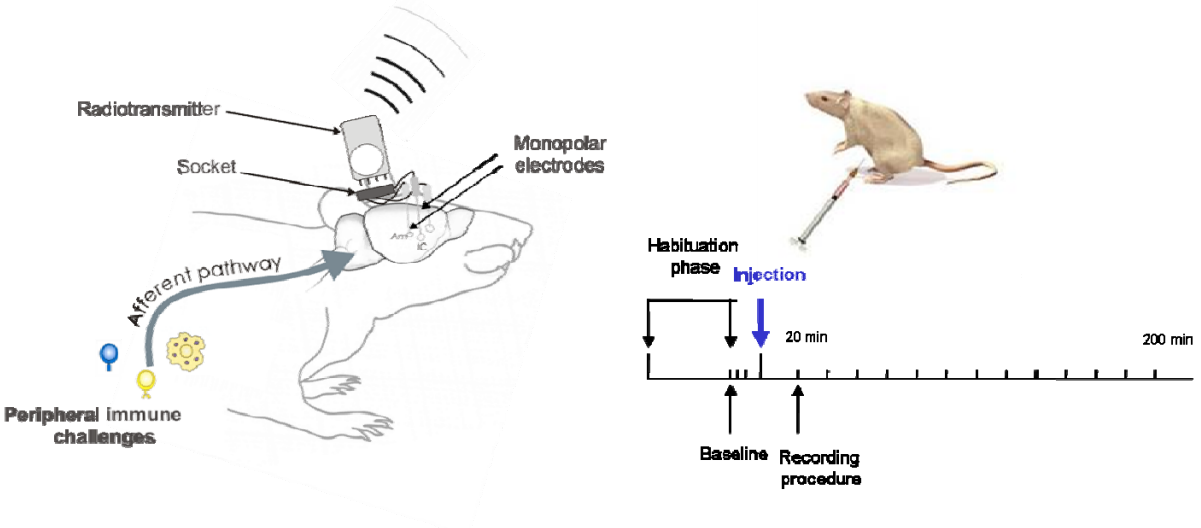


**Figure 5.** Experimental design for the EEG recording procedure. The animals were placed in the recording room for 40 min as a habituation phase. Three baseline recordings of the Am and IC were performed one hour before injection time. Then, the animals were injected i.p. with the selective immunomodulating agents. 20 min after injection, the EEG recording procedure started and lasted for 200 min. A record of 5 min was performed each 15 min.

### *Statistical analysis*

Data were analysed using SPSS software (Version 14.0, Chicago, IL, USA) and the level of significance was set at $p \leq 0.05$. Neural activity data and body temperature data were analysed by repeated measures ANOVA with "time" as repeated measures within subject factor and "group" as between-subject factor. Additionally, independent *t*-tests were performed for each time point.

## Results

### *Changes in EEG power after single and repeated injection of lipopolysaccharide (LPS)*

Peripheral injection of LPS induced increases of the EEG power in the insular cortex (IC) (Fig. 6D, black curve) but not in the amygdala (Am) (Fig. 6A, black curve) at different time point compared to saline treated group. Repeated measures ANOVA revealed a significant "group" effect between saline and LPS treated animals in the IC ($F_{(1,14)} = 5.56$, $p \leq 0.05$). Independent *t*-tests revealed specific increases of the EEG power in the IC 140 and 185 min after one i.p. injection of LPS (140 min and 185 min, $p \leq 0.05$).

After inducing LPS tolerance (Fig. 6A and 6D, grey curve), repeated measures ANOVA revealed a significant "group" effect between saline and LPS tolerance groups in the Am ($F_{(1,14)} = 13.13$, $p \leq 0.01$) and the IC ($F_{(1,14)} = 16.27$, $p \leq 0.01$). Independent *t*-tests revealed that several significant increases of the EEG power were observed in Am (35 min, 50 min, 65 min, 80 min, 95 min, 110 min and 155 min, $p \leq 0.05$ and 140 min and 185 min, $p \leq 0.07$) and IC (35

min, 65 min, 80 min, 95 min, 110 min, 140 min, 155 min and 185 min, $p \leq 0.05$). These results suggested that EEG power in the Am did not change after a single injection of LPS, although the EEG power in IC increased of 30-35% from the baseline 140 min after single LPS injection compared to saline group. However, when the same animals were injected with increasing doses of LPS over a period of 5 days in order to induce LPS tolerance, the EEG power consisted in increases of 20 % of the mean power values in the Am and in increases of 25-30% of the mean power values in the IC compared to saline group. These increases of EEG power after induced LPS tolerance started already 35 min in Am and IC after the fifth injection of LPS. These data suggested that Am and IC were differently stimulated after a single or repeated LPS injection, and that LPS tolerance provides a different pattern of EEG power compared to a single administration of the same immunostimulating agent. This was confirmed when the absolute EEG power of Am and IC (Fig. 6) was expressed as a spectrum of 6 frequency bands: 0.6-4.0 ($\delta$-band), 4.0-8.0 ($\theta$-band), 8.0-9.5 ($\alpha$1-band), 9.5-13 ($\alpha$2-band), 13-19 ($\beta$1-band) and 19-30 ($\beta$2-band) (Fig. 7) 95 min and 140 min after single LPS injection or induced LPS tolerance. In the Am (Fig. 7A and 7B), no significant differences were observed in the 6 frequency bands between LPS and saline treated group, but significant changes of $\alpha$1 at 95 min ($p \leq 0.05$) and $\alpha$1, $\beta$2 at 140 min ($p \leq 0.05$) were observed when compared induced LPS tolerance to saline treated group. In the IC (Fig. 7A and 7B), significant changes were observed 140 min after a single LPS injection for $\theta$, $\alpha$1, $\alpha$2, $\beta$1 and $\beta$2 ($p \leq 0.05$) between the LPS group and saline group. Significant changes of $\delta$, $\theta$, $\alpha$1 and $\beta$2 ($p \leq 0.05$) at 95 min and $\delta$, $\alpha$1, $\alpha$2 and $\beta$2 ($p \leq 0.05$) at 140 min were observed when compared the induced LPS tolerance frequency bands to saline frequency bands. These data demonstrate that Am and IC provided different pattern of EEG power and changes of frequency bands depending if the immune stimulus (LPS) was administered in a naïve animal or in a tolerant animal.

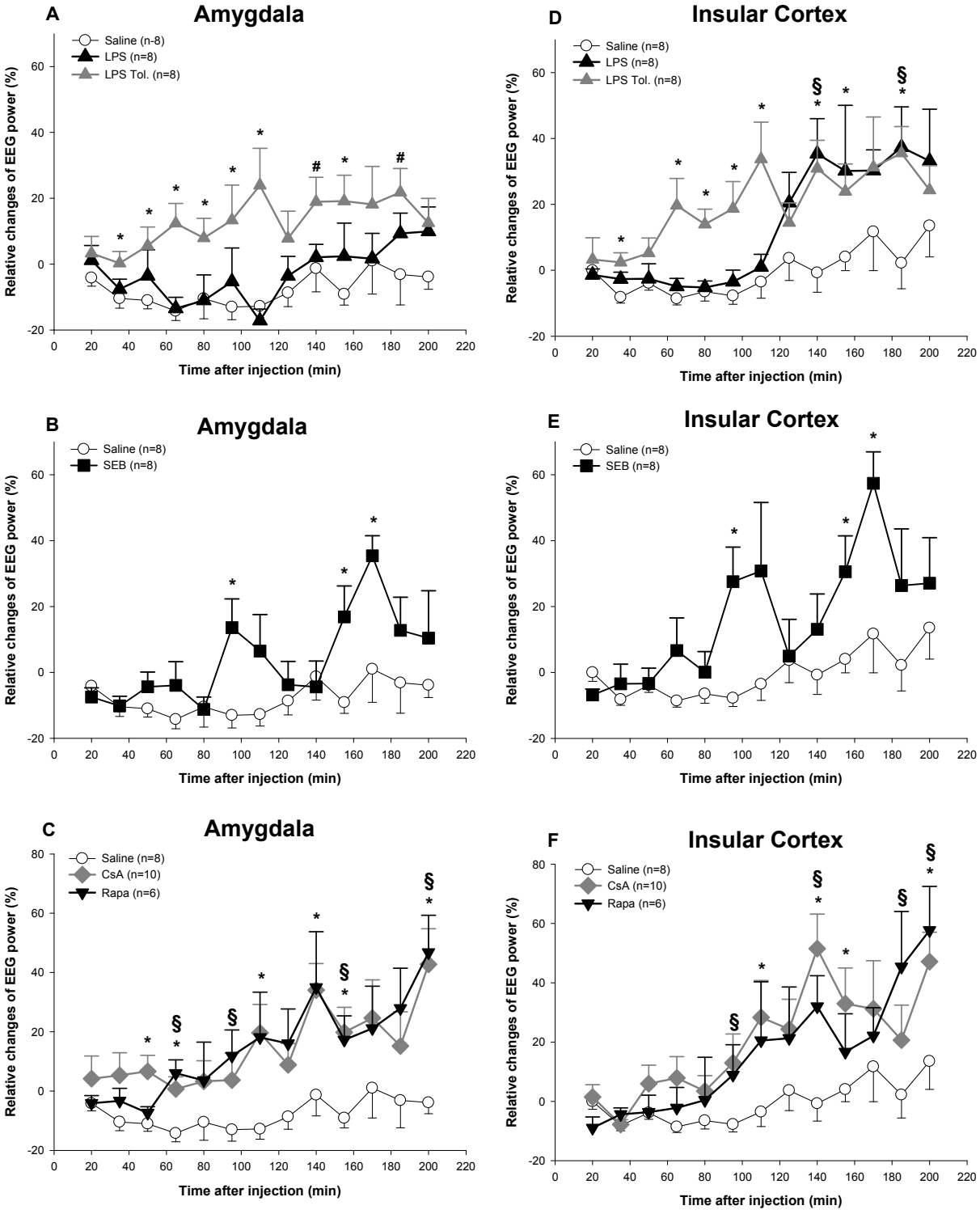


**Figure 6.** Changes in absolute EEG power in the Amygdala (A, B and C) and Insular cortex (D, E and F) after i.p. injection of 0.1 mg/kg LPS, 1.0 mg/kg SEB, 3 mg/kg rapamycin (Rapa), 20 mg/kg cyclosporin A (CsA) and induction of LPS tolerance with 5 consecutive injections (LPS Tol). Data are shown as mean and SEM, n = 6 to 10 animals per group. Independant *t*-test, * $p \leq 0.05$ (LPS Tol. vs. Saline, SEB vs. Saline and CsA vs. Saline), § $p \leq 0.05$ (LPS vs. Saline and Rapa vs. Saline), # $p \leq 0.07$.

***Changes in EEG power after administration of Staphylococcal enterotoxin B (SEB)***

Peripheral injection of SEB induced changes of EEG power in the Am and IC and the pattern of EEG can be described as two peaks of neural activity which occurred at the same time in Am and IC (Fig. 6B and 6E). Repeated measures ANOVA revealed a significant "group" effect between saline and SEB groups in the Am ($F_{(1,14)} = 4.67$, $p \leq 0.05$) and the IC ($F_{(1,14)} = 7.39$, $p \leq 0.05$). Post hoc independent *t*-tests revealed a first significant increase of the EEG power 95 min in Am (95 min, $p \leq 0.05$) and in IC (95 min, $p \leq 0.05$) after SEB injection compared to saline treated group. A second significant increase of the EEG power was observed in an interval of 155 min to 170 min in the Am (155 min, 170 min, $p \leq 0.05$) and in the IC (155 min, 170 min, $p \leq 0.05$) after administration of SEB compared to saline treated animals. The spectral analysis of the absolute EEG power after peripheral injection of SEB (Fig. 7A and 7B) showed significant differences of $\delta$ ($p \leq 0.05$) at 95 min and $\beta 2$ ($p \leq 0.05$) at 140 min in Am compared to saline group. In addition, changes of $\delta$ and $\theta$ ($p \leq 0.05$) 95 min and $\beta 2$ ($p \leq 0.05$) 140 min after a single injection of SEB were observed when compared to saline treated animals (Fig. 7C and 7D). These data suggest that changes in the absolute EEG power and changes in the different frequency bands occurred at the same time and in the same manner in the Am and the IC after a single peripheral injection of SEB. However, the comparison of the EEG power in Am and IC after induced LPS tolerance (Fig. 6A and 6D) or single peripheral injection of SEB (Fig. 6B and 6E) or LPS (Fig. 6A and 6D) showed patterns of neural activity which completely differed one to each other. These differences of EEG power in Am and IC after induced LPS tolerance and single LPS or SEB injection became more evident when the absolute EEG signal was expressed in 6 frequency bands (Fig. 7). These data strongly demonstrated how specific can be the sensing process of the CNS after peripheral injection of two different immunostimulating agents (LPS and SEB) or after induction of two different immune statuses (naïve vs. tolerance) by the same immunostimulating agent (LPS).

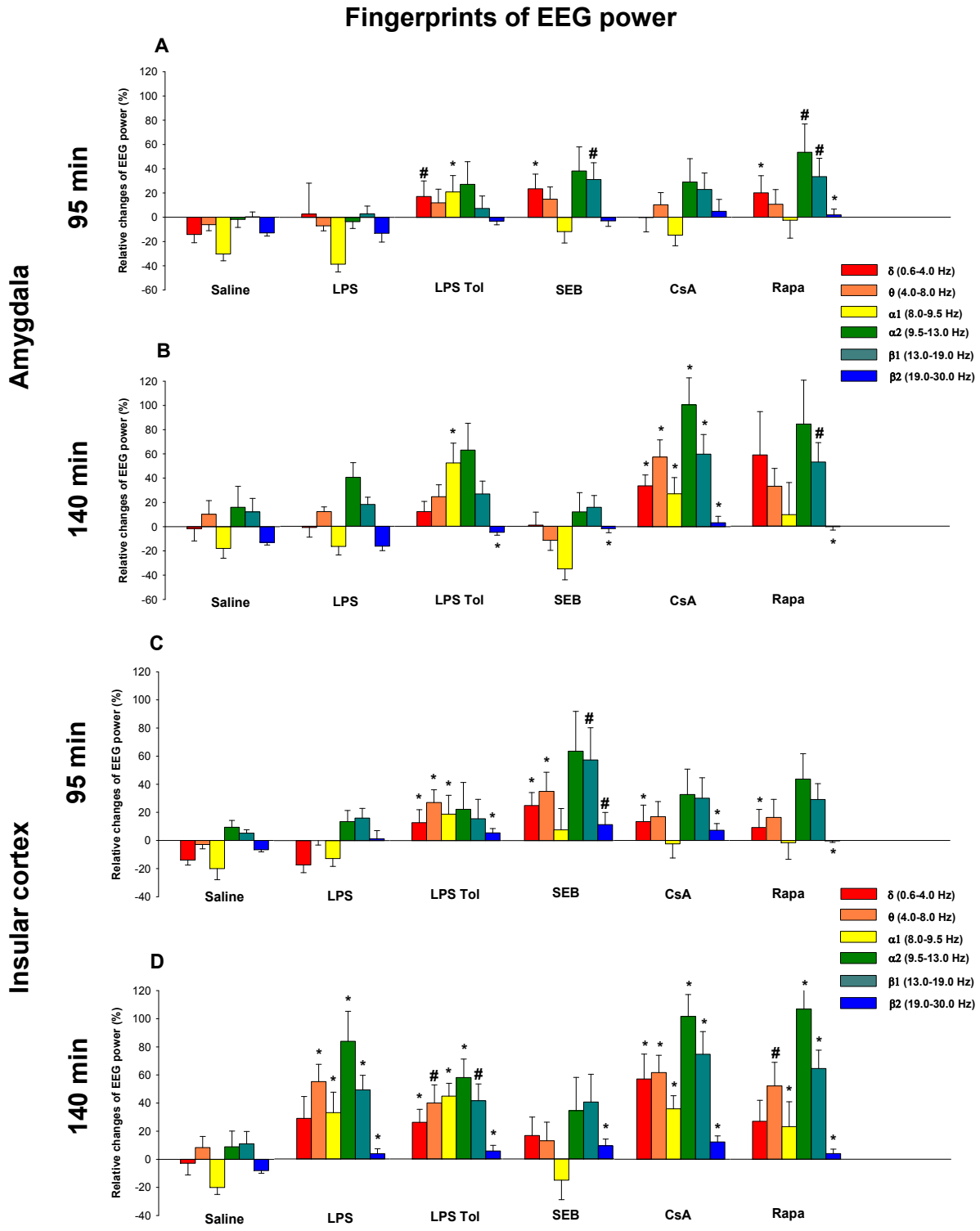


**Figure 7.** Changes of EEG power of each frequency band in the amygdala (A and B) and the insular cortex (C and D) 95 min and 140 min after i.p. injection of 0.1 mg/kg LPS, 1.0 mg/kg SEB, 3 mg/kg Rapamycin (Rapa), 20 mg/kg Cyclosporin A (CsA) and induction of tolerance to LPS with 5 consecutive injections (LPS Tol.). Data are shown as mean and SEM, n = 6 to 10 animals per group. Independant *t*-test, * $p \leq 0.05$ (LPS Tol. versus Saline, LPS versus Saline, SEB versus Saline, CsA versus Saline and Rapa versus Saline), # $p \leq 0.07$.

***Changes in EEG power after administration of cyclosporin A (CsA) or rapamycin (Rapa)***

Peripheral injection of CsA and Rapa induced increases of EEG power in Am and IC. Repeated measures ANOVA revealed a significant "group" effect between saline and CsA groups in the Am ($F_{(1,14)} = 9.96$, $p \leq 0.01$) and the IC ($F_{(1,14)} = 11.49$, $p \leq 0.01$). Independent *t*-tests revealed significant increases of the EEG power in the Am (50 min, 65 min, 110 min, 140 min, 155 min and 200 min, $p \leq 0.05$) and in the IC (110 min, 140 min, 155 min and 200 min, $p \leq 0.05$) after peripheral injection of CsA compared to saline treated group (Fig. 6C and 6F). Repeated measures ANOVA revealed a significant "group" effect between saline and CsA groups in the Am ($F_{(1,14)} = 11.61$, $p \leq 0.01$) and the IC ($F_{(1,14)} = 8.33$, $p \leq 0.01$). Independent *t*-tests revealed significant increases of EEG power in the Am (65 min, 95 min, 155 min and 200 min, $p \leq 0.05$) and in the IC (95 min, 140 min, 185 min and 200 min, $p \leq 0.05$) after peripheral injection of Rapa compared to saline treated group (Fig. 6C and 6F). The spectral analysis of the absolute EEG power showed significant changes of $\delta$ and $\beta 2$ ($p \leq 0.05$) at 95 min only in the IC after peripheral injection of CsA compared to saline treated animals (Fig. 7C). In addition, significant changes of $\delta$, $\theta$, $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ ($p \leq 0.05$) at 140 min in Am and IC were observed after peripheral injection of CsA compared to saline treated group (Fig. 7B and 7D). The spectral analysis of the absolute EEG power after peripheral injection of Rapa showed significant changes of $\delta$ and $\beta 2$ ($p \leq 0.05$) at 95 min in the Am and the IC (Fig. 7A and 7C). In contrast, significant changes of $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ ($p \leq 0.05$) were observed only in the IC at 140 min compared to saline treated group (Fig. 7B and 7D). These data suggest that the CNS is able to detect peripherally administered immunosuppressive agents (CsA and Rapa). The EEG power (Fig. 6C and 6F) and the spectral analysis of the absolute EEG power (Fig. 7) in the Am and the IC showed similar patterns. In this case, the detection process of CNS seems to be not as specific as for the discrimination between two different immunostimulating agents (LPS vs. SEB) or for the discrimination between two different immune statuses induced by the same immunostimulating agent (naïve vs. tolerant).

# Discussion

The results show that the EEG power in Am and IC were differently affected after injection of different immunomodulating agents. Specific changes in amplitude and kinetic of the EEG power were observed after a single injection of LPS, SEB and during LPS tolerance.

A single injection of LPS induced significant increases of EEG power in the IC whereas no alterations were observed in the Am. Peripheral injection of LPS has been showed to result in elevated body temperature and increased plasma levels of pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α), ACTH and corticosterone (Givalois et al., 1994; West and Heagy, 2002; Zhou et al., 2003). In addition, LPS stimulates the secretion of prostaglandins, reactive oxygen species and activate coagulation components (Schletter et al., 1995). Increase of c-Fos has been reported in several brain regions after peripheral injection of LPS (Dallaporta et al., 2007; Elmquist et al., 1993; Lacroix and Rivest, 1997; Sagar et al., 1995). Specially, c-Fos expression has been observed in the nucleus tractus solitarii (NTS), the medial and central nucleus of amygdala and the paraventricular nucleus of hypothalamus (PVH) and lateral hypothalamus after peripheral LPS injection. These brain areas provide specific neural projections to the Am and the IC. These neural inputs mainly relay visceral information that may inform the CNS from peripheral changes (Allen et al., 1991; Barnabi and Cechetto, 2001; Cechetto, 1987; Cechetto and Saper, 1987; Shi and Cassell, 1998). Peripheral injection of LPS also induces cytokine mRNA expression in the brain and activates hypothalamus-pituitary-adrenal (HPA) axis (Beishuizen and Thijs, 2003; Chen et al., 2005; Pitossi et al., 1997). Therefore, several lines of evidence may explain the changes of EEG power observed in the IC after peripheral injection of LPS. However, the lack of EEG changes in the Am after a single injection of LPS still remains unclear.

Consecutive injections of LPS induced immune tolerance as demonstrated by a lack in the secretion of pro-inflammatory cytokines and reduced febrile response (Oberbeck et al., 2003, Pacheco-López et al., 2008, West and Heagy, 2002). In addition, c-Fos immunoreactivity was shown to be reduced in the preoptic area of the hypothalamus in LPS-tolerant animals (Navarro et al., 2007). Interestingly, the present study revealed changes in EEG power in the Am and the IC after LPS injection in endotoxin-tolerant animals. Increased cytokine mRNA expression in the brain was reported during endotoxin

tolerance (Chen et al., 2005). The latter may explain why changes of EEG power were observed in the Am and the IC during endotoxin tolerance.

Increased EEG power was observed in the Am and the IC after peripheral injection of SEB. SEB stimulates T cells and induces a strong increase in Th1 cytokine production (Huang and Koller, 1998). In addition, peripheral injection of SEB has been shown to induce c-Fos expression in the PVH, Am and NTS (Serrats and Sawchenko, 2006) and activates the HPA axis, resulting in increased plasma levels of ACTH and corticosterone (Goehler et al., 2001; Kusnecov et al., 1999; Shurin et al., 1997). These data might explain why changes of EEG power were observed in the Am and the IC after peripheral injection of SEB.

In the present chapter, it has been shown that the CNS was able to detect changes in the immune status after administration of different immunostimulating agents (LPS or SEB) or after inducing LPS tolerance. But in addition to a detection process, the tele-stereo-EEG technique provides evidence of a specific "sensing" process that take place between the immune system and the CNS.

Peripheral injection of the immunosuppressive agents induced changes of EEG power in the Am and the IC but the pattern of the EEG power after peripheral injection of CsA or Rapa was similar (Fig. 6C and 6F). These results may indicate that the CNS was not able to specifically differentiate these two drugs, while CsA and Rapa have two distinct signalling pathways to specifically inhibit T cells proliferation (CaN inhibition vs. mTOR inhibition). CsA binds to a cytosolic receptor called cyclophilin A (CyPA). The CsA-CyPA complex interacts and inhibits the $Ca^{+}$-dependent serine-threonine phosphatase, calcineurin (CaN) (Halloran, 1996). Rapamycin binds to a cytosolic protein called FKBP12, the major FK506 binding protein. This complex targets and inhibits the function of mTOR protein (Abraham and Wiederrecht, 1996; Halloran, 1996). Furthermore, the results indicated that the pattern of the EEG power in the Am and the IC was strongly different after peripheral injection of an immunosuppressive agent compared to the pattern of EEG after peripheral injection of an immunostimulating agent.

The spectrum analysis of the absolute EEG power after peripheral immune challenge (Fig. 7) triggers the idea of a specific "sensing" process that may take place between the CNS and the immune system. Indeed, the EEG power of the 6 different frequency bands did not change at the same time with the same amplitude. For each immunomodulating agents (LPS, LPS Tol, SEB, CsA and Rapa), not all the 6 frequency bands were different from the

saline treated control. Each immune challenge had its own combination of changes in frequency bands. Such a combination can be associated to a code which may provide the EEG identity card of the present immune status. However, the meaning of such specific changes in frequency bands after different immune challenge still remains unclear and has to be further investigated.

The present study shows for the first time that the CNS may sense which immunomodulating agent challenges the immune system by generating specific patterns of electrical activity that can be associated to "fingerprints"of neural activity.

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# Chapter 3.

# Expression of the activation marker c-Fos in the amygdala and the insular cortex after peripheral administration of immunomodulating agents

# Introduction

The specific changes of neural activity observed in the Am and IC after peripheral injection of different immunomodulating agents shows increases of EEG at specific time points depending on the injected immune stimuli. In the present chapter, activation of Am and IC after i.p. injection of either an immunostimulating or an immunosuppressive agent was anatomically investigated. In that purpose, imaging c-Fos protein by immunohistochemistry (IHC) was established. The c-Fos protein represents an immediate early gene lasting only few hours after transcription (Dragunow and Faull, 1989; Hughes and Dragunow, 1995). The expression of c-Fos is low and undetectable in quiescent cells, but is rapidly induced at the transcriptional level within minutes of extracellular stimulation. c-Fos expression is related to cell excitation but not inhibition (Dragunow and Faull, 1989; Hughes and Dragunow, 1995). Therefore, while c-Fos positive cells provide definitive evidence that the cell, in some way, is involved in the target brain area activity, the absence of c-Fos expression provides no relevant information to the functional involvement of a cell (Sagar et al., 1988; Dragunow and Faull, 1989; Sheng and Greenberg, 1990; Hughes and Dragunow, 1995).

The neural circuit of behavioral conditioning of immune function based on the model of conditioning taste aversion/avoidance (CTA) comprises sensory and hedonic neural pathways, including the nucleus tractus solitarii (NTS), the parabrachial nucleus (PBN), medial thalamus, Am and IC (Yamamoto et al., 1994; Yamamoto, 2007). In addition, a novel CS-US pairing induces c-Fos immunoreactivity (IR) in IC and Am. For example, novel saccharin induces larger increases in c-Fos expression in the central nucleus of amygdala (CeA) and IC compared to familiar saccharin. This pattern is not observed in the basolateral nucleus of amygdala (BLA), PBN and NTS suggesting a specific role of CeA and IC for novel CS-US association (Cubero et al., 1999; Koh et al., 2003; Koh and Bernstein, 2005). Am and IC were shown to be involved in behavioral conditioning that mediate conditioned enhancement of antibody production (Ramirez-Amaya and Bermudez-Rattoni, 1999) or conditioned suppression of splenocyte proliferation and cytokine production induced by pairing saccharin (CS) with cyclosporin A (CsA) (US) (Pacheco-Lopez et al., 2005). Therefore, in the present study, c-Fos IHC was performed in the CeA, BLA and IC at three different time points (120, 240 and 360 min) after peripheral injection of CsA.

In addition, c-Fos IHC was performed in the Am and the IC after peripheral injection of an immunostimulating agent (LPS). Increase of c-Fos has been previously observed in the NTS, area postrema (AP), PBN, locus coeruleus (LC), paraventricular nucleus of the hypothalamus (PVN), ventromedial preoptic area (VPO) and CeA after LPS injection (Elmquist et al., 1993; Sagar et al., 1995; Lacroix and Rivest, 1997; Dallaporta et al., 2007). However, most of these studies report c-Fos expression in a qualitative way. In the present experiment, c-Fos expression was quantified by stereology (optical fractionator). This technique allowed the experimentator to quantify the total number of c-Fos expression in an unbiased way. The selected time points to quantify c-Fos expression in the Am and the IC were 150 and 200 min after administration of LPS.

Additionally, plasma cytokine levels were measured after CsA and LPS injection at the different time points mentioned above. It has been reported that increase of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) is observed in the plasma and the spleen after peripheral injection of LPS (Givalois et al., 1994; Zhou et al., 2003; Chen et al., 2005). Recent studies showed that IL-1β, IL-6 and TNF-α are potent activators of the HPA axis, even though their ability to bypass or cross the BBB and signal to the brain still remains unclear (Banks et al., 1989; Besedovsky et al., 1991; Besedovsky and del Rey, 1992; Banks, 2006; Quan and Banks, 2007; Dantzer et al., 2008). The aim of the experiment was to quantitatively compare the amount of c-Fos expression in the Am and the IC after administration of an immunostimulating agent (LPS) to the amount of c-Fos expression in the same brain areas after administration of an immunosuppressive agent (CsA).

# Materials & Methods

### *Animals*

Male Dark Agouti rats, weighing between 250-300 g, were obtained from Harlan Netherland (Horst, The Netherlands). Animals were individually housed under an inverted 12:12h light/dark schedule (lights off at 7 am) with food and water available *ad libitum*. The experiments were carried out following the current Swiss regulations for animal experimentation (Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance) and were approved by the local animal ethics committee (Kantonales Veterinäramt Zürich).

***Experimental protocol***

The animals were divided in three different treatment groups: (1) the LPS group was injected with lipopolysaccharides (LPS) from E. Coli (serotype: B5, 0.1 mg/kg, i.p., Sigma Aldrich, Germany), (2) the CsA group received an injection of cyclosporine A (CsA, 20 mg/kg, i.p., Sandimmun, Novartis, Switzerland), and (3) the control group received an injection of an equivolume of sterile saline solution (Vehicle). At 120 min, 240 min and 360 min after CsA and saline injections and at 150 min and 200 min after LPS and saline injections, the animals were deeply anesthetized with isoflurane. Blood was collected by heart punction and centrifuged at 10 000 rpm during 5 min at 4°C in order to collect the plasma. Animals were transcardially perfused with low molarity PBS followed by high molarity PBS containing 4% paraformaldehyde. The brains were removed, postfixed for 24 h and cyroprotected by immersion in 30% sucrose until slicing.

***Cytokine determination in plasma***

Plasma cytokine concentrations were quantified using commercially available bead-based multiplex assays (Bio-Plex cytokine assay, Bio-Rad Laboratories AG, Reinach, Switzerland). Plasma dilutions were incubated with fluorescence-labelled beads that are coupled to monoclonal antibodies against rat IL-1β, IL-2, IL-6, TNF-α and IFN-γ. Upon incubation with the detection-antibodies against these cytokines, samples were incubated with Streptavidin-PE (Becton Dickinson, Allschwill, Switzerland). Two hundred beads per sample were analyzed on a flow cytometer (LSR II, Becton Dickinson Immunocytometer Systems, Allschwill, Switzerland). The fluorescence intensity measured is proportional to the cytokine concentration in the sample. Absolute cytokine concentrations were calculated based on the median fluorescence of the cytokine standard dilutions. The detection limits of the assays were determined to be: 0.1 pg/ml (IL-1β, IL-2 and IL-6), 0.5 pg/ml (IFN-γ) and 1.4 pg/ml (TNF-α), respectiviely.

***c-Fos immunohistochemistry***

Serial 40-µm coronal sections were cut through the central nucleus of the amygdala (CeA), basolateral nucleus of the amygdala (BLA), the insular cortex (IC) and dentate gyrus (DG) using a vibrotome (Leica VT1000S, Leica Microsystems, Nussloch, Germany). Free floating sections were incubated for 30 min in PBS containing 0.5% $H_2O_2$ to block endogenous

peroxidase. After rinsing in PBS, sections were incubated at room temperature for 1 h in PBS with 0.3% Triton X-100 (PBS-T) containing 5% normal goat serum (NGS). Sections were then incubated at 4 °C for 72 h with rabbit polyclonal anti-c-Fos IgG (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS-T containing 2% NGS. Subsequently, sections were rinsed and incubated for 2 h with anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) diluted in PBS-T containing 2% NGS, followed by 1% avidin-biotin complex (Vectorstain Elite ABCkit, Vector Laboratories). Finally, sections were washed in 0.1M Tris-HCl (pH 7.4) and the immunoreaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (1.25%) and 0.08% $H_2O_2$ in Tris-HCl.

***Stereology***

For counting c-Fos immunoreactive cells, a microscope (DM5500B, Leica Microsystems, Heerbrugg, Switzerland) equipped with a motorized stage (SCAN, Märzhäuser, Wetzlar, Germany) was used. A digital camera (Microfire CCD, Optronics, Goleta, CA, USA) connected to a computer was mounted on top of the microscope. The computer was equipped with Mercator Pro software (Explora Nova, La Rochelle, France). The optical fractionator method was used to count the c-Fos-positive cells in an unbiased way by stereology (Howard and Reed, Unbiased stereology, 2005). The first section was randomly selected and the section sampling fraction (ssf) was $1/4^{th}$ for CeA, BLA and DG, and $1/12^{th}$ for IC (Table 1). Sections were analyzed using a 63x oil immersion objective (PL Fluotar, Leica). The optical fractionator was used at regular predetermined dx and dy distances ((dx × dy) = 175 µm x 175 µm for CeA and BLA, 250 µm x 250 µm for DG and 300 µm x 300 µm for IC). The area associated with each frame (a/f) was 2500 $\mu m^2$. The height sampling fraction (hsf) was corresponding to 60% of the section thickness.

| | hsf | asf | ssf |
|---|---|---|---|
| CeA | 0.6 | 0.08 | $1/4^{th}$ |
| BLA | 0.6 | 0.08 | $1/4^{th}$ |
| IC | 0.6 | 0.04 | $1/12^{th}$ |
| DG | 0.6 | 0.03 | $1/4^{th}$ |

**Table 1.** Stereological parameters used for the optical fractionator.

The total number of cells in CeA, BLA, DG and IC was estimated according to Q (number of cells counted in all the slides) and the following optical fractionator formula:

$$N = \frac{1}{hsf} \times \frac{1}{asf} \times \frac{1}{ssf} \times Q$$

The coefficient of error (CE) for the estimated number of cells in each area was calculated based on the Gunderson and Jensen (Gj) prediction of CE (Howard & Reed, Unbiased stereology, 2005). The CE for the different estimated cell number ranged between 0.04 and 0.08.

***Statistical analysis***

The data were analysed using SPSS software (Version 14.0, Chicago, IL, USA) and the level of significance was set at $p \leq 0.05$. The Levene's test was used in order to check the homogeneity of variance at each time point. Independent sample t-tests were performed to compare treated group to saline group for each time point.

# Results

***Cytokine determination in plasma and central c-Fos quantification after peripheral injection of LPS***

Plasma cytokine concentrations were measured 150 min and 200 min after peripheral injection of LPS (Fig. 8). As expected, significant increases in plasma levels of IL-1β, IL-6, TNF-α and IFN-γ ($p \leq 0.05$) compared to saline treated animal were found. IL-2 levels were increased but the difference did not reach statistical significance.

Immunohistochemical assessment of the early-immediate gene protein c-Fos showed an increase of immuno-reactive (IR) cells in the central nucleus of amygdala (CeA) at 150 min and 200 min, and in the basolateral nucleus of amygdala (BLA) at 200 min after peripheral injection of LPS (Fig. 9A, 9B, 9E and 9C). The distribution of c-Fos in the CeA was concentrated in a cluster, whereas in the BLA, the c-Fos distribution was more homogeneous. Stereological quantification of c-Fos IR cells showed significant increases at 150 min and 200 min in CeA ($p \leq 0.05$), and only at 200 min in BLA ($p \leq 0.05$) compared to saline treated animals (Fig. 10). No significant changes of c-Fos IR cells were observed in insular cortex (IC) and dentate gyrus (DG).

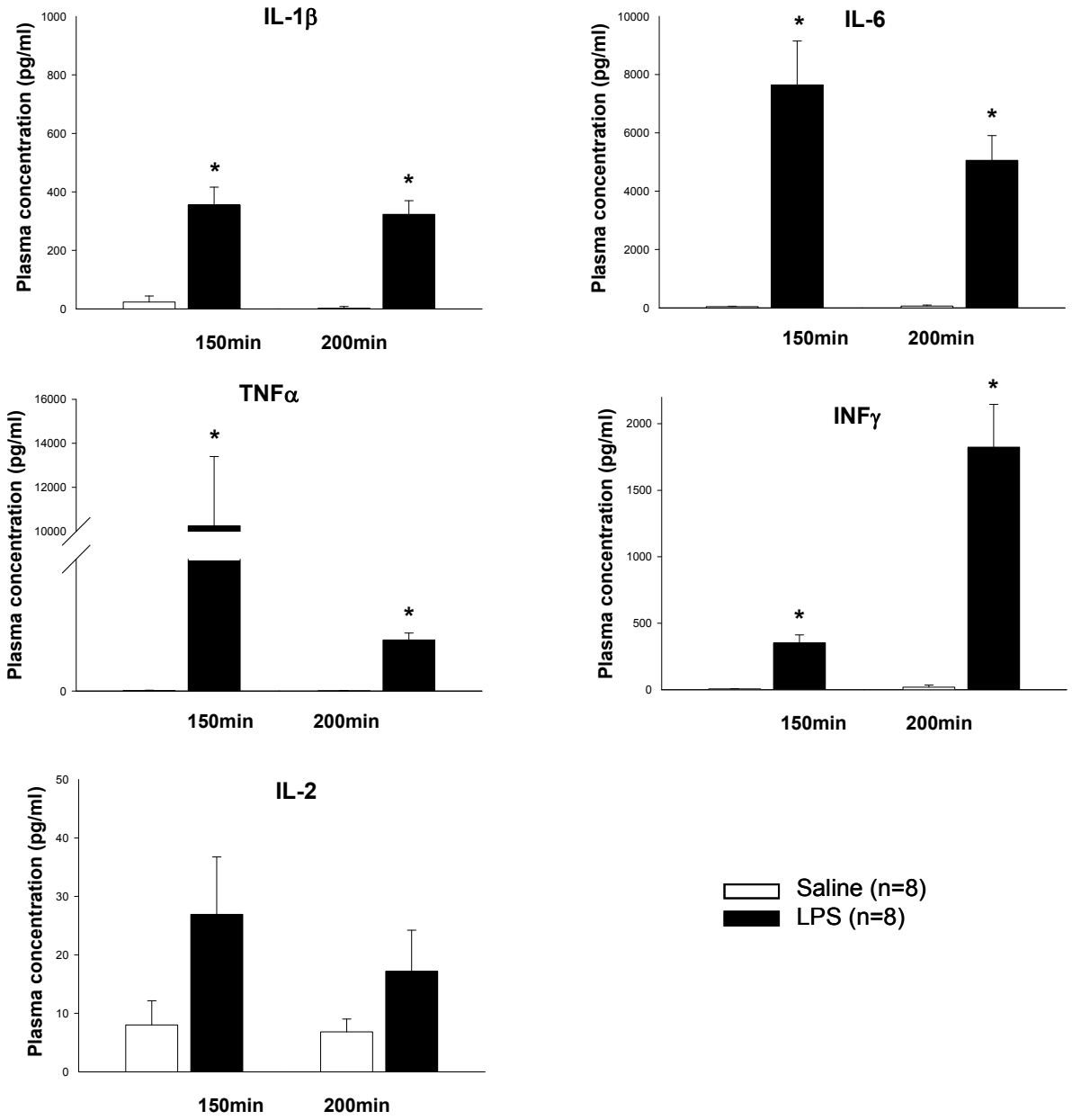


**Figure 8.** Plasma cytokine concentrations at 150 and 200 min after peripheral injection of saline or LPS (0.1 mg/kg). Data are shown as mean and SEM, n = 8 animals per group. Student's *t*-test, * $p \leq 0.05$.

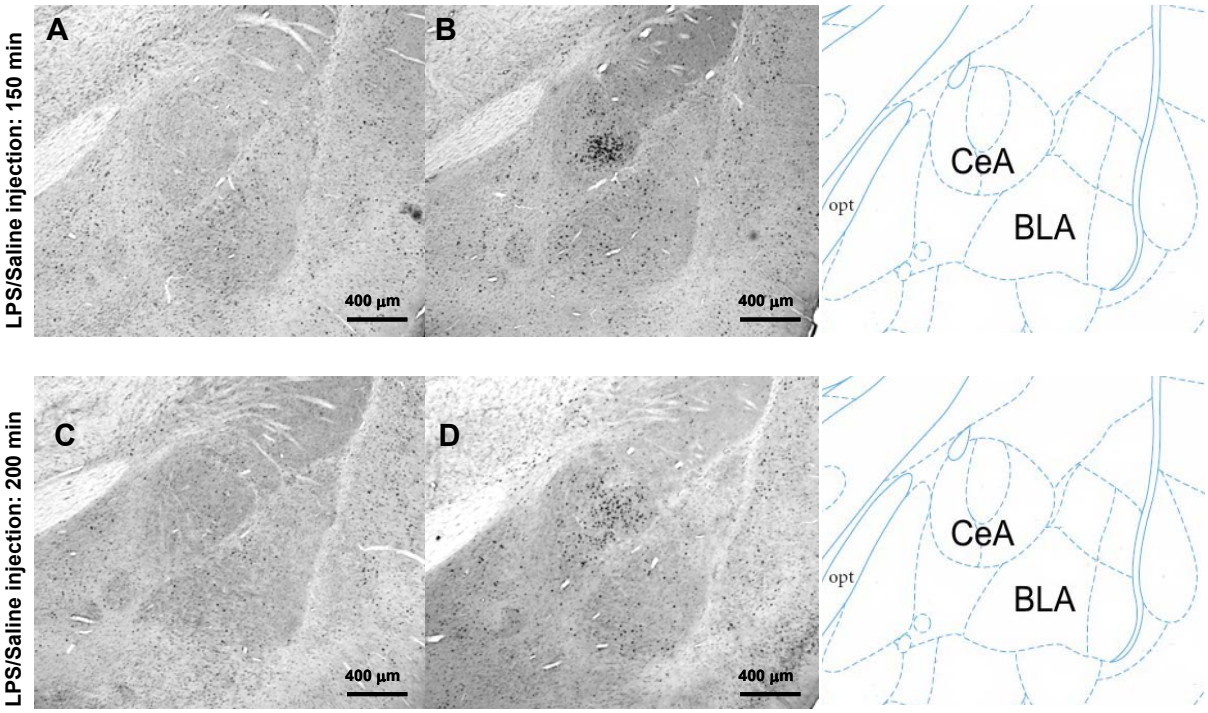


**Figure 9.** c-Fos immunohistochemistry in the central nucleus of amygdala and the basolateral nucleus of the amygdala at 150 min (A and B) and 200 min (C and D) after LPS injection (B and D) or saline injection (A and C). **CeA:** central nucleus of amygdala, **BLA:** basolateral nucleus of amygdala, **opt:** optical tract.

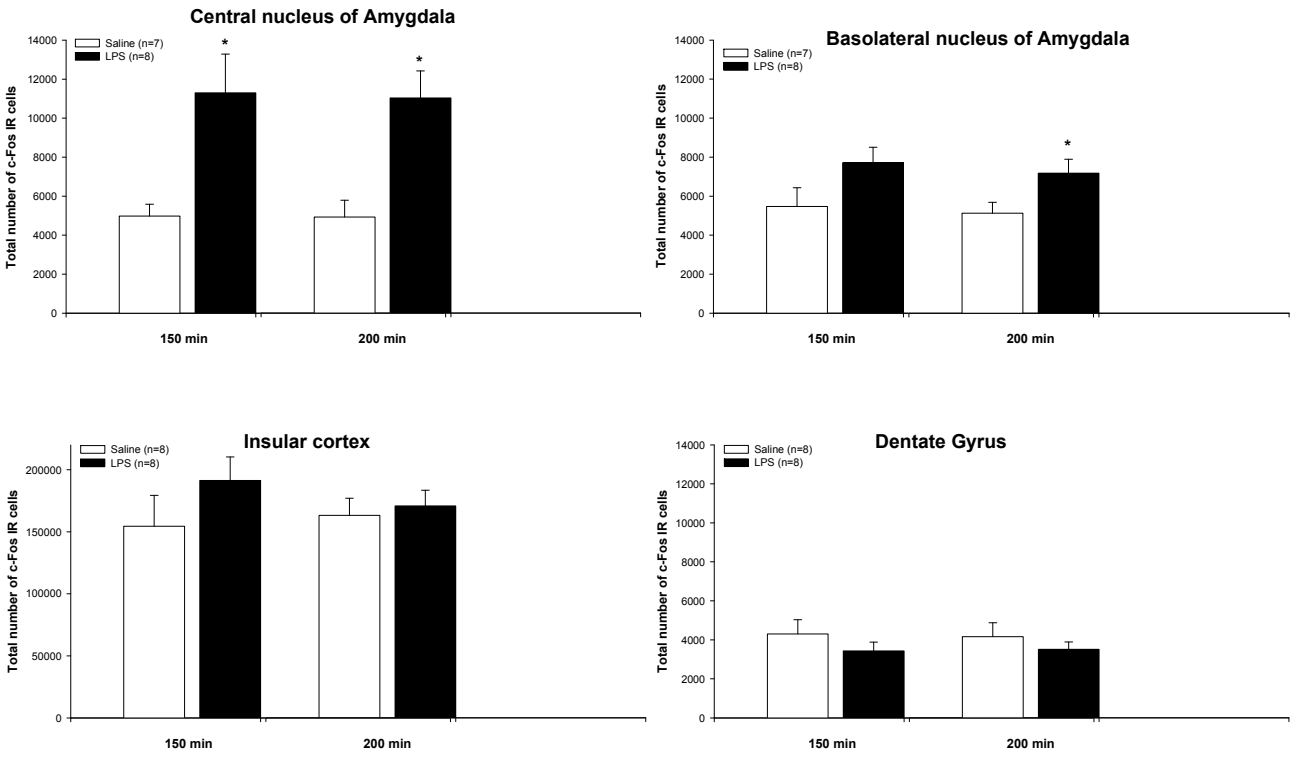


**Figure 10.** Total number of c-Fos IR cells in CeA, BLA, IC and DG at 150 and 200 min after peripheral injection of saline or LPS (0.1 mg/kg). Data are shown as mean and SEM, n = 7 to 8 animals per group. Student's *t*-test, * $p \leq 0.05$.

### *Cytokine determination in plasma and central c-Fos quantification after peripheral injection of CsA*

Plasma cytokine concentration was measured 120, 240 and 360 min after peripheral injection of CsA (Fig. 11). As expected, no significant differences were observed in plasma levels of IL-1β, IL-2, IL-6, TNF-α and IFN-γ at these three time points compared to saline treated animal.

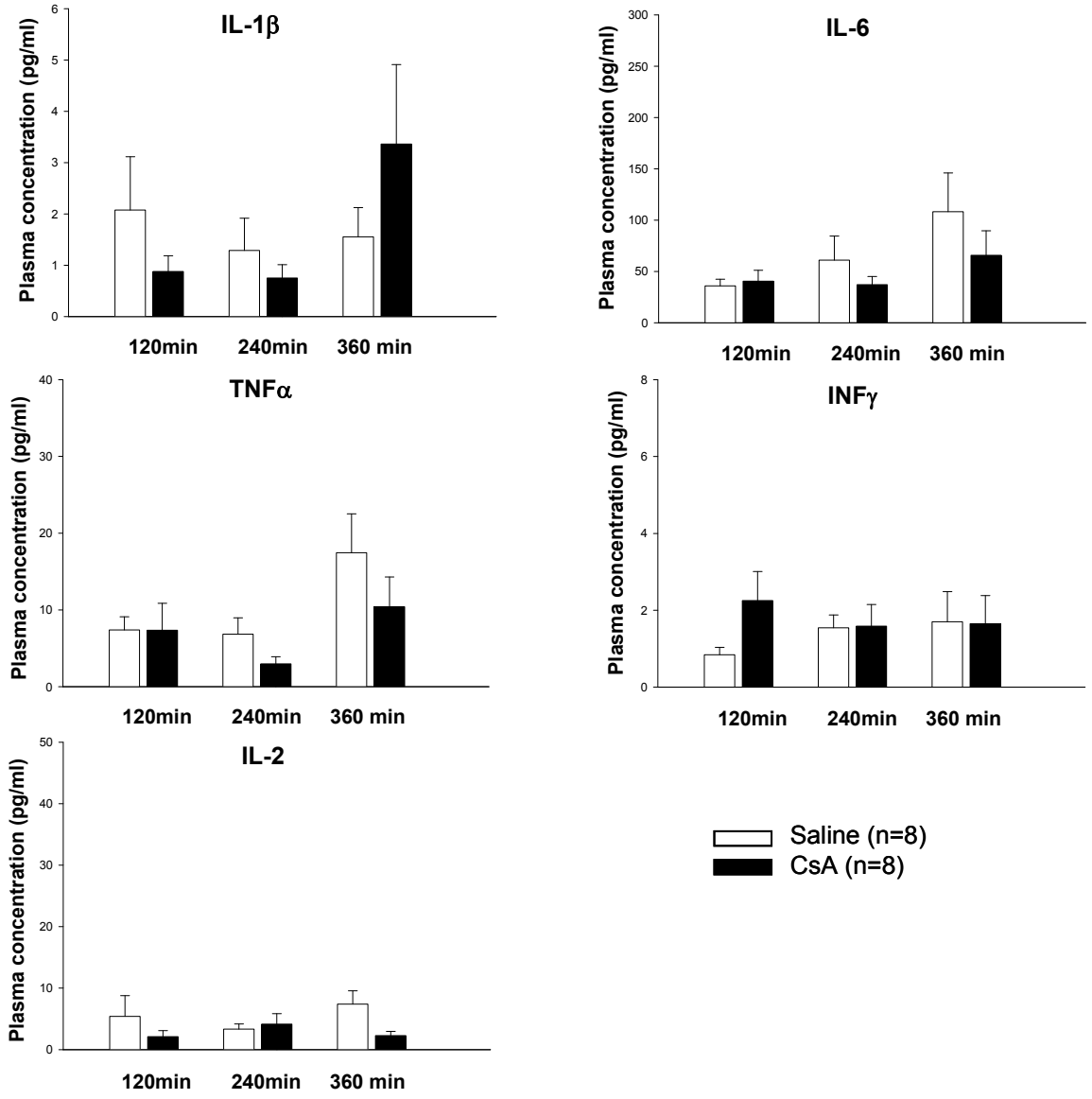


**Figure 11.** Plasma cytokine concentrations at 120, 240 and 360 min after peripheral injection of saline or CsA (20 mg/kg). Data are shown as mean and SEM, n = 8 animals per group. Student's *t*-test, * $p \leq 0.05$.

Immunohistochemical assessment of the early-immediate gene protein c-Fos showed an increase of immuno-reactive (IR) cells in the central nucleus of amygdala (CeA) at 120, 240 and 360 min, and in the basolateral nucleus of amygdala (BLA) at 120 min after peripheral

injection of CsA (Fig. 12A, 12B, 12E and 12C). The distribution of c-Fos in the CeA was concentrated in a cluster, whereas in the BLA, the c-Fos distribution was more homogeneous.

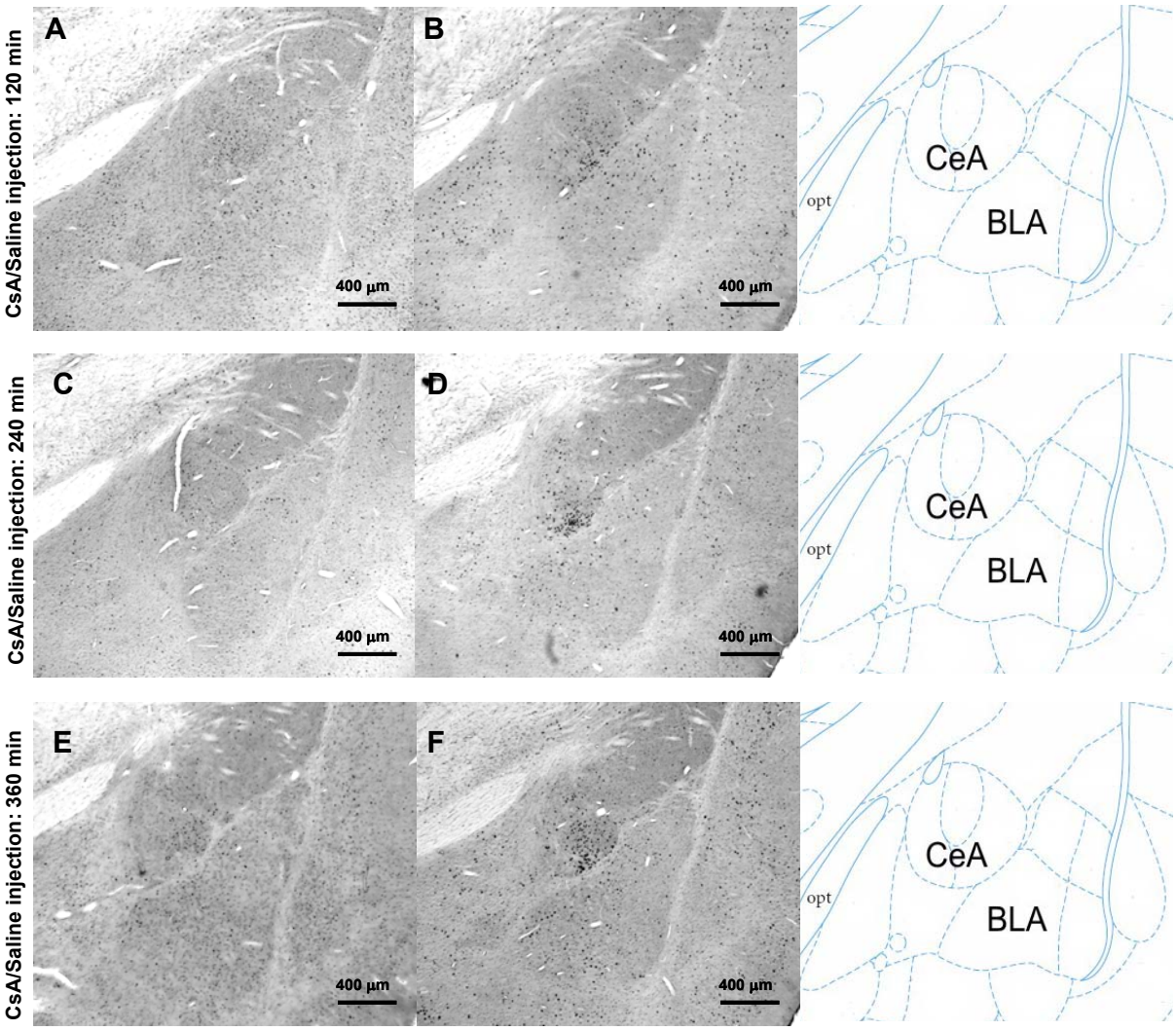


**Figure 12.** c-Fos immunohistochemistry in the CeA and BLA at 120 min (A and B), 240 min (C and D) and 360 min (E and F) after CsA injection (B, D and F) or saline injection (A, C and E). **CeA:** central nucleus of amygdala, **BLA:** basolateral nucleus of amygdala, **opt:** optical tract

Stereological quantification of c-Fos IR cells showed significant increase at 120 min, 240 min and 360 min in CeA ($p \leq 0.05$), and only at 120 min in BLA ($p \leq 0.05$) compared to saline treated animals (Fig. 13). No changes of c-Fos IR were observed in the DG and the IC 120, 240 and 360 min after CsA injection compared to saline treated animals.

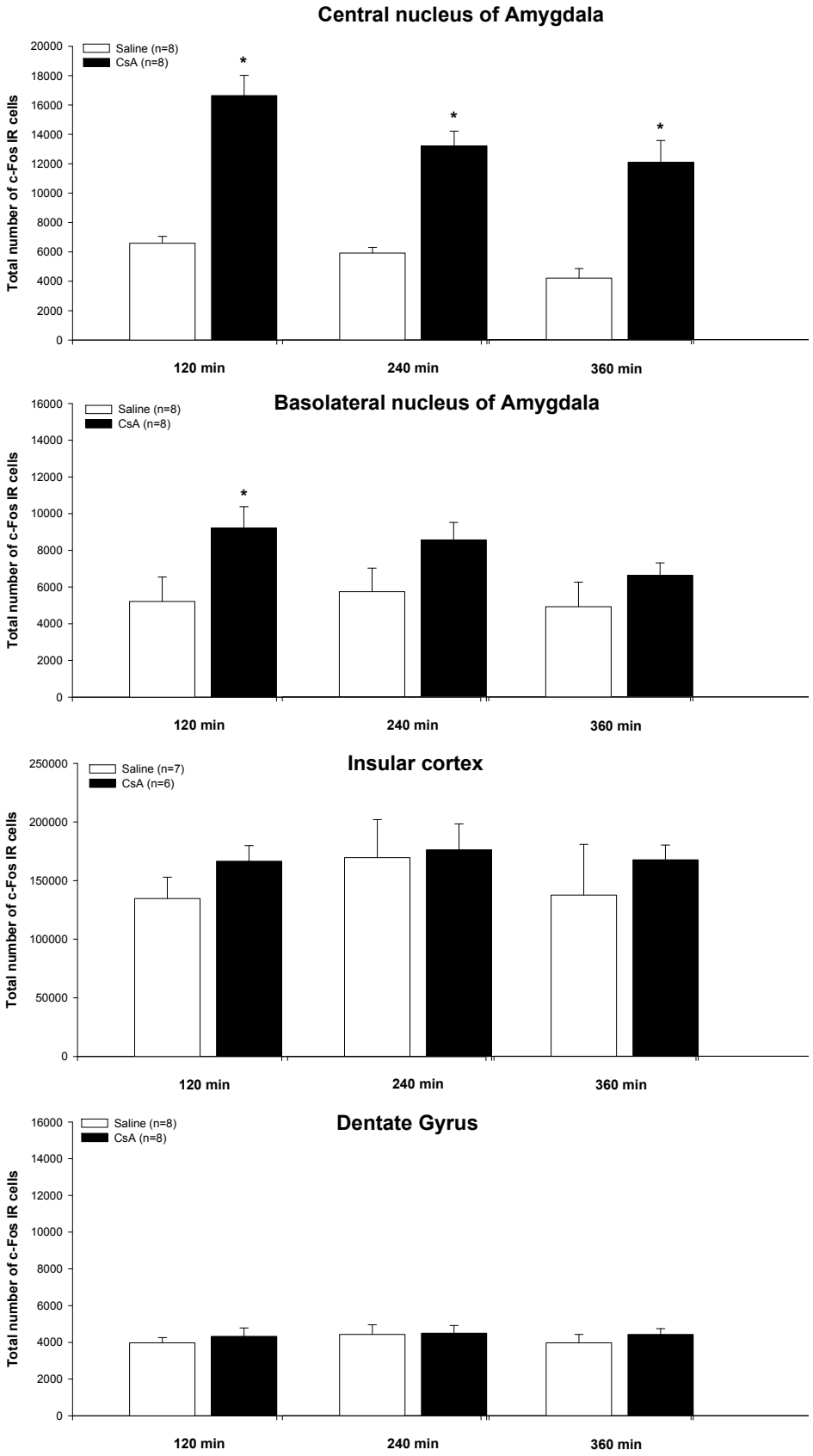


**Figure 13.** Total number of c-Fos IR cells in CeA, BLA, IC and DG at 120, 240 and 360 min after peripheral injection of saline or CsA (20 mg/kg). Data are shown as mean and SEM, n = 8 animals per group. Student's *t*-test, * $p \leq 0.05$.

# Discussion

The aim of the experiment was to quantify the amount of c-Fos in the Am and the IC after administration of either an immunostimulating agent (LPS) or an immunosuppressive agent (CsA). The present study showed increases of c-Fos IR cells numbers in the central nucleus of amygdala (CeA) 150 and 200 min after peripheral injection of LPS. The total number of c-Fos IR cells also increased in the basolateral nucleus of the amygdala (BLA) 200 min after peripheral injection of LPS. No changes in the number of c-Fos IR were observed in the dentate gyrus (DG) of the hippocampus and the IC. It has been reported that lesions of the hippocampus have no effect on acquisition of the conditioned taste avoidance/aversion (CTA) (Yamamoto et al., 1995). Based on the CTA neural circuit (chapter 1, Fig. 3), the DG was selected in this experiment as a control area for the background level of c-Fos. Therefore, no changes in the amount of c-Fos were expected between the LPS treated group and the saline treated group.

In the periphery, significant increases in plasma levels of IL-1β, IL-6, TNF-α and IFN-γ cytokines were observed 150 and 200 min after i.p. injection of LPS. In addition, mRNA expression of IL-1β, IL-6 and TNF-α was increased in the Am at 150 min and 200 min after peripheral injection of LPS (Data not shown). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are potent activators of the HPA axis, even though their ability to bypass or cross the BBB and signal to the brain still remains unclear (Banks et al., 1989; Besedovsky et al., 1991; Besedovsky and del Rey, 1992; Banks, 2006; Quan and Banks, 2007; Dantzer et al., 2008). In addition, LPS treatment induces IL-1β IR in immune cells that associate with perivascular cells or neuronal elements like dendritic cells (Goehler et al., 2006). This neuro-immune cells interaction could directly and specifically influence neurons in the area postrema. It was also demonstrated that peripheral injection of IL-1β induces c-Fos protein in vagal primary afferent neurons (Goehler et al., 1998; Goehler et al., 1999). Therefore, the peripheral increase of cytokine observed in the present experiment may directly, through the area postrema or the BBB, or indirectly, through stimulation of the vagal afferent fibers, signal to the CNS. The previous cytokine data, the c-Fos data described in the NTS, AP, PBN, LC and PVN and the involvement of different afferent neuro-immune pathways after peripheral LPS administration (Elmquist et al., 1993; Sagar et al., 1995; Lacroix and Rivest, 1997; Dallaporta et al., 2007) may explain why increases of c-Fos expression were observed

in CeA at 150 and 200 min and in BLA at 200 min. However, the fact that no changes of c-Fos levels were observed in the IC after peripheral administration of LPS still remains unclear.

In the present chapter, increase of c-Fos IR cells was observed at 120, 240 and 360 min in CeA and at 120 min in BLA after peripheral injection of CsA. No changes in the number of c-Fos IR were observed in the dentate gyrus (DG) and the insular cortex (IC). Additionally, mRNA cytokine measurements in the Am showed increases of IL-1β, IL-2, IL-6, TNF-α and IFN-γ mRNA 360 min after peripheral injection of CsA (Riether C., PhD manuscript 2008). Am and IC were shown to be involved in behavioral conditioning that mediate conditioned suppression of splenocyte proliferation and cytokine production induced by pairing saccharin (CS) with cyclosporin A (CsA) (US) (Pacheco-Lopez et al., 2005). It has been reported that the PBN which receives general visceral information can relay the US through three different neural routes: a first direct route to the CeA, a second route (diencephalic route) to the BLA involving the thalamus and a third route (cortical route) to the BLA involving the IC (Yamamoto et al., 1997; Sakai and Yamamoto, 1999; Yamamoto, 2007). This connectivity between the PBN, thalamus, CeA, BLA and IC in the model of CTA may explain the increases of c-Fos in the CeA and BLA after intraperitoneal administration of CsA. However, the fact that no changes of c-Fos level were observed in the IC still remains unclear.

The analysis of peripheral cytokine concentration showed no significant changes after i.p. injection of CsA compared to saline injected animal. The present data clearly suggest that plasma cytokines may not be the relevant information that may signal to the brain through different afferent pathway after peripheral injection of CsA. Therefore, secretion of alternative molecules or induction of side effect after CsA injection have to be considered in order to explain the increase of c-Fos in the Am. Neverthless, local increases of cytokines in various tissues after CsA administration that may stimulate the afferent fibers of the vagus nerve which may relay the information to the brain or a direct effect of CsA in the brain can not be excluded.

The overall c-Fos data suggest a key role of the Am in the detection process of either an immunostimulating agent (LPS) or an immunosuppressive agent (CsA). Based on the implication of a neural pathway in signalling LPS injection to the CNS and the neural connectivity between the PBN, thalamus, CeA, BLA and IC in the model of conditioned

taste avoidance/aversion, the vagus nerve was considered to be involved in signalling CsA injection to the CNS. Therefore, selective vagal deafferentation will be performed in the next chapter, and c-Fos levels will be quantified in NTS, Am and IC after peripheral injection of CsA.

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# Chapter 4.

# Effect of selective vagal deafferentation on the afferent signalling of cyclosporin A to the brain

# Introduction

The brain is able to monitor peripheral immune stimulations by several pathways (Quan and Banks, 2007; Dantzer et al., 2008): a neural afferent pathway mainly through the vagus nerve (Bluthe et al., 1994; Watkins et al., 1994), a humoral pathway through the circumventricular organs and the choroid plexus (Quan et al., 1998; Vitkovic et al., 2000), a cytokine transporter pathway at the blood-brain barrier (BBB) (Banks, 2006) and cytokine receptors which are located on perivascular macrophages and endothelial cells of brain venules (Schiltz and Sawchenko, 2002; Konsman et al., 2004).

The vagus nerve, with relays in brain stem nuclei, has been proposed as the main neural afferent pathway during the immunosensory process, prior to the immune information reaching the forebrain structures (Goehler et al., 2000). Vagal sensory nerves terminate in the dorsal vagal complex (DVC), located in the brainstem. The DVC contains a sensory component, the area postrema (a circumventricular organ), and the nucleus tractus solitarii (NTS), as well as a motor component, the subjacent dorsal motor nucleus of the vagus. Several lines of evidence support the DVC as a potentially important entryway for information from activated immune cells in the periphery (Gaykema et al., 2007). LPS treatment induces IL-1β IR in immune cells that associate with perivascular cells or neuronal elements like dendritic cells (Goehler et al., 2006). This neuro-immune cell interaction could directly and specifically influence neurons in the area postrema.

In addition, peripheral injection of LPS has been shown to induce increases of c-Fos levels in the NTS and amygdala (Am) and subdiaphragmatic vagotomy significantly reduces the level of c-Fos in the NTS and amygdala after i.p. injection of LPS compared to sham control animals (Konsman et al., 2000; Ge et al., 2001; Marvel et al., 2004). The inactivation of the DVC also blocked LPS-induced social withdrawal but not the LPS-induced fever response (Konsman et al., 2000; Ge et al., 2001). Investigation of the peripheral IL-1 receptor distribution using biotinylated of IL-1 receptor antagonist (bIL-1ra) demonstrated that glomus cells of paraganglia associated with subdiaphragmatic, cervical and thoracic vagus nerve express binding sites for bIL-1ra (Goehler et al., 1997). These paraganglia may then detect local increases in tissue IL1-β along the course of the vagus nerve in order to relay this information to the brain. Further studies demonstrate that IL1-β IR is expressed in dentritic cells and macrophages within the connective tissues associated with the

abdominal vagus 45 min after i.p. injection of LPS. In addition, i.p. injections of IL1-β induce c-Fos expression in vagal primary afferent neurons (Goehler et al., 1998; Goehler et al., 1999). Together, these studies suggest a strong involvement of the vagus nerve in the afferent communication between the immune and the central nervous systems after peripheral injection of LPS.

In chapter 3, it was shown that peripheral injection of LPS induced increases in plasma concentration of pro-inflammatory cytokines and c-Fos IR cells in the CeA and the BLA. In contrast, peripheral injection of CsA failed to induce alterations in plasma cytokines although increases in c-Fos IR cells in the CeA and BLA were observed. In addition, the pattern of EEG in the Am and the IC showed that the CNS is able to specifically sense the peripheral immune stimuli by providing EEG "fingerprints". These data suggested that the brain is able to detect peripheral injection of an immunosuppressive agent (CsA) although no peripheral changes of cytokines were observed. The aim of the following experiments was to elucidate whether the vagus nerve is involved in the afferent signalling to the CNS after peripheral administration of an immunosuppressive agent (CsA). In that purpose, selective vagal deafferentation was performed and c-Fos expression was quantified by stereology in the NTS, CeA and IC after peripheral injection of CsA.

# Materials & Methods

### *Animals*

Male Dark Agouti rats, weighing between 250-300 g, were obtained from Harlan Netherland (Horst, The Netherlands). Animals were individually housed in metallic grid cage under an inverted 12:12h light/dark schedule (lights off at 7 am) with food and water available *ad libitum*. The experiments were carried out following the current Swiss regulations for animal experimentation (Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance) and were approved by the local animal ethics committee (Kantonales Veterinäramt Zürich).

### *Surgery*

*Vagal rootlet transection:* Under anesthesia (isoflurane), the rat was placed supine in a head holder. With the aid of an operating microscope, the basal aspect of the occipital bone was exposed by blunt dissection after tying off and sectioning the superior thyroid artery and

removing the posterior wing of the hyoid bone with its attached musculature. The posterior lacerated foramen was expanded medially by thinning the occipital bone with a dental burr and then removing the remaining bone posterior to the level of the hypoglossal canal with fine forceps. This exposure revealed the vagus nerve as it penetrates through the dura mater and separates into two groups of nerve rootlets. The rootlets in the ventral group are smaller and more numerous, and many extend caudally before penetrating the ventrolateral medulla. The dorsal group consists of two to four rootlets that reach the dorsolateral surface of the medulla at the level of the posterior lacerated foramen. Once the dura is lanced, the dorsal (afferent) rootlets can be sectioned with iris scissors. In addition to the vagus, afferent transection includes both vagal and glossopharyngeal axons, because in the rat these two cranial nerves join in a common sheath at the level of the posterior lacerated foramen. All rats received rootlet section on the left side. After the selective nerve section was completed, the cavity was packed gently with sterile Gelfoam to reduce CSF drainage, and the wound was closed in a single layer (Fig. 14).

*Unilateral Subdiaphragmatic vagotomy:* After the rootlet surgery, unilateral (left) abdominal vagotomy was carried out to lesion the afferent and efferent fibers connected contralateral to the side of the rootlet section. Using laparotomy, the left cervical vagus nerve was exposed. The left trunk was identified just below the diaphragm. Two 5-O silk sutures, aimed to restrict regeneration of transacted fibers and to facilitate later anatomic verification of the vagotomy, were tied around the vagal trunk 2-4 mm apart, and the nerve between the sutures was cut. Subsequently, the abdominal muscles were then sutured with 3-O vicryl suture, and the skin was closed with 5-O vicryl suture. A mixture of ketamine/xylaxine was provided s.c. as sedative before ending gas anesthesia. Animals have been provided with analgesic Rimadyl (Pfizer, USA) before ending the gas anesthesia. The sham procedure consisted of similarly opening the skull and abdomen to expose the vagal rootlets and abdominal vagus, but no further manipulating them. Experimental animals received s.c. injection of 5 ml physiological saline for re-hydratation and two injections of analgesic (5 mg/kg, Rimadyl, s.c., Pfizer USA) at 24 and 48h post-surgery. The animals were monitored daily in order to assess health status. Fluid diet (condense milk) was provided for 2 days and wet-mashed diet (powder diet + condense milk) was offered on day 3, 4 and 5 post-surgery. Thereafter, the animals received regular lab chow.

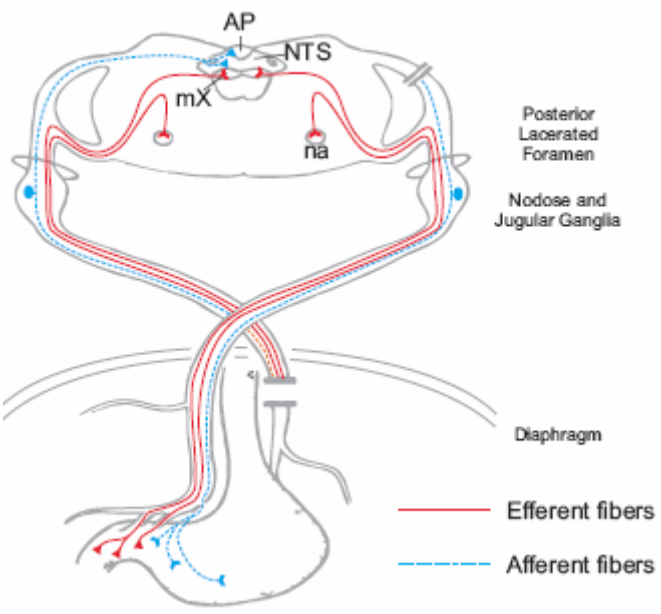


**Figure 14.** Selective vagal deafferentation (SDA): schematic representation of SDA surgical procedure. **AP**; area postrema, **mX**; dorsal motor nucleus of the vagus, **na**; nucleus ambiguous.

### *Cholecystokinin test*

The cholecystokinin (CCK) test was used to confirm the vagotomy. CCK satiation depends on abdominal vagal afferent fibers (Monnikes et al., 1997). 1 mg CCK (Cholecystokinin Octapeptide, Bachem Switzerland) was reconstituted with 1 ml 0.9% saline and stored in aliquots (24 µl = 25 µg) at -25°C. 12 h before lights out, food was withdrawn and rats were weighed and handled. Immediately before use, CCK stock solution was diluted 1:150 with sterile PBS (50 µl + 75 ml PBS). The injected dose was 4 µg/kg for the selective vagal deafferentiated group (SDA) and the sham groups. Injections started 15 min before lights out. Immediately after injections, feedings cups were given back and manually weighed 30 min, 1 h and 2 h after the CCK injection. The order of the drug and vehicle was counter-balanced between 2 test days for each group and each test day was separated by at least one day during which no injections of drug or vehicle were made.

### *Experiment protocol*

The animals were divided in four different treatment groups. (1) The sham cyclosporine A (Sham CsA) and (2) the SDA cyclosporine A (SDA CsA) groups were treated with cyclosporine A (20 mg/kg, Sandimmun, Novartis, Switzerland). (3) The sham vehicle (Sham veh.) and (4) the SDA vehicle (SDA veh.) groups were treated with equivolumes of vehicle

(chremophor/EtOH + NaCl). At 120 min after CsA or vehicle injections, the animals were deeply anesthetized with isoflurane. Animals were transcardially perfused with low molarity PBS followed by high molarity PBS containing 4% paraformaldehyde. The brains were removed, postfixed for 24 h and cyroprotected by immersion in 30% sucrose for 72 h.

***c-Fos immunohistochemistry***

Serial 40-µm coronal sections were cut through the central nucleus of the amygdala (CeA), the insular cortex (IC), dentate gyrus (DG) and the nucleus tractus solitarii (NTS) using a vibrotome (Leica VT1000S, Leica Microsystems, Nussloch, Germany). Free floating sections were incubated for 30 min in PBS containing 0.5% $H_2O_2$ to block endogenous peroxidase. After rinsing in PBS, sections were incubated at room temperature for 1 h in PBS with 0.3% Triton X-100 (PBS-T) containing 5% normal goat serum (NGS). Sections were then incubated at 4 °C for 72 h with rabbit polyclonal anti-c-Fos IgG (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS-T containing 2% NGS. Subsequently, sections were rinsed and incubated for 2 h with anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) diluted in PBS-T containing 2% NGS, followed by 1% avidin-biotin complex (Vectorstain Elite ABCkit, Vector Laboratories). Finally, sections were washed in 0.1M Tris-HCl (pH 7.4) and the immunoreaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (1.25%) and 0.08% $H_2O_2$ in Tris-HCl.

***Stereology***

For counting c-Fos immunoreactive cells, a microscope (DM5500B, Leica Microsystems, Heerbrugg, Switzerland) equipped with a motorized stage (SCAN, Märzhäuser, Wetzlar, Germany) was used. A digital camera (Microfire CCD, Optronics, Goleta, CA, USA) connected to a computer was mounted on top of the microscope. The computer was equipped with Mercator Pro software (Explora Nova, La Rochelle, France). The optical fractionator method was used to count the c-Fos positive cells in an unbiased way by stereology (Table 2). The first section was randomly selected and the section sampling fraction (ssf) was $1/2^{nd}$ for NTS, $1/4^{th}$ for CeA and $1/12^{th}$ for IC. Sections were analyzed using a 63x oil immersion objective (PL Fluotar, Leica). The optical fractionator was used at regular predetermined dx and dy distances ((dx × dy) = 175 µm x 175 µm for CeA, 200 µm x 200 µm for NTS and 300 µm x 300 µm for IC). The area associated with each frame (a/f) was

2500 µm². The height sampling fraction (hsf) was corresponding to 60% of the section thickness.

| | hsf | asf | ssf |
|---|---|---|---|
| CeA | 0.6 | 0.08 | 1/4th |
| IC | 0.6 | 0.04 | 1/12th |
| NTS | 0.6 | 0.06 | 1/2th |

**Table 2.** Stereological parameters used for the optical fractionator.

The total number of cells in CeA, IC and NTS was estimated according to Q (number of cells counted in all the slides) and the following optical fractionator formula:

$$N = \frac{1}{hsf} \times \frac{1}{asf} \times \frac{1}{ssf} \times Q$$

The coefficient of error (CE) for the estimated number of cells in each area was calculated based on the Gunderson and Jensen (Gj) prediction of CE (Howard & Reed, Unbiased stereology, 2005). The CE for the different estimated cell number ranged between 0.04 and 0.09.

### *Statistical analysis*

The data were analysed using SPSS software (Version 14.0, Chicago, IL, USA) and the level of significance was set at $p \leq 0.05$. The Levene's test was performed to check the homogeneity of variance at each time point. Independent sample *t*-test was performed to compare CsA group to vehicle group.

# Results

### *Confirmation of the vagal deafferentation*

The CCK test showed a significant reduction of the cumulative food intake ($p \leq 0.05$) 30 min after injection of CCK for the sham animals compared to SDA animals (Fig. 15). CCK is a hormone secreted by cells of the small intestine and neurons in the central and enteric nervous system (Gulley et al., 2005). It evokes numerous digestive functions and in particular reduction of food intake. CCK acts through two G-protein coupled receptors, CCK1 and CCK2. These receptors are distributed in various peripheral regions and in particular in the peripheral nervous system including vagal afferents (Monnikes et al., 1997; Gulley et al., 2005). The reduction of food intake after CCK injection depends of the vagus

nerve signalling. The result clearly suggested that the reduction of food intake did not take place in SDA animals after CCK injection. Therefore, these data clearly indicated that selective vagal deafferentation successfully took place in each SDA animal.

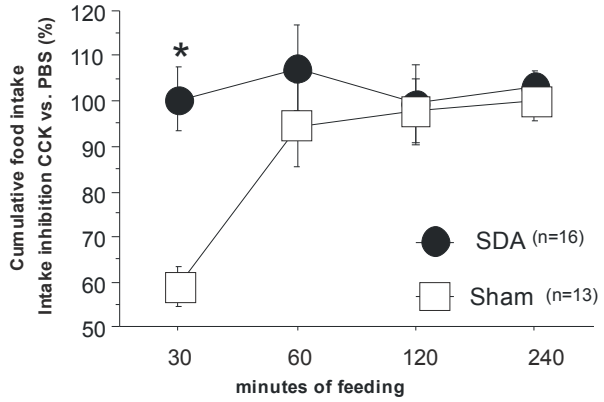


**Figure 15.** Selective vagal deafferentation (SDA) effects on CsA-induced c-Fos IR in the brain. SDA functional verification via cholecistokinin (CCK) vagal mediated anorexic effects. Data are shown as mean ± S.E.M. Student's *t*-test, * $p \leq 0.05$.

### *Impact of selective vagal deafferentation on c-Fos expression after peripheral injection of CsA*

Stereological quantification of c-Fos IR cells in the NTS 120 min after peripheral injection of CsA showed a significant increase of c-Fos staining in the SDA CsA group compared to SDA Veh group ($p \leq 0.05$). Numbers of c-Fos IR cells in the NTS was also increased in the Sham CsA compared to the Sham Veh group but the difference did not quite reach statistical significance ($p \leq 0.07$) (Fig. 17A). The representative distribution of c-Fos indicated that the activated cells were mainly located in the area postrema (AP) and in the rostral part of NTS (Fig. 16 and 17A).

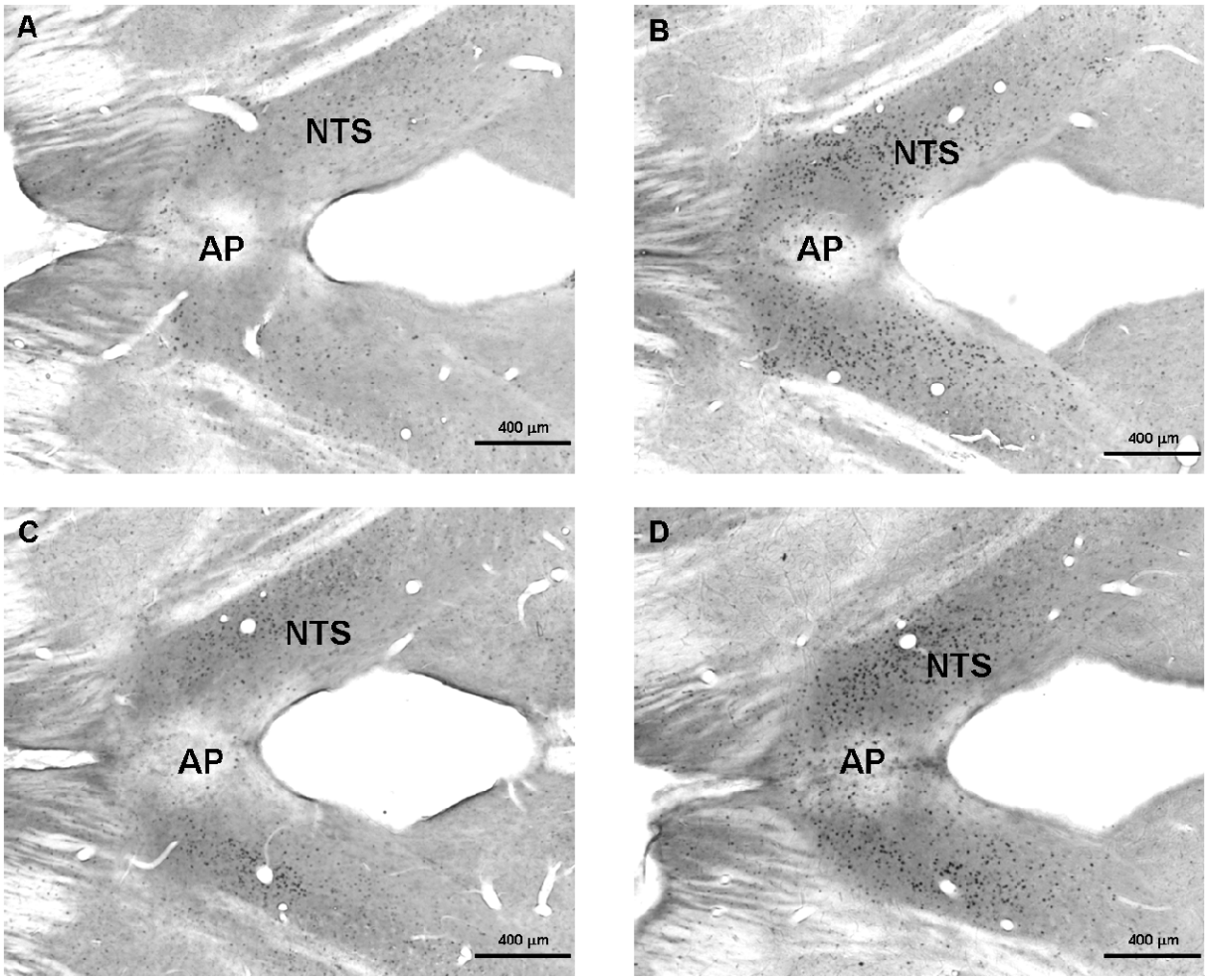


**Figure 16.** c-Fos immunohistochemistry in the area postrema (AP) and nucleus tractus solitarii (NTS) of sham Veh. (A), sham CsA (B), SDA Veh (C) and SDA CsA (D).

The IHC investigation in CeA showed significant increase of c-Fos IR cells for the SDA CsA treated group compared to SDA Veh group ($p \leq 0.05$). A significant increase of c-Fos IR cells was observed for Sham CsA treated group compared to Sham Veh group ($p \leq 0.05$). The representative distribution of c-Fos (Fig. 17B) indicated that the activated cells were mainly located in the capsular part of CeA. In addition, an increase of c-Fos IR cells in the IC was observed for the SDA Veh and Sham Veh when compared to SDA CsA ($p \leq 0.05$) and Sham CsA ($p \leq 0.07$), respectively (Fig. 17C). No significant differences were observed between the four experimental groups after c-Fos quantification in DG. In summary, peripheral injection of CsA induced significant changes in numbers of c-Fos IR cells in the NTS, CeA and IC. However, no differences were observed between Sham CsA treated animals and SDA CsA treated animals.

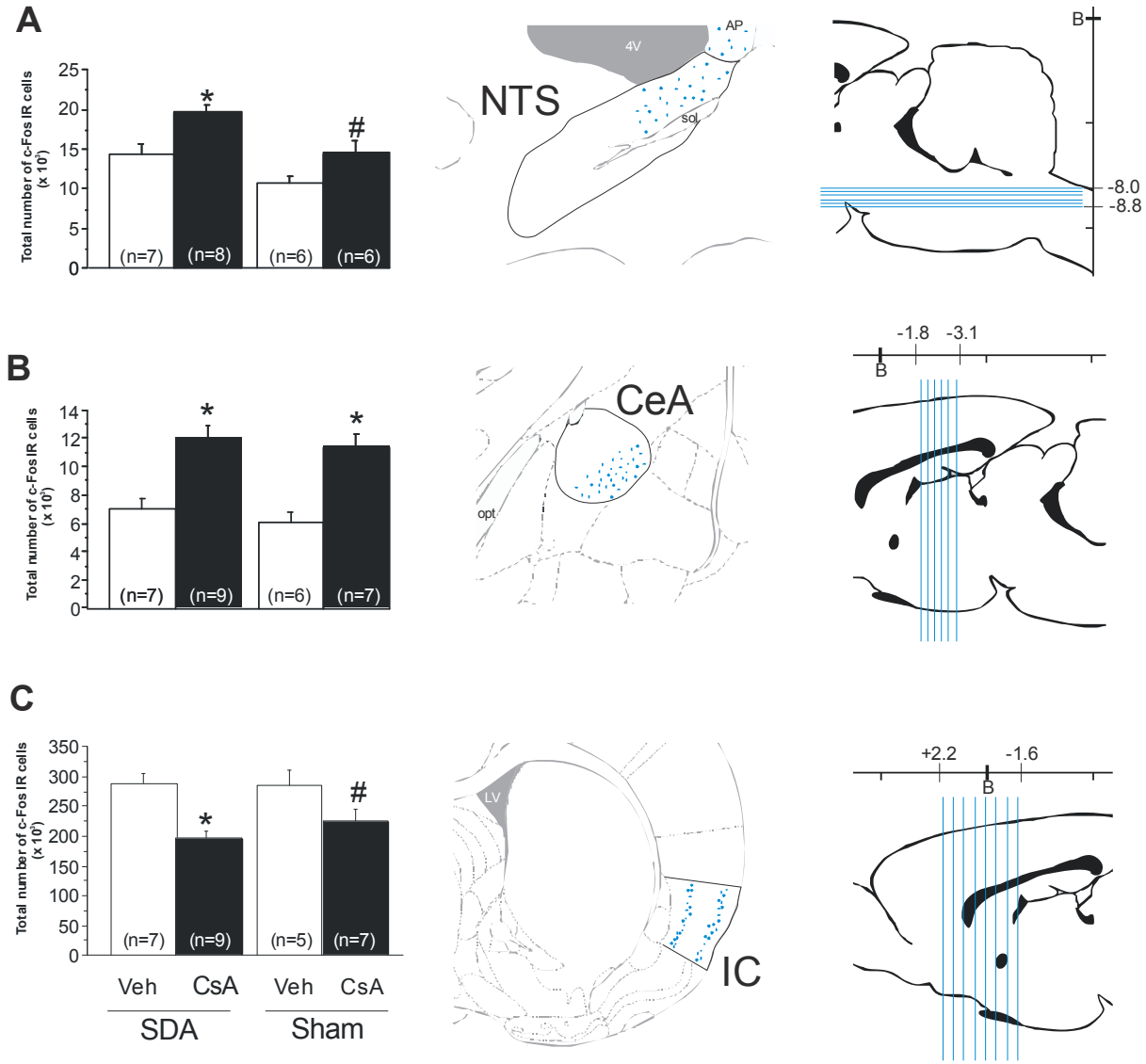


**Figure 17.** Impact of selective vagal deafferentation (SDA) on CsA-induced c-Fos IR in the brain. Stereological estimation of total number of c-Fos IR cells in (A) the nucleus tractus solitarii (NTS), (B) the central nucleus of the amygdala (CeA) and (C) the insular cortex (IC). Data are shown as mean and SEM Student's *t*-test, * $p \leq 0.05$, # $p \leq 0.07$. Middle panel displays approximate localization of c-Fos IR cells. Respective right panels show anatomical landmarks and parameters employed for systematically and randomly sampling. **B**; bregma, **AP**; area postrema, **4V**; fourth ventricle, **sol**; solitary tract, **opt**; optical tract; **LV**; lateral ventricle.

# Discussion

Based on several data showing the involvement of the vagus nerve in signalling peripheral injection of LPS to the CNS (Konsman et al., 2000; Ge et al., 2001; Marvel et al., 2004), it has been hypothesised that the vagus nerve may also be involved in signalling peripheral injection of CsA to the CNS. The CCK test clearly indicated that selective vagal deafferentation successfully took place in each SDA animal. Vagal sensory nerves terminate in the dorsal vagal complex (DVC), located in the brainstem. The DVC contains a sensory

component, the area postrema (a circumventricular organ), and the nucleus tractus solitarii (NTS) (Goehler et al., 2000). The quantification of c-Fos by stereology demonstrated that independently of selective vagal deafferentation, the same amount of c-Fos expression was quantified in the NTS, CeA and IC after peripheral injection of CsA. The vagus nerve seems to be not essential in signalling CsA to the CNS. Therefore, alternative afferent pathways have to be investigated in order to explain the increase of c-Fos expression in the NTS and Am after CsA administration.

The description in the literature of a neuro-immune site, the area postrema, where immune cells could directly interact with perivascular cells or neuronal elements like dendritic cells may be an alternative pathway of signalling CsA to the CNS (Goehler et al., 2006). The amygdala (Am) and insular cortex (IC) receive ascending visceral informations from the ventroposterior parvicellular nucleus of the thalamus (VPpc), the parabrachial nucleus (PBN), the nucleus tractus solitarii (NTS) and the lateral hypothalamic area (LHA) (Cechetto and Saper, 1987; Krushel and van der Kooy, 1988; Allen et al., 1991; Shi and Cassell, 1998; Barnabi and Cechetto, 2001). The representative distribution of c-Fos indicated that the activated cells were mainly located in the area postrema (AP) and in the rostral part of NTS (Fig. 16 and 17A). Through the direct neuro-immune cell interaction in the area postrema (AP), the NTS may relay the CsA stimulus to the Am and the IC. This alternative pathway which is independent of the vagal afferent fibers may explain why no differences have been observed in NTS, CeA and IC after quantification of c-Fos IR cells in vagotomised animals (SDA) compared to non-vagotomised animals (Sham) after peripheral injection of CsA.

A second alternative pathway could be a direct effect of CsA in the CNS. In fact, only a small ratio of the CsA administered amount is able to reach the central nervous system (Scheinman et al., 1990; Jeruss et al., 1998). Approximately 95% of absorbed CsA bound to plasma protein, erythrocytes and leukocytes. The CsA-protein complexes are then too large to pass trough the junctions of blood brain barrier (Begley, 1992). Nevertheless, some studies indicate that CsA inhibits acetylcholinesterase (AChE) activity in several parts of the brain. It has been shown that AChE activity was reduced in cerebral cortex, striatum, hippocampus, hypothalamus and cerebellum after single dose of CsA per orally (20-45 mg/kg) or several i.p. injections of CsA (Herink et al., 2002; Herink et al., 2003; Mazzanti et al., 2007).

A third alternative pathway could be a neural pathway independent of the vagal afferent pathway. Some studies indicate that CsA induced hypertension due to an activation of renal and other subdiaphragmatic visceral afferent fibers that reflexively increase efferent sympathetic nerve activity (Lyson et al., 1993; Lyson et al., 1994). The signalling process of increase in blood pressure after peripheral injection of CsA uses mainly two different afferent neural pathways: the subdiaphragmatic vagal afferent fibers and the low thoracic dorsal spinal roots (Lyson et al., 1994). The low thoracic dorsal spinal roots could be a second neural pathway that may be involved in signalling peripheral injection of CsA to the CNS.

Finally, further investigations have to be conducted in order to validate or not one of these three afferent pathways that may explain how the administration of CsA can induce increase of c-Fos expression in the brain. Certainly, all of the described afferent pathways may play a role in signalling CsA to the CNS and the route of injection and the injected concentration of CsA may be experimental criteria that select which one of these three afferent pathways is mainly used to signal to the brain. In addition, if a direct effect of CsA in the CNS may be excluded, peripheral relevant mediators that may stimulate the afferent fibers of a neural pathway or reach directly the brain after CsA injection have to be considered. It has been described that the CsA-CyPA complex formed after CsA injection interacts and inhibits the CaN (Halloran, 1996). This protein is a critical component of the TcR-linked signal transduction pathway leading to cytokine gene transcription (Halloran, 1996). This CaN inhibition may be a sufficient criterion that may be interpreted as a change in the immune status. In addition, the activation of renal and subdiaphragmatic visceral fibers seems to be specifically related to CaN inhibition (Zhang et al., 2000). Therefore, CaN inhibition inducing hypertension as a side effect of CsA injection could be a relevant component of the CsA stimulus that may signal to the brain.

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# Chapter 5.

# General discussion

## Overview

In the model of behavioral conditioning of immunosuppression, a US (cyclosporin A: CsA) is associated to a CS (drinking solution: saccharin). The neural substrates involved in behaviorally conditioned immunosuppression by CsA were identified (Pacheco-Lopez et al., 2005), showing that the insular cortex (IC), the amygdala (Am) and the ventromedial part of hypothalamus (VMH) modulate the conditioned immunosuppressive effects on the immune system. More specifically, these results indicate that IC is essential to acquire and evoke the conditioned response in the underlying paradigm. In contrast, the Am seems to mediate the input of visceral information necessary at acquisition time, while the VMH appears to participate in the efferent output pathway to the immune system to evoke the behaviorally conditioned immune response.

In a first part (chapter 2 and 3), the aim of the study was to focus on the afferent pathway of the behavioral conditioning of immune function, when the US (e.g. SEB, LPS, CsA, Rapa or during LPS tolerance) signals to the CNS, and to determine how specific can be the sensing process, resulting in a specific EEG pattern, of an injected US. The results demonstrated that peripheral injection of immunostimulating (LPS, SEB or induced LPS Tol.) or immunosuppressive (CsA or Rapa) agents (Fig. 6) induced specific EEG response in the Am and the IC. In addition, the distribution pattern of the EEG in 6 frequency bands ($\delta$, $\theta$, $\alpha1$, $\alpha2$, $\beta1$ and $\beta2$) in the Am and the IC can be associated to "fingerprints" which were specific to the immunomodulating agents that challenged the immune system (Fig. 7). The changes of EEG pattern observed in the Am after peripheral injection of CsA were supported by c-Fos quantification in the Am. The c-Fos data showed increases of c-Fos IR cells in the CeA and BLA after CsA injection. However, changes of EEG signal were observed in the IC after CsA administration but the c-Fos quantification in the IC did not support this result. Indeed, no increases of c-Fos expression were observed in the IC after peripheral CsA administration. The changes of EEG pattern in the IC after peripheral injection of LPS was also not supported by c-Fos quantification in the IC. The c-Fos data showed no increases of c-Fos IR cells in the IC after LPS administration. In addition, increases of c-Fos expression were observed in the CeA and BLA after peripheral LPS injection, whereas no changes of the EEG pattern were observed in the Am after LPS administration.

In a second part (chapter 4), the aim of the study was to elucidate one afferent pathway involved in signalling a peripheral injection of CsA to the CNS. The vagal neural

afferent pathway was considered as the major entryway in the CNS for the CsA signalling process. However, the results demonstrated that in contrary of the LPS signalling process which is mainly dependent of the vagal afferent fibers, the CsA signalling process may be independent of the vagal afferent pathway. This finding suggested that alternative afferent signalling pathways have to be considered in order to explain how CsA signal to the CNS.

## The central nervous system specifically detects peripheral immune changes and generates "fingerprints" of neural activity

Peripheral immune stimulation has repeatedly shown to alter brain activity (Saphier et al., 1987a, b; Saphier, 1989; Saphier et al., 1990; Valles et al., 2002; Beishuizen and Thijs, 2003; Chen et al., 2004; Chen et al., 2005; Dallaporta et al., 2007; Teeling et al., 2007). However, the capacity of the CNS to detect or specifically "sense" different immunomodulating agents remains unclear. Based on previous work done for the behavioral conditioning of immune function, the insular cortex (IC) and the Amygdala (Am) were the selected brain regions. These two brain regions are integrative and associative brain areas that receive ascending visceral informations from the ventroposterior parvicellular nucleus of the thalamus (VPpc), the parabrachial nucleus (PBN), the nucleus tractus solitarii (NTS) and the lateral hypothalamic area (LHA) (Cechetto, 1987; Cechetto and Saper, 1987; Krushel and van der Kooy, 1988; Allen et al., 1991; Shi and Cassell, 1998; Barnabi and Cechetto, 2001).

In the present study, the peripheral immune system was challenged either with (a) immunostimulating agents like lipopolysaccharides (LPS) of E. Coli and Staphylococcal enterotoxin B (SEB) or with (b) immunosuppressive agents like cyclosporin A (CsA) or rapamycin (Rapa). LPS is a major component of the outer membrane of Gram-negative bacteria that stimulates the secretion of pro-inflammatory cytokines (IL-1$\beta$, IL-6 and TNF-$\alpha$). SEB is a bacterial superantigen from Gram-positive bacteria that activates T cells and mainly stimulates the secretion of Th1-cytokines (e.g., IL-2 and IFN-$\gamma$). CsA elicits its immunosuppressive effects based on calcineurin (CaN) inhibition and Rapa induces an immunosuppressive effects based on a CaN-independent mechanism, mTOR inhibition (chapter 2). The results suggest that the CNS is able to detect which immunostimulating agent challenges the system. Besedovsky and Edwin Blalock already proposed that the immune system functions as a sense organ which informs the CNS about infection and

injury. The results of the tele-stereo-EEG method described in chapter 2 confirmed the concept of a sensing process that takes place between the immune system and the CNS after injection of different immune stimuli.

The specific sensing process after peripheral injection of different immunostimulating agents (LPS or SEB) may be explain by the fact that peripheral injection of LPS induces an increase of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) (chapter 3), ACTH and corticosterone (Givalois et al., 1994; West and Heagy, 2002; Zhou et al., 2003). LPS also stimulates secretion of prostaglandins, reactive oxygen species and activate coagulation components (Schletter et al., 1995; Blatteis et al., 2004; Perlik et al., 2005; Crane and Buller, 2007). Peripheral injection of SEB stimulates the proliferation of T cells and the release of Th1 cytokines (IL-2 and IFN-γ) in plasma (Miethke et al., 1992; Huang and Koller, 1998). In addition, peripheral injection of SEB has been shown to induce c-Fos expression in the PVH, Am and NTS (Serrats and Sawchenko, 2006) and activates the HPA axis, resulting in increased plasma levels of ACTH and corticosterone (Goehler et al., 2001; Kusnecov et al., 1999; Shurin et al., 1997). c-Fos expression has been observed in the nucleus tractus solitarii (NTS), the paraventricular nucleus of hypothalamus (PVH) and lateral hypothalamus after peripheral LPS injection. These brain areas provide specific neural projections to the Am and the IC. These neural inputs mainly relay visceral information to the Am and the IC that may inform the CNS from peripheral changes (Cechetto, 1987; Cechetto and Saper, 1987; Allen et al., 1991; Shi and Cassell, 1998; Barnabi and Cechetto, 2001). Peripheral injection of LPS also induces mRNA cytokines expression in the brain and activates hypothalamus-pituitary-adrenal (HPA) axis (Pitossi et al., 1997; Beishuizen and Thijs, 2003; Chen et al., 2005). In addition to a single LPS challenge, LPS tolerance was induced in a group of animal. In contrast to LPS naïve animals, LPS tolerant animals do not show increases in plasma pro-inflammatory cytokines (Oberbeck et al., 2003, Pacheco-López et al., 2008). It has been described that c-Fos expression is reduced in the preoptic area of the hypothalamus after induced LPS tolerance (Navarro et al., 2007). However, mRNA cytokine expression still occurs in the brain during endotoxin tolerance (Chen et al., 2005). Therefore, the differences, in "how", "when" and "where" the immune status is changed after peripheral administration of LPS or SEB or after induction of LPS tolerance, may explain why the pattern of EEG is specific to the injected immunostimulating agent.

In parallel, peripheral injection of immunosuppressive agents showed increases of the EEG power in the Am and the IC but the pattern of the EEG power after peripheral injection of CsA or Rapa was similar (Fig 6C and 6F). The results may indicate that the CNS was able to detect but not able to specifically differentiate these two immunosuppressive drugs, while CsA and Rapa have two distinct signalling pathways to inhibit T cells proliferation. CsA binds to a cytosolic receptor called cyclophilin A (CyPA). The CsA-CyPA complex interacts and inhibits the $Ca^{+}$ -dependent serine-threonine phosphatase, calcineurin (CaN) (Halloran, 1996). Rapamycin binds to a cytosolic protein called FKBP12, the major FK506 binding protein. This complex targets and inhibits the function of mTOR protein (Abraham and Wiederrecht, 1996; Halloran, 1996). In addition, the tele-stereo-EEG technique suggests that the EEG pattern of an immunosuppressive agent is different to the EEG pattern of an immunostimulating agent.

Immunohistochemistry (IHC) of c-Fos performed in the central nucleus of amygdala (CeA), basolateral nucleus of amygdala (BLA) and the IC at different time points after peripheral injection of LPS or CsA confirmed the afferent signalling process to the CNS (chapter 3). However, the investigations of the EEG signal and c-Fos expression after peripheral administration of CsA or LPS showed contradictory results. These contradictions between the EEG data and the c-Fos data may be explained by the fact that EEG signal reveals changes of electrical potentials which reflect inhibitory signals as well as excitatory signals. c-Fos expression is related to cell excitation but not inhibition (Dragunow and Faull, 1989; Hughes and Dragunow, 1995). Therefore, while c-Fos positive cells provide definitive evidence that the cell is involved in the targeted brain area activity, the absence of c-Fos expression provides no relevant information to the functional involvement of a cell (Sagar et al., 1988; Dragunow and Faull, 1989; Sheng and Greenberg, 1990; Hughes and Dragunow, 1995). In sum, the EEG signal reflects the overall changes induced by a stimulus in the CNS, whereas c-Fos investigation just reflects a part of the overall changes induced by a stimulus. In addition, c-Fos IHC performed in chapter 3 was not coupled to an IHC of glial or neuronal marker. A triple IHC (c-Fos, glial and neuronal marker) would have helped to differentiate whether c-Fos expression would have been more concentrated in glial cells or neuronal cells.

The combined results of chapter 2 and 3 showed for the first time that the CNS is able to sense which immunomodulating agent challenges the immune system by providing specific pattern of EEG that can be associated to "fingerprints"of neural activity.

## Implication of the vagus nerve in the afferent communication between the immune and central nervous systems after CsA injection

Four major afferent signalling pathways have been described in the literature and may explain how the CNS detect or "sense" modifications of the immune status: neural afferent pathway mainly through the vagus nerve (Bluthe et al., 1994; Watkins et al., 1994), a humoral pathway through the circumventricular organs and the choroid plexus (Quan et al., 1998; Vitkovic et al., 2000), a cytokine transporter pathway at the blood-brain barrier (BBB) (Banks, 2006) and finally cytokine receptors which are located on perivascular macrophages and endothelial cells of brain venules (Schiltz and Sawchenko, 2002; Konsman et al., 2004). The vagal afferent pathway is one of the most studied pathways, especially after peripheral injection of LPS. Vagal sensory nerves terminate in the dorsal vagal complex (DVC), located in the brainstem. The DVC contains a sensory component, the area postrema (a circumventricular organ), and the nucleus of the solitary tract (NTS). Several lines of evidence support the involvement of the vagus nerve in signalling LPS challenge to the CNS (Gaykema et al., 1998; Gaykema et al., 2007). Peripheral Injection of LPS induces increase of IL-1β which in turn stimulates the vagal afferent fibers. Peripheral injection of IL1-β induces c-Fos expression in vagal primary afferent neurons (Goehler et al., 1998). In addition, LPS treatment induces IL-1β IR in immune cells that associate with perivascular cells or neuronal elements like dendritic cells (Goehler et al., 2006). This neuro-immune cells interaction could then directly and specifically influences neurons in the area postrema.

Peripheral injection of CsA induced no significant changes of plasma cytokine concentration although increases in c-Fos IR cells in CeA, BLA and IC were observed (chapter 3). In addition, the EEG pattern of CeA and IC showed that the CNS is able to detect the peripheral injection of the CsA immunosuppressive drug (chapter 2). Based on the model of LPS stimulates vagal afferent fibers, the hypothesis of an involvement of the vagus nerve in the signalling process of CsA to the CNS was tested in chapter 4. The results of c-Fos IR cells quantification indicated that CsA does not stimulate the vagal afferent

fibers in order to signal to the Am and IC. Indeed, select vagal deafferentation did not reduce the level of c-Fos IR cells in NTS, Am and IC after peripheral injection of CsA (chapter 4). This interesting finding suggests the involvement of alternative afferent signalling pathways for CsA.

One alternative pathway could be a direct effect of CsA in the CNS. In fact, only a small ratio of the CsA administered amount is able to reach the CNS (Scheinman et al., 1990; Jeruss et al., 1998). Approximately 95% of absorbed CsA bound to plasma protein, erythrocytes and leukocytes. The CsA-protein complexes are then too large to pass trough the junctions of blood brain barrier (Begley, 1992). Nevertheless, crossing the junctions of blood brain barrier may be dose dependent. Some studies indicate that CsA inhibits acetylcholinesterase (AChE) activity in several parts of the brain. It has been shown that AChE activity was reduced in cerebral cortex, striatum, hippocampus, hypothalamus and cerebellum after single dose of CsA per orally (20-45 mg/kg) or several i.p. injections of CsA (Herink et al., 2002; Herink et al., 2003; Mazzanti et al., 2007).

A second alternative signalling pathway could be a direct neuro-immune interaction that may occur in the area postrema (part of the circumventricular organs) between immune cells like T cells and perivascular cells or neuronal elements like dendritic cells as previously described for the LPS (Goehler et al., 2006). The immunosuppression effect of CsA comes from the specific CaN inhibition that takes place in T cells. The consequence of this inhibition is to block the transcription of IL-2 in T cells (Halloran, 1996). In addition, no peripheral changes of cytokines were observed in chapter 3 after peripheral injection of CsA. Blocking the transcription of IL-2 by CaN inhibition may then be the relevant signal that could signal to neuronal elements in the area postrema.

A third alternative pathway could be a neural pathway independent of the vagal afferent pathway. Some studies indicate that CsA induced hypertension due to an activation of renal and other subdiaphragmatic visceral afferent fibers that reflexively increase efferent sympathetic nerve activity (Lyson et al., 1993; Lyson et al., 1994). The signalling process of an increase in blood pressure after peripheral injection of CsA uses mainly two different afferent neural pathways: the subdiaphragmatic vagal afferent fibers and the low thoracic dorsal spinal roots (Lyson et al., 1994). The low thoracic dorsal spinal roots could be a second neural pathway that may be involved in signalling peripheral injection of CsA to the CNS.

Finally, further investigations have to be conducted in order to validate or not one of these three afferent pathways that may explain how the administration of CsA can induce increase of c-Fos expression in the brain. Certainly, all of the described afferent pathways may play a role in signalling CsA to the CNS and the route of injection and the injected concentration of CsA may be experimental criteria that select which one of these three afferent pathways is mainly used to signal to the brain. In addition, if a direct effect of CsA in the CNS may be excluded, peripheral relevant molecules that may stimulate the afferent fibers of a neural pathway or reach directly the brain after CsA injection still need to be identified. In chapter 3, peripheral injection of CsA did not induce any changes of plasma cytokine concentration. Therefore, the relevancy of plasma cytokine that may stimulate the afferent fibers of the vagus nerve in signalling CsA to the brain has to be reconsidered. Nevertheless, local increases of cytokines in various tissues after CsA administration that may stimulate the afferent fibers of a neural pathway which may relay the information to the brain can not be excluded. It has been also described that the CsA-CyPA complex formed after CsA injection interacts and inhibits the CaN (Halloran, 1996). This protein is a critical component of the TcR-linked signal transduction pathway leading to cytokine gene transcription (Halloran, 1996). This CaN inhibition in T cells may be a sufficient criterion that may be interpreted as a change in the immune status. In addition, the CaN inhibition inducing hypertension could be another relevant component of CsA stimulus that may signal to the brain. However, some studies show that Rapa, which induces immunosuppression through a different molecular mechanism than CsA, does not induce hypertension (Lyson et al., 1993; Zhang and Victor, 2000). The EEG data in chapter 2 also suggest that CaN inhibition may not be the relevant information which signals to the CNS. If we compare the pattern of EEG after peripheral injection of CSA or Rapa, these two patterns are similar and Rapa does not induce CaN inhibition (Abraham and Wiederrecht, 1996). Therefore assuming the specificity of the CNS in sensing peripheral injection of different immunomodulating agents, common criteria that may be changed after CsA and Rapa administration have to be identified in order to explain a similar pattern of EEG observed in chapter 2.

## Concluding remarks

Over the last three decades, several lines of evidence demonstrated the presence of a bi-directional communication that take place between the CNS and the immune system. A lot of studies described the role of cytokine in mediating information from the immune system to the CNS. In turn, neuro-endocrine mediators such as corticosterone or prolactin under the control of the CNS may modulate the activity of the immune system. In the present dissertation, the immune system was considered as a sensory organ that provides specific information of the host's immune status to the CNS. The results demonstrated that the CNS specifically detects changes of the immune status induced by immunostimulating agents (LPS and SEB) or immunosuppressive agents (CsA and Rapa). In addition, the spectral analysis of the EEG signal demonstrated that each immunomodulating agent had its own combination of changes in frequency bands. Such a combination can be associated to a code which may provide the EEG identity card of the present immune status. However, the meaning of such specific changes in frequency bands after different immune challenge still remains unclear and has to be further investigated. These specific changes in the frequency bands may reflect and be associated to a combination of physiological changes such as the cytokine concentration, immune cell activity, HPA axis activity and sympathetic activity after peripheral immune challenge that may form the peripheral code and be the relevant information that may signal to the CNS through different afferent communication pathway (Fig. 18). In sum, the present manuscript provides additional insights into the communication process between the immune and central nervous systems. Our data suggest that the CNS is able to specifically "sense" changes in the immune status by generating EEG "fingerprints" that may reflect peripheral modifications of the immune system activity after administration of different immunomodulating agents. The Tele-stereo-EEG technique may also be an additional tool to investigate more specifically this bi-directional communication between the CNS and the immune system. Finally, the understanding and the meaning of the observed changes in $\theta$, $\tau$, $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ may be on the clinical point of view an additional and very helpful tool to establish the diagnosis of patient faster and more specific to the type of disease (Fig. 18).

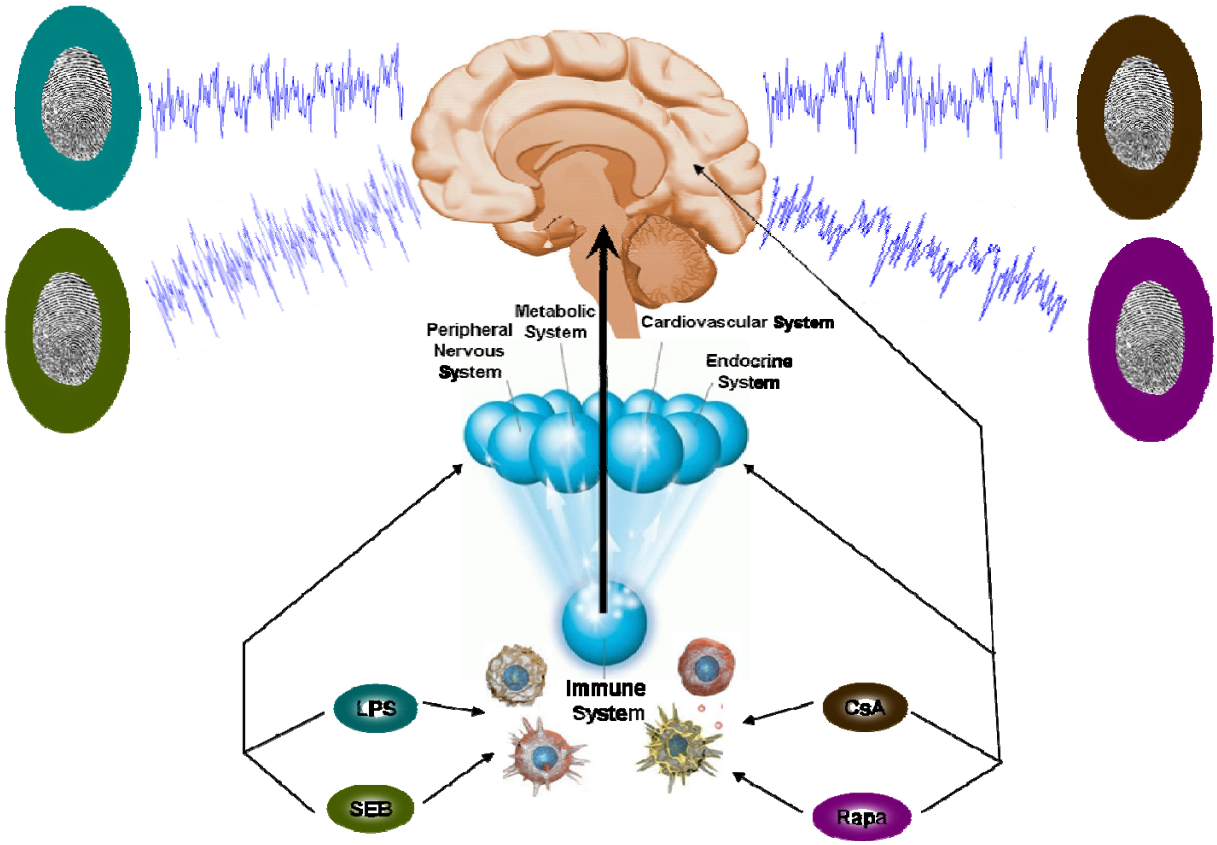


**Figure 18.** Generation of EEG "Fingerprints" after peripheral administration of different immune stimuli inducing specific physiological changes.

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## PEER-REVIEWED PUBLICATIONS

Niemi MB, Pacheco-López G, Engler H, Riether C, **Doenlen R** and Schedlowski M (2008) "Neuro-Immune Associative Learning" in: Handbook of Neurochemistry and Molecular Neurobiology: Neuroimmunology Ed. A. Lajtha, A Galoyan and H.O. Besedovsky, Springer Press, pp. 123-150.

Pacheco-Lopez G, Niemi MB, Engler H, Engler A, Riether C, **Doenlen R**, Espinosa E, Oberbeck R and Schedlowski M (2008) Weakened taste-LPS association during endotoxin tolerance. Physiol. Behav. 93(1-2): 261-266.

Riether C*, **Doenlen R***, Pacheco-López G, Niemi MB, Engler A, Engler H and Schedlowski M (2008) Behavioral conditioning of immune functions: How the CNS controls peripheral immune responses by evoking associative learning processes. Rev. Neurosci. 19(1): 1-17. (* equal contribution)

Engler H, **Doenlen R**, Riether C, Pacheco-Lopez G, Engler A, Niemi MB, del Rey A, Besesdovsky HO, Feldon J and Schedlowski M (2009) Time-dependent alterations of peripheral immune parameters after nigrostriatal dopamine depletion in a rat model of Parkinson's disease. BBI 23 (4): 518-528.

Pacheco-López G*, Riether C*, **Doenlen R**, Engler H, Niemi MB, Engler A, Kavelaars A, Heijnen CJ and Schedlowski M (2009) Learned inhibition of calcineurin activity in T lymphocytes. FASEB J 23 (4): 1161-7. (* equal contribution)

## MANUSCRIPTS IN PREPARATION

**Doenlen R**, Kruegel U, Riether C, Engler H, Engler A, Niemi MB, Schedlowski M and Pacheco-Lopez G. Specific Brain activity patterns in response to different peripheral immunostimulating agents.

Pacheco-Lopez G, **Doenlen R**, Kruegel U, Engler A, Riether C, Engler H, Niemi MB and Schedlowski M. Neural correlates of peripheral immunosuppression.

## PUBLISHED ABSTRACTS

**Doenlen R**, Kruegel U, Riether C, Engler H, Niemi MB, Schedlowski M and Pacheco-Lopez G (2007) Fingerprints of neural activity after peripheral immunes challenges. Exp. Dermatology, 16 (4), 358.

**Doenlen R**, Kruegel U, Riether C, Engler H, Niemi MB, Schedlowski M and Pacheco-Lopez G (2007) Brain activity patterns in response to different peripheral immunomodulators. Brain Behav Immun, 21 (4), e1–e42.